UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

DAIANA MORELI SOARES DOS SANTOS

Antimicrobial and anti-caries effects of 4% titanium tetrafluoride varnish under a microcosm biofilm model on dentin

Efeitos antimicrobiano e anti-cárie do verniz tetrafluoreto de titânio a 4% sob um modelo de biofilme microcosmo em dentina

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Orientadora: Profª Drª Ana Carolina Magalhães

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“A vida é como andar de bicicleta. Para manter seu equilíbrio você deve continuar em movimento.”

Albert Einstein

ABSTRACT

Antimicrobial and anti-caries effects of 4% titanium tetrafluoride varnish under a microcosm biofilm model on dentin

The study aimed: 1) to compare the effect of two different nutrients supply models (static and semi-dynamic) on the microcosm biofilm viability and dentin carious lesions formation; 2) to compare micro-CT versus TMR to measure dentin demineralization; and 3) to evaluate the effect of 4% TiF_4 varnish on the viability and metabolism of a microcosm biofilm and on development of dentin carious lesions. Microcosm biofilm was produced using pooled human saliva mixed with McBain saliva for the first 8 h; thereafter, only McBain saliva with 0.2% sucrose was applied daily (37°C, 5% CO_2), for a total time of 5 days. In the study 1, the static model consisted of 24-wells microplate, where bovine root dentin samples were submitted to biofilm formation. The semi-dynamic model, consisted of artificial mouth with continuous flow of McBain saliva with 0.2% sucrose (0.15 ml/min, 37°C) during 10 h a day (for the other 14 h, no flow was applied). Biofilm viability was measured by fluorescence and dentin demineralization by TMR. For the studies 2 and 3, bovine root dentin samples were treated for 6 h: A) 4% TiF_4 (pH 1.0, 2.45% F); B) 5.42% NaF (pH 5.0, 2.45% F); C) 2% CHX gel – positive control D) placebo or E) untreated – negative control. Treated samples were submitted to biofilm formation under static model as described above. Demineralization was measured using micro-CT (study 2) and TMR (studies 2 and 3). In the study 3, biofilm was analyzed with respect to viability by fluorescence and CFU counting for total microorganisms, total streptococci, *mutans streptococci* and Lactobacillus sp., and lactic acid and EPS production. In study 1, biofilm viability was lower for the static model (0.420±0.138) compared to semi-dynamic one (0.944±0.599). Both models were able to provoke dentin demineralization; however, the static model produced a higher number of typical subsurface lesions (83%) compared to the semi-dynamic (45%). In study 2, both fluorides were able to reduce dentin demineralization. Data obtained from micro-CT and TMR presented a significant and positive correlation (ΔZ: $r=0.78$ $p<0.0001$ and LD: $r=0.57$ $p<0.0001$) In study 3, all treatments reduced the biofilm viability, but not the CFU counting, except NaF that significantly reduced the number of

Lactobacillus sp. compared to control. No treatment was able to decrease the lactic acid production neither EPS production, except CHX that reduced the amount of insoluble EPS. Fluorides were able to reduce dentin demineralization compared to control, but TiF_4 had the best effect in reducing mineral loss and lesion depth (reduction of ΔZ: 70% and LD: 45%). In conclusion, 1) the nutrient supply model may have influence on the biofilm viability and the profile of dentin carious lesions; 2) micro-CT may be a suitable non-destructive method to measure dentin demineralization; and 3) despite TiF_4 varnish has no relevant antimicrobial effect, it is the best option to reduce the development of dentin carious lesions under this model.

Key words: Biofilm. Demineralization. Dentin. Fluorides.

RESUMO

Efeitos antimicrobiano e anti-cárie do verniz tetrafluoreto de titânio a 4% sob um modelo de biofilme microcosmo em dentina

O estudo objetivou: 1) comparar o efeito de dois modelos diferentes de disponibilidade de nutrientes (estático e semi-dinâmico) sobre a viabilidade do biofilme microcosmo e formação de lesões de cárie em dentina; 2) comparar micro-CT versus TMR para mensurar a desmineralização da dentina; e 3) avaliar o efeito do verniz TiF_4 4% na viabilidade e metabolismo do biofilme microcosmo e no desenvolvimento de lesões de cárie em dentina. O biofilme microcosmo foi produzido utilizando saliva humana misturada com saliva de McBain durante as primeiras 8 h; depois, apenas saliva de McBain com sacarose 0,2% foi aplicada diariamente (37°C, 5% de CO_2), totalizando 5 dias. No estudo 1, o modelo estático consistiu de placa de 24 poços, onde amostras de dentina radicular bovina foram submetidas à formação do biofilme. O modelo semi-dinâmico, consistiu de boca artificial com fluxo contínuo de saliva de McBain com sacarose 0,2% (0,15 ml/min, 37°C) durante 10 h por dia (nas demais 14 h, não foi aplicado fluxo). A viabilidade do biofilme foi mensurada por fluorescência e a desmineralização da dentina por TMR. Para os estudos 2 e 3, as amostras de dentina radicular bovina foram tratadas por 6 h: A) TiF_4 4% (pH 1,0, 2,45% F); B) NaF 5,42% (pH 5,0, 2,45% F); C) gel CHX 2% - controle positivo D) placebo ou E) não tratado - controle negativo. As amostras tratadas foram submetidas à formação do biofilme sob modelo estático conforme descrito acima. A desmineralização foi mensurada utilizando micro-CT (estudo 2) e TMR (estudos 2 e 3). No estudo 3, o biofilme foi analisado quanto à viabilidade por fluorescência e contagem das UFC para microrganismos totais, Streptococcus totais, *Streptococcus mutans* e Lactobacillus, e quanto à produção de ácido lático e PEC. No estudo 1, a viabilidade do biofilme foi menor para o modelo estático (0,420±0,138) comparado ao semi-dinâmico (0,944±0,599). Ambos os modelos provocaram desmineralização da dentina; entretanto, o modelo estático produziu maior número de lesões de subsuperfície (83%) comparado ao semi-dinâmico (45%). No estudo 2, ambos os fluoretos reduziram a desmineralização da dentina. Dados obtidos por micro-CT e TMR apresentaram uma correlação significativa e

positiva (ΔZ: r=0,78 p<0,0001 e LD: r=0,57 p<0,0001). No estudo 3, todos os tratamentos reduziram a viabilidade do biofilme, mas não a contagem de UFC, exceto o NaF que reduziu o número de Lactobacillus comparado ao controle. Nenhum tratamento diminuiu a produção de ácido lático e PEC, exceto a CHX que reduziu PEC insolúvel. Os fluoretos reduziram a desmineralização da dentina comparado ao controle, mas o TiF_4 apresentou o melhor efeito em reduzir perda mineral e profundidade da lesão (redução de ΔZ: 70% e LD: 45%). Em conclusão, 1) o modelo de disponibilidade de nutrientes pode influenciar a viabilidade do biofilme e o perfil das lesões de cárie em dentina; 2) micro-CT pode ser um método não destrutivo adequado para mensurar desmineralização da dentina; e 3) apesar do verniz TiF_4 não apresentar efeito antimicrobiano relevante, é a melhor opção para reduzir o desenvolvimento de lesões de cárie em dentina neste modelo.

Palavras-chave: Biofilme. Dentina. Desmineralização. Fluoretos.

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1 INTRODUCTION

1 INTRODUCTION

Dental biofilm is defined as a community of microorganisms, arranged three-dimensionally and included in an extracellular matrix, on tooth surface. The first stage of biofilm formation involves the precipitation of the acquired pellicle, an acellular and organic layer composed mainly of salivary proteins (HANNIG; HANNIG; ATTIN, 2005; HANNIG et al., 2007). This layer is responsible for determining the primary colonization of microorganisms on the tooth surface (CHEAIB et al., 2015). The microorganisms multiply and form colonies, generating an extracellular matrix, which is determined by the substrates present in the environment, mainly from the host's diet in case of supragingival biofilm. Through the extracellular matrix, other microorganisms can adhere to the biofilm via link with the extracellular polysaccharides (EPS) (KOO; FALSETTA; KLEIN, 2013). With time, biofilm reaches the homeostasis with the oral cavity; however, any instability with the host, such as frequent exposure to sucrose, can lead to the development of carious lesions due to the increase prevalence of acidogenic and aciduric bacteria (MARSH, 1994, 2004; DO; DEVINE; MARSH, 2013).

In vitro models have been applied to study the dynamic of biofilm on the tooth surface (SALLI; OUWEHAND, 2015). Among the models, there are the monospecies (one type of bacteria), the multispecies (more than one type, generally 2-3 species) and the microcosm (hundred species from a external source) biofilms. The microcosm biofilm is produced from the inoculum of pooled human saliva or dental biofilm together with a medium rich in nutrients. It has been considered the closest biofilm model to the *in vivo* reality, making possible to more accurately mimic the complexity of a real dental biofilm *in vitro* (SISSONS, 1997; TANG et al., 2003).

Biofilm models can be further classified according to the availability of nutrients, such as: 1) static model, which consists of limited supply of nutrients over time (e.g.: agar plates or multiple well plates); and 2) dynamic model that allows a continuous nutrients supply over time (e.g.: flow cell/chamber or artificial mouth) (SALLI; OUWEHAND, 2015; SIM; DASHPER; REYNOLDS, 2016; MASKE et al., 2017). However, there is lack of information about the effect of the availability of nutrients on the viability of a microcosm biofilm and its capacity of inducing caries.

Therefore, the 1st aim of this work was to analysis the effect of the availability of nutrients (static and semi-dynamic models) on the viability of a microcosm biofilm and its capacity of inducing carious lesions in dentin (Article 1).

Other important point is that the model must allow not only the analysis of the biofilm, but also of the tooth. To measure tooth demineralization, transverse microradiography (TMR) is considered the gold-standard method. It is able to measure demineralization produced *in vitro* and *in situ* by 2D images, quantifying mineral loss, lesion depth and the pseudo-intact surface layer (CENCI et al., 2009; MORON et al., 2013). On the other hand, micro-computed tomography (micro-CT) provides 3D images and mineral density profiles of an entire specimen without the time-consuming procedures of specimen preparation for TMR (LO; ZHI; ITTHAGARUN, 2010; HAMBA et al., 2012). Despite an expensive method, micro-CT has the biggest advantage of being non destructive (DAVIS; EVERSHED; MILLS, 2013). It has been often applied for the analysis of enamel de-remineralization (HAMBA et al., 2012; DAVIS; EVERSHED; MILLS, 2013; SHAHMORADI et al., 2016; FREE et al., 2017; ROVARIS et al., 2018); but less frequently for dentin de-remineralization (MEI et al., 2013; CHIEN et al., 2016; PIRES et al., 2018). Lo, Zhi and Itthagarun (2010) evaluated dentin demineralization using micro-CT and compared it with TMR and polarized light microscopy (PLM). However, the authors used different specimens for each analysis and did not statistically correlate the data. Therefore, the 2nd aim of this work was to apply TMR and micro-CT to measure dentin demineralization by a microcosm biofilm, under different preventive treatments using fluorides (Article 2).

Dental caries become a significant problem in public health when reaches dentin, impacting negatively in quality of life and often requiring restorative treatments (PITTS et al., 2017). Accordingly, root caries lesions (RCLs) are often diagnosed in elderly due to the symptoms of hyposalivation by the use of medicaments and due to the root exposure caused by brushing or chronic periodontitis (SAUNDERS; MEYEROWITZ, 2005). The reported global annual root caries incidence ranges from 10.1 to 40.6% (HAYES; BURKE; ALLEN, 2017). Therefore, attention to prevent this type of lesion is highly recommended and because of this this dissertation has the main focus on dentin carious lesions.

Fluorides have been often applied to prevent RCLs; 5% sodium fluoride (NaF) varnish and 38% silver diamine fluoride (SDF) prevent the emergence of new root caries in 64 and 71%, respectively (MAGALHÃES, 2017). The advantage of the varnish is the prolonged effect provided due to the presence of resinous base, which allow a long time of contact with the tooth surface (MARINHO et al., 2004, 2013). Furthermore, it does not produce tooth color change as done by SDF application.

Our research group has given a lot of attention to titanium tetrafluoride (TiF_4) varnish, since it has shown to be more effective in reducing enamel demineralization and in increasing enamel remineralization compared to NaF varnish under *in vitro* and *in situ* models (MAGALHÃES et al., 2008; COMAR et al., 2012, 2017b). Its mechanism of action occurs by the formation of an acid-resistant layer on the tooth surface rich in titanium oxide and hydrated titanium phosphate, providing mechanical protection and higher fluoride uptake compared to NaF, which in turn increase the acid resistance of the dental enamel (COMAR et al., 2017a).

Despite the mechanism of action of TiF_4 is well known, there is lack information about its potential as antimicrobial. Furthermore, no study has attempted to test the effect of TiF_4 varnish on the prevention of dentin demineralization by cariogenic challenges, but only under erosive challenges (COMAR et al., 2015; MAGALHÃES et al., 2016; MARTINES DE SOUZA et al., 2017). A recent study of our group tested the antimicrobial and anti-caries effects of TiF_4 varnish on enamel under a microcosm biofilm model. TiF_4 varnish showed to have a negligible antimicrobial effect, but it was able to reduce enamel carious lesion formation (SOUZA et al., 2018).

Considering the negative impact of carious lesions in dentin and the increase incidence of RCLs as well as the promising results of TiF_4 in enamel, the 3rd aim of this study was to compare the effect of TiF_4 varnish with NaF varnish and chlorhexidine (CHX) gel on the viability and metabolism of a microcosm biofilm formed on dentin and its impact on the carious lesion formation (Article 3). All Works were previously approved by the Local Ethical Committees (ANNEXES A and B).

Therefore, the null hypothesis of the 3 works presented in this dissertation were: 1) the models for nutrients supply (static versus semi-dynamic) do not differ with respect to microcosm biofilm viability and to their capacity of inducing dentin

carious lesions; 2) micro-CT do not differ from TMR in the ability to measure dentin demineralization; 3) a) the antimicrobial effect of TiF_4 varnish is similar to those from NaF varnish and CHX gel; and b) the anti-caries effect of TiF_4 varnish is similar to those from NaF varnish and CHX gel.

2 ARTICLES

2 ARTICLES

2.1 ARTICLE 1

Article accepted by the Journal of Applied Oral Science (ANNEX C)

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Comparison between static and semi-dynamic models for microcosm biofilm formation on dentin

Daiana Moreli Soares dos SANTOS (ORCID ID 0000-0002-9113-4076), Juliana Gonçalves PIRES (ORCID ID 0000-0002-7146-0456), Aline Silva BRAGA (ORCID ID 0000-0002-9331-1531), Priscila Maria Aranda SALOMÃO (ORCID ID 0000-0002-8580-9348), Ana Carolina MAGALHÃES (ORCID ID 0000-0002-6413-5348)

Universidade de São Paulo, Faculdade de Odontologia de Bauru, Departamento de Ciências Biológicas, Bauru, São Paulo, Brasil.

Corresponding address:
Ana Carolina Magalhães
Departamento de Ciências Biológicas – Faculdade de Odontologia de Bauru – Universidade de São Paulo
Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP: 17012-901 – Brasil
Phone/Fax. + 55 14 32358497
e-mail: acm@fob.usp.br

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ABSTRACT

Microcosm biofilm has been applied to induce carious lesions in dentin. However, no study has been done comparing the impact of the type of model for providing nutrients to microcosm biofilm formation on dentin. **Objective:** This study compared the performance of two kinds of models (static and semi-dynamic) on the biofilm formation and the dentin carious lesions development. **Material and Methods:** In both models, biofilm was produced using inoculum from pooled human saliva mixed with McBain saliva for the first 8 h (5% CO_2 and 37°C). Afterwards, for the static model, the samples were placed in 24-wells microplate containing McBain saliva with 0.2% sucrose, which was replaced at 24 h. In the semi-dynamic model, the samples were submitted to artificial mouth system with continuous flow of McBain saliva with 0.2% sucrose (0.15 ml/min, 37°C) during 10 h a day (for the other 14 h, no flow was applied, similarly to the static model). After 5 days, biofilm viability was measured by fluorescence and dentin demineralization by transverse microradiography. **Results:** Biofilm viability was significantly lower for the static compared to semi-dynamic model, while dentin demineralization was significantly higher for the first one ($p<0.05$). The static model was able to produce a higher number of typical subsurface lesions compared to the semi-dynamic model ($p<0.05$). **Conclusions:** The type of model (static and semi-dynamic) applied in the microcosm biofilm model may have influence on the formed biofilm and severity/profile of dentin carious lesions.

Keywords: Biofilms. Demineralization. Dental caries. Dentin.

INTRODUCTION

Dental caries is a disease that affects millions of people around the world.[1] It is generated by the instability created between the host and the microorganisms from dental biofilm due to the high and frequent consume of sugar, especially sucrose.[2] Other factors may interfere on biofilm development and increase the risk of root caries lesions, especially for adults and elderly individuals, who present low salivary flow and root exposure due to chronic periodontitis. It has been already established that the prevalence of carious lesions, involving dentin, increases with age.[3]

To better understand the dynamic of biofilm on dentin and to test the protective effect of antimicrobial agents, *in vitro* models of dental caries formation have been applied.[4] The microcosm biofilm has been considered the closest biofilm model to the *in vivo* reality, making possible to more accurately simulate the complexity of a real dental biofilm *in vitro*.[5,6]

Biofilm models can be further classified according to the availability of nutrients, as: 1) static model, which consists of limited supply of nutrients over time (e.g.: agar plates or multiple well plates); and 2) dynamic model that allows a continuous nutrients supply over time (e.g.: constant depth biofilm fermenter or artificial mouth).[4-8] However, there is no study comparing the impact of the type of model for providing nutrients (static and semi-dynamic) to microcosm biofilm on the development of carious lesion in dentin.

Therefore, the aim of this study was to compare two models (static and semi-dynamic) with respect to the viability of a microcosm biofilm and to its capacity of producing carious lesion in dentin. The null hypothesis is that the models do not differ with respect to biofilm viability and capacity of inducing dentin demineralization.

MATERIAL AND METHODS

Saliva collection

This study was firstly approved by the local Ethical Committee (CAAE: 58330616.7.0000.5417). Saliva was collected from 2 healthy donors only (the amount of saliva was enough for the experiment), who have followed the inclusion criteria: 1) normal salivary flow (stimulated saliva flow >1 ml/min and non-stimulated saliva flow >0.3 ml/min), 2) with previous history of caries, but not caries active (no active white spot and/or cavitated lesions), 3) without gingivitis/periodontitis (gum bleeding or tooth mobility) and 4) who did not ingest antibiotics 3 months prior to the experiment. The donors were not allowed to brush their teeth in the last 24 h and to ingest food or drinks in the last 2 h before saliva collection.[9,10] Saliva was collected under stimulation by chewing a gum for 10 min during the morning. The human saliva pool (70%) was mixed with glycerol (30%) and frozen at -80°C.[9,10]

Tooth sample preparation

Thirty-six dentin samples were prepared from eighteen bovine roots (4 mm x 4 mm, buccal and lingual surfaces) by using a semi-precision cutting machine (Buehler; Lake Bluff, Illinois, USA) and polished using a metallographic polishing machine (Arotec; Cotia, São Paulo, Brazil) and water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler; Lake Bluff, Illinois, USA). The average surface roughness was measured using contact profilometer and Mahr Surf XCR 20 software (Mahr; Göttingen, Lower Saxony, Germany), to standard the dentin surface for biofilm formation between the groups. Samples with Ra means <0.2 or >0.4 µm were excluded. The Ra means were further applied for randomly allocation of the samples into the groups by using the random function of Excel. Two thirds of

the root dentin surfaces were protected with wax to obtain control areas for the TMR analysis. The samples were then sterilized using ethylene oxide.

Microcosm biofilm formation

The microcosm biofilm was formed under two models (18 samples for each model, n=6 *per* biological replicate) for 5 days:

Static model

For the static model the samples were placed into 24-wells microplate. Human saliva solution was defrosted and mixed with McBain artificial saliva[11] in a proportion of 1:50.[9,10] During the first 8 h of inoculation, the solution of human saliva and McBain saliva was added to each well containing a root dentin sample (v=1.5 ml), which was incubated at 5% CO_2 and 37°C. Thereafter, the culture medium was removed and the root dentin samples were washed using phosphate-buffered saline twice (PBS, v=2 ml/well, each time). Fresh culture medium of McBain saliva containing now 0.2% sucrose was added into the wells (v=1.5 ml/well). The microplate were incubated at 5% CO_2 and 37°C for further 16 h, completing the first day of biofilm formation. During the next 4 days, the culture medium was daily removed, the root dentin samples were washed twice using PBS (v=2 ml/well, each time), McBain saliva with 0.2% sucrose was replaced (v=1.5 ml/well) and the microplate were stored at 5% CO_2 and 37°C.

Semi-dynamic model

For the semi-dynamic model the samples were placed into either microplate or artificial mouth. Human saliva solution was defrosted and mixed with McBain artificial saliva[11] in a proportion of 1:50.[9,10] During the first 8 h of inoculation, the solution of human saliva and McBain saliva was added to each well containing a root dentin sample (v=9 ml). The 6-wells microplate was incubated at 5% CO_2 and 37°C. Thereafter, the culture medium was removed and the root dentin samples were washed using PBS twice (v=9 ml/well, each time). Fresh culture medium of McBain saliva containing now 0.2% sucrose was added into the wells (v=9 ml/well). The 6-wells microplate was incubated at 5% CO_2 and 37°C for further 16 h, completing the first day of biofilm formation. During the next 4 days, we applied artificial mouth with continuous flow of McBain saliva containing 0.2% sucrose during 10 h a day (from 8 am to 6 pm, flow of 0.15 ml/min at 37°C and aerobic environment). Overnight (14 h a day), the samples were stored in 6-wells microplate with fresh McBain saliva containing 0.2% sucrose (v=9 ml/well) under 5% CO_2 and 37°C. Between the changes, the samples were washed twice using PBS (v=9 ml/well). In this model, 6-wells microplate was applied since the samples should be attached to acrylic disks to be placed into artificial mouth.

The biofilm cultivation was repeated three-independent times (n=6 independent samples for each type of model *per* replicate). Figure 1 shows the experimental design.

Bacterial viability analysis

Samples from static and semi-dynamic models (36 in total) were transferred to new 24 and 6 well microplates and exposed to 1 and 9 ml of MTT dye (0.5 mg MTT in 1 ml PBS) *per* well, respectively, for 4 h at 5% CO_2 and 37°C. The bacteria metabolically active are able to reduce MTT to purple formazan. After 4 h, the dye was removed and 1 and 9 ml of dimethyl sulfoxide (DMSO) was added in each well, respectively, to solubilize the formazan crystals in the absence of light for 30 min. Two hundred microliters from each sample were then transferred to a 96-well microplate, and the absorbance was measured using a microplate reader (Fluorstar Optima - BMG Labtech; Ortenberg, Baden-Württemberg, Germany) at 540 nm.[12] The final values were adjusted to the initial volume.

Transverse microradiography (TMR)

Dentin samples were sectioned perpendicularly to the wax (to allow the presence of sound and demineralized area in the fragment). Two fragments from each sample (approximately 500 µm thickness each) were manually polished using 600 grit papers, until the approximate thickness of 100-120 µm, and fixed in a sample-holder together with an aluminum calibration step wedge with 14 steps. A microradiograph was taken using an x-ray generator (Softex; Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The developed plate was analyzed using a transmitted light microscope fitted with a 20x objective (Zeiss; Oberkochen, Baden-Württemberg, Germany), a CCD camera (Canon; Tokyo, Japan) and a computer. The mineral content was calculated based on the formula described by Angmar, Carlström & Glas[13] (1963). The integrated mineral loss (ΔZ, %vol.µm) and lesion depth (LD, µm) were calculated as well as the semi-intact surface layer was detected.

Statistical analysis

Data were statistically analyzed using software Graph Pad Instat for Windows (GraphPad Software; San Diego, California, USA). The normal distribution and homogeneity were checked using Kolmogorov & Smirnov and Bartlett tests, respectively. The biofilm viability data (absorbance) were compared using Mann-Whitney test. ΔZ and LD data were compared using unpaired t test. For the association between the type of model and the percentage of subsurface lesions created, Fisher's Exact Test was done. The level of significance was set at 5% (n=18).

RESULTS

The biofilm viability was significantly lower for the static model (0.420±0.138) compared to semi-dynamic model (0.944±0.599) (Figure 2). On the other hand, the static model produced dentin lesions with higher values of the integrated mineral loss and lesion depth compared to the semi-dynamic model (Table 1).

The semi-intact surface layer was often seen in samples from the static model (83%, n=15/18) compared to those from the semi-dynamic model (45%, n=8/18), which means that the static model was able to produce a significant higher number of typical initial subsurface carious lesions (Fisher's Exact Test, p=0.0354). Figure 3 shows representative TMR images of dentin lesions produced by each model.

DISCUSSION

Despite the advantages of the microcosm biofilm,[4,6] most of the studies have applied this model to produce enamel lesions only,[14-16] highlighting the need of further studies on dentin carious lesion formation. We applied a period of 5 days of biofilm formation in according to Maske, et al.[17] (2015), after performing a pilot to define the best period to induce initial carious lesions in dentin (without cavitation). Researchers often choose one model to supply nutrients to the microorganisms, being either artificial mouth (an example of dynamic model) or multiple well plates (an example of static model).[4,7,8] No attention has been given to compare the impact of the type of model for providing nutrients (static and semi-dynamic) on the viability of microcosm biofilm and severity of carious lesion formation in dentin.

In the present study, biofilm produced using semi-dynamic model showed greater viability compared to those from the static model, which might be due to: 1) continuous flow of nutrients 10 hours *per* day, allowing more microbial growth and/or 2) the capacity of the model to wash metabolites (such as acids) that could be cytotoxic for the bacteria. The second hypothesis may also help to explain the less aggressive dentin lesions induced by this model.

The continuous flow in the semi-dynamic model was simulated only during 10 h, since some periods of flow absence would simulate the nighttime, where salivary flow rate physiologically decreases to zero. It is important to clarify that no atmosphere control was provided during 10 h of artificial mouth, which might have favored the growth of aerobic and facultative bacteria during this period, justifying the presence of less demineralized lesions. This is a limitation of our artificial mouth, which should be taken into account in the interpretation of the data.

On the other hand, the static model had atmosphere control,[18,19] which may justify the different performances between the models with respect to carious lesions formation in dentin. Static model produced larger dentin lesions than the semi-dynamic model. There are also two hypotheses to explain this result: 1) the differences in the type of microorganisms prevalent in both biofilm (probably the number of anaerobic/facultative bacteria was higher for the static model), which should be further investigated and 2) the washing effect provided by the semi-dynamic model, while for the static one the metabolites (acids) could stay in contact with the dentin surface for longer time. Future studies shall be focused on the analysis of biofilm thickness as well as the differences in microbiome and metabolome between microcosm biofilms formed under both models on dentin.[20]

Other interesting finding of the present study was the higher number of typical subsurface lesions produced by the static compared to the semi-dynamic model. This is in agreement with Owens, et al.[21] (2017), who showed that semi-dynamic biofilm model induced less evident subsurface layer, while Arthur, et al.[22] (2013) found well-defined subsurface lesions for the static model. The flow in semi-dynamic model may have also washed away free calcium and phosphate from biofilm, reducing their availability to precipitate on the lesion surface.

Considering the limitations of the design and the interpretations of the results, the null hypothesis can be rejected. Both models are able to produce viable cariogenic biofilm and dentin carious lesions; however, semi-dynamic model tends to produce more lesions with loss of surface integrity than the static one, which can be consequence of the availability of nutrients in each system. The response of both models to antimicrobial agents shall be analyzed in the future, especially with respect to the type of microorganisms prevalent in both biofilms and their impact on carious lesions formation in dentin.

CONCLUSION

The type of model applied to supply nutrients may have influence on the microcosm biofilm viability and the production of carious lesions in dentin.

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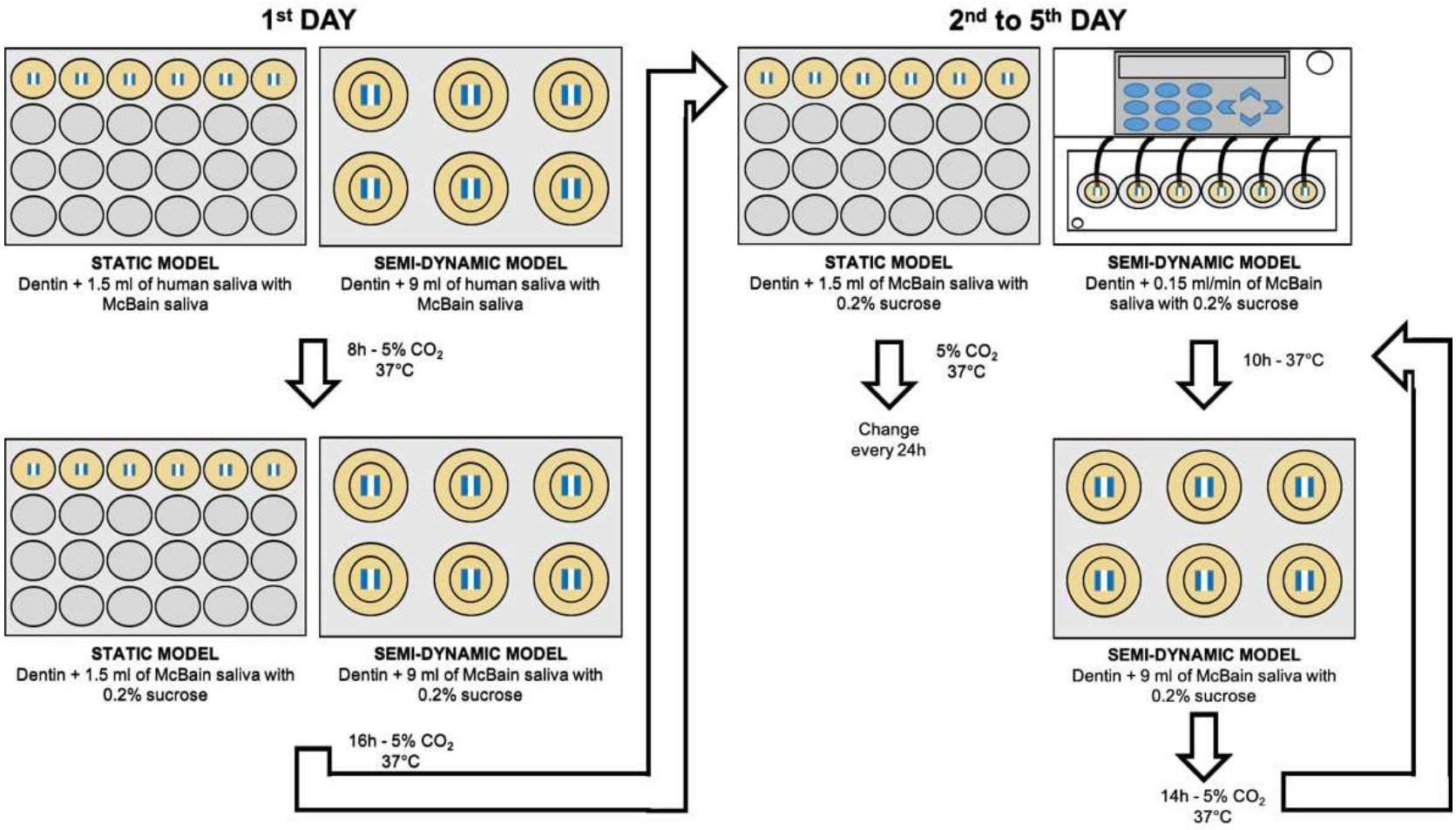

Figure 1. Experimental design

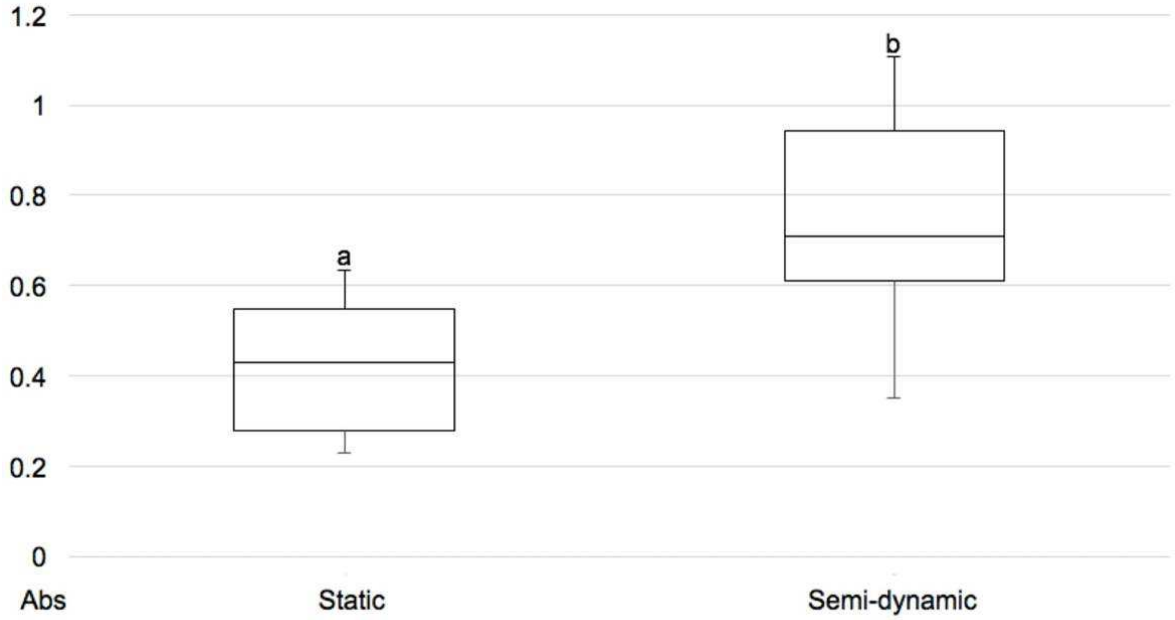

Figure 2. Boxplot of the biofilm viability (absorbance) according to the different models for microcosm biofilm formation. High absorbance values mean high biofilm viability. Different lowerscript letters indicate statistical significance (Mann-Whitney test, $p<0.05$)

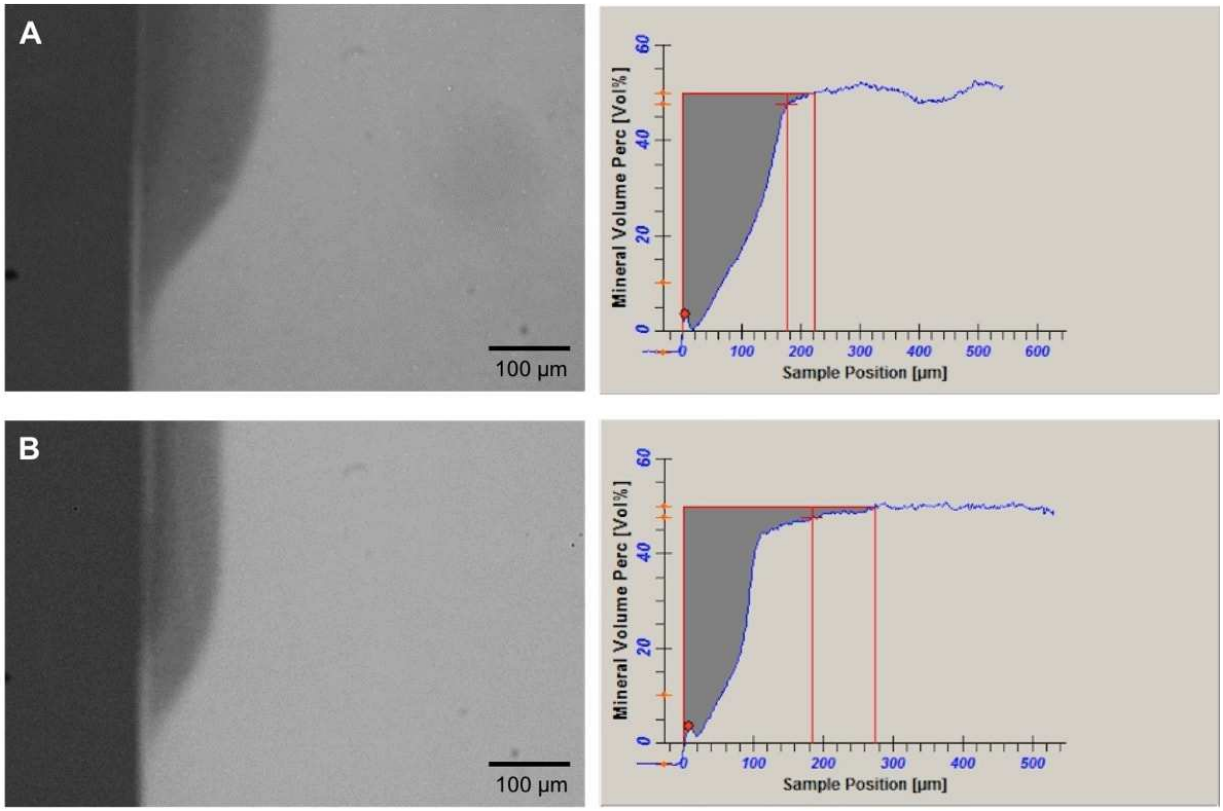

Figure 3. Representative TMR pictures (20x) of the artificial root dentin carious lesions created using microcosm biofilm under A) Static model and B) Semi-dynamic model, showing a more demineralized lesion for the first model

Table 1. Mean and standard deviation of integrated mineral loss (ΔZ, %vol.µm) and lesion depth (LD, µm) of dentin carious lesions produced using static and semi-dynamic models (microcosm biofilm, 5 days)

Models	**ΔZ (%vol.µm)**	**LD (µm)**
Static	4355±685[a]	160.3±16.7[a]
Semi-dynamic	3469±545[b]	129.3±13.2[b]

*Different letters in the same column show significant differences among the models (ΔZ: Unpaired T test, p=0.0002. LD: unpaired T test, p<0.0001, n=18). Higher values mean more demineralized lesions.

2.2 ARTICLE 2

Article submitted to the European Journal of Oral Sciences (ANNEX D)

Micro-CT versus TMR analysis of dentine treated with different fluorides and demineralized under a microcosm biofilm model

Daiana Moreli Soares dos Santos[a], Aline Silva Braga[a], Marta Rizk[b], Annette Wiegand[b], Ana Carolina Magalhães[a*]

[a]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo. Bauru, Brazil.

[b]Department of Preventive Dentistry, Periodontology and Cariology, University Medical Center Göttingen. Göttingen, Germany.

Running title: Micro-CT analysis of demineralized dentine

***Correspondent author:** Ana Carolina Magalhães. Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo. Al. Dr. Octávio Pinheiro Brisolla, 9-75, Jardim Brasil, 17012-901, Bauru, São Paulo, Brazil. Phone: +55 14 3235-8497. E-mail: acm@usp.br

Abstract

The study aimed 1) to compare the effect of TiF_4 with NaF and CHX on the prevention of dentine demineralization under a microcosm biofilm and 2) to apply micro-CT versus TMR to measure dentine demineralization. Bovine root dentine specimens were treated for 6 h: A) 4.0% TiF_4 varnish (pH 1.0, 2.45% F); B) 5.42% NaF varnish (pH 5.0, 2.45% F); C) 2% CHX gel D) placebo varnish or E) untreated. Treated dentine specimens were exposed to human saliva mixed with McBain saliva for 8 h. Thereafter, McBain saliva containing 0.2% sucrose was daily applied on the specimens for the formation of a microcosm biofilm, during 5 d. Demineralization was measured using micro-CT and TMR. The data were submitted to Kruskal-Wallis/Dunn tests and Pearson correlation ($p<0.05$). Fluoride varnishes were able to significantly reduce dentine mineral loss and lesion depth, except NaF that did not differ from control with respect to lesion depth measured by TMR. CHX failed in reducing dentine demineralization. Data obtained from both methods presented a significant and positive correlation. Fluorides, in special TiF_4, are still the best option to reduce dentine demineralization. Micro-CT may be a suitable non-destructive method to measure dentine demineralization.

Key words: dentine; fluoride; micro-computed tomography; microcosm biofilm; transverse microradiography

Introduction

Root caries lesions are common findings in elderly; the reported global annual root caries incidence ranges from 10.1 to 40.6% (1). Fluorides are often applied to control root caries lesions. Five percent sodium fluoride (NaF) varnish is able to prevent the formation of new root caries lesions in a range of 64% compared to control (2). The advantages of varnishes are the prolonged contact time with dental hard tissue and the low incidence of side effects (3, 4). Alternatively, antimicrobial agents (such as chlorhexidine – CHX) may also be applicable (5) especially for patients with special needs (6, 7).

Titanium tetrafluoride (TiF_4) has regained interest by dental researches due to the development of an experimental varnish that has been shown to be more effective against enamel caries compared to NaF varnishes *in vitro* and *in situ* (8–11). However, nothing is known about its effect on root caries lesions. There are some old studies about the effect of TiF_4 on dentine, but as solution and not as varnish (12, 13). Therefore, the first aim of this study was to compare the effect of TiF_4 varnish with NaF varnish (with similar F concentration) and CHX gel on the prevention of root dentine demineralization under a cariogenic microcosm biofilm.

Transverse microradiography (TMR) is considered the gold-standard method to measure tooth demineralization (2D image) produced *in vitro* and *in situ*, since it is able to quantify mineral loss and lesion depth as well as the thickness and the degree of mineralization of the surface layer (14, 15). Micro-computed tomography (micro-CT) provides 3D images and mineral density profiles of an entire specimen without the time-consuming procedures of specimen preparation for TMR (16, 17). Despite being an expensive method, it has the biggest advantage of being non destructive (18). Micro-CT has been often used for the analysis of enamel de-remineralization (17–26); but less frequently for dentine de-remineralization (19, 27–31); however, there is no study validating the use of micro-CT for the analysis of dentine carious lesions.

LO et al. (16) measured dentine demineralization using micro-CT and compared it with TMR and polarized light microscopy (PLM). However, the authors used different specimens for each analysis and did not statistically correlate the data. Therefore, the second aim of this paper was to use micro-CT analysis for measuring

root caries lesions as alternative to TMR, applying the same specimens for both analyses.

Material and Methods

Ethical aspects and saliva collection

This study was firstly approved by the local Ethical Committee (CAAE: 58330616.7.0000.5417). Then, the subjects read, agreed and signed the informed consent for saliva collection. Saliva was donated by 2 healthy subjects (26 and 28 yr old), who matched the following inclusion criteria: 1) normal salivary flow (stimulated saliva flow > 1 ml/min and non-stimulated saliva flow > 0.3 ml/min), 2) with previous history of caries, but not caries active (no active white spot and/or cavitated lesions), 3) without gingivitis/periodontitis (gum bleeding or tooth mobility) and 4) who did not ingest antibiotics 3 months prior to the experiment. The number of subjects was based on previous works (11, 32). The donors were not allowed to brush their teeth in the last 24 h and to ingest food or drinks in the last 2 h before saliva collection (11, 32).

Saliva was collected under stimulation by chewing a gum for 10 min during the morning. After collection, saliva was diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 ml were stored at -80°C (33).

Tooth specimen preparation and treatment

Eighty root dentine specimens were prepared from bovine permanent incisors using two diamond discs and a 4 mm spacer coupled to a precision cutting machine (Buehler, Enfield, CT, USA). Two specimens (buccal and lingual surfaces, 4 mm x 4 mm each) were cut from the cervical portion of the root. Specimens were then polished with 600 grit papers (Extec, Enfield, CT, USA) for 5-10 s, at low speed and under irrigation, using a metallographic polishing machine (Arotec, Cotia, SP, Brazil). Thereafter, the specimens were cleaned using ultrasound device (T7 Thornton, Indaiatuba, SP, Brazil) for 2 min and analyzed with respect to the average surface roughness (Ra) by using contact profilometer coupled to Marh Surf XCR20 software (Marh, Göttingen, Lower Saxony, Germany).

Two third of each specimens surface were protected with nail polish (Risqué, Taboão da Serra, SP, Brazil) to ensure two control areas (sound dentine) for the

TMR analysis. Specimens were subjected to sterilization by exposure to the ethylene oxide gas for 4 h under pressure of 0.5±0.1 Kgf/cm^2 (Acecil Central de Esterilização, Campinas, SP, Brazil).

The specimens were allocated to the following experimental groups according to Ra mean values (0.308±0.042 µm) (n=4 for each independent repetition of the biofilm formation, biological quadruplicate, n final=16): A) 4.0% TiF_4 varnish (pH 1.0, 2.45% F); B) 5.42% NaF varnish (pH 5.0, 2.45% F); C) 2% CHX gel – positive control (Maquira Indústria de Produtos Odontológicos, Maringá, PR, Brazil) D) placebo varnish or E) untreated – negative control. The varnishes were produced by FGM as previously described (11).

During the treatment period (6 h), the dentine specimens were stored in remineralizing solution (34). Thereafter, the varnishes and gel were removed with cotton swab and acetone solution diluted 1:1 in water (8, 9, 11) and the nail polish was applied again before biofilm formation.

Microcosm biofilm formation

Human saliva was defrosted and mixed with MCBAIN (35) artificial saliva in a proportion of 1:50 (11, 32). The solution of human saliva and McBain saliva was added to each well containing a treated dentine specimen (v=1.5 ml/well) in a 24-well plate, which was incubated at 5% CO_2 and 37°C, for 8 h. The dentine specimens were washed twice with phosphate buffer solution (PBS) (v=2 ml/well each time) and then fresh McBain saliva with 0.2% sucrose were added (v=1.5 ml/well) and the plate incubated under the same conditions for further 16 h. After this period, the McBain saliva with 0.2% sucrose was replaced once a day, totalizing 5 d of biofilm growth at 5% CO_2 and 37°C (32).

Micro-CT analysis

The micro-CT analysis was performed using Skyscan 1272 (Bruker Microct, Kontich, Antwerp, Belgium) at 90 kV and 111 µA on wet specimens. A filter (Al 0.5+Cu 0.038 mm) was used to reduce the beam-hardening artifacts. Each specimen was scanned at 180° rotation with a rotation step of 0.35° and averaging 3 readings with a total of 2 h scanning. Data were acquired in TIFF format with the binning 1x1, resulting in the resolution of 4904x3280 pixels and 1.4 µm isotropic voxel sizes. Two

phantom specimens with a known mineral density (0.25 and 0.75 g/cm^3 calcium hydroxyapatite) were scanned under identical conditions for the calibration.

The data were reconstructed using the NRecon (Bruker Microct, Kontich, Antwerp, Belgium), applying a beam hardening correction (BHC) of 10 and ring artifacts reduction of 35. The images were calibrated and rotated in Data Viewer (Bruker Microct, Kontich, Antwerp, Belgium) to mimic the orientation of the specimen during the cutting procedure for TMR analysis. A region with thickness of 120 µm around the center of the specimen was chosen and all 2D images in this region were stacked to one projection image to be compared to the TMR scan by Image J program (National Institutes of Health, Bethesda, Maryland, USA) (17). Integrated mineral loss (ΔZ, % min vol. µm) and lesion depth (LD, µm) were obtained from the gray values considering that sound dentine contains 50% mineral by volume and that the lesion ends when dentine presents 95% of the mineral content (equivalent to 47.5%) (15).

TMR analysis

After the micro-CT analysis, the dentine specimens were cut perpendicularly to the nail polish (to allow the presence of sound and demineralized area in the fragment) for the TMR analysis. Two slices from each specimen were manually polished with 600 grit papers (Extec, Enfield, CT, USA), until the approximate thickness of 100-120 µm. The fragments were immersed in propylene glycol 24 h prior to the analysis to avoid shrinkage during the irradiation.

Microradiographs were made of the dentine specimens in combination with "step wedge" (14 slices, ±30 µm thick, 99.9% Al) for calibration, using glass plates that were exposed to x-ray Cu Kα (20 KV and 20 mA) for 13 min. The glass plates were revealed (4 min, 20°C), fixed (7 min, 20°C) and washed using running water (10 min). The plates were analyzed using a transmitted light microscope fitted with a 20x objective (Axioplan, Zeiss, Oberkochen, Baden-Württemberg, Germany) and a CCD camera (XC-77 CE, Sony, Tokyo, Japan) coupled to a computer with software for image acquisition and calculation (TMR 2012 and TMR 2006, Inspector Research BV, Amsterdam, Netherlands). Integrated mineral loss (ΔZ, % min vol. µm) and lesion depth (LD, µm) were obtained in the same manner as described for the micro-CT analysis (15).

Statistical analysis

Graph Pad Software (San Diego, CA, USA) was used for statistical analysis of the data from TMR and micro-CT analyses. Firstly, the data were checked for normal distribution and homogeneity using Kolmogorov-Smirnov and Bartlett tests, respectively. Thereafter, the treatments were compared using Kruskal-Wallis followed by Dunn test. Additionally, the TMR and micro-CT data were submitted to Pearson correlation. The level of significance adopted in all tests was set at 5%.

Results

Both fluoride varnishes were similarly effective in reducing mineral loss (ΔZ) and lesion depth (LD) compared to CHX, placebo varnish and control ($p<0.0001$). The only exception was NaF that did not differ from control with respect to lesion depth when analyzed by TMR. CHX failed in reducing dentine demineralization, being similar to placebo varnish and control. Table 1 shows the median and interquartile interval of ΔZ and LD obtained from both methods.

ΔZ and LD obtained from TMR and micro-CT analyses presented a positive and significant correlation ($r=0.78$ $p<0.0001$ and $r=0.57$ $p<0.0001$, respectively).

Discussion

NaF and CHX have been widely used in dentistry for the prevention and treatment of dentine carious lesions (2, 36, 37); however, CHX has been shown to have a greater antimicrobial effect than anti-caries effect, at least when compared to fluoride (11). It may also cause some side effects as tooth discoloration, mucosa desquamation, taste changes and calculus (38, 39). On the other hand, TiF_4 varnish has shown superior effect compared to NaF varnish on enamel de-remineralization (8–11) due to the formation of a glaze like-layer rich in calcium fluoride (due to the low pH of the varnish), titanium oxide and hydrated titanium phosphate, which is acid resistant (40). Besides, it has cytotoxic potential on fibroblasts similar to NaF, which is widely applied in mouth (41). However, nothing is known about its protective effect against root dentine caries.

Our model was able to show that CHX was ineffective in reducing root carious lesion development in accordance with previous *in vitro* studies (42, 43), despite it has shown antimicrobial effect on microcosm biofilm, which was analyzed by

fluorescence (viability assay) using confocal microscopy (unpublished data). We speculate that its antimicrobial effect may be stronger against non-cariogenic bacteria that might also be present in microcosm biofilm, justifying the lack of anti-caries effect in the present study.

Despite no significant difference was found between TiF_4 and NaF, dentine specimens treated with TiF_4 (% of protection: 75% for mineral loss and 41.6% for lesion depth) showed numerically less demineralization compared to those from NaF group (% of protection: 41.8% for mineral loss and 27.6% for lesion depth). The lack of statistical difference between both fluorides might be justified by the high cariogenic challenge provoked by the model compared to previous works done by our research group (8–10). Another reason might be the dental substrate - dentine - rich in organic content, which in turn could present different interaction with fluoride. In contrast, we have found differences between TiF_4 and NaF on enamel under similar model (11). This result should be confirmed under *in situ* and *in vivo* models, which are closer to the clinical conditions.

As stated above, TMR is the gold standard method to measure tooth demineralization *in vitro*, but it presents some disadvantages that have prompted the researchers to look for new methods to analyse dental caries as micro-CT. This method is non-destructive and offers the complete information about the full volume of the specimen. Furthermore, it can be applied *in vivo* as well (16, 17, 44). On the other hand, micro-CT requires long measurements for data with good quality and its software is not suitable for analysis comparable to the current TMR standard (16).

Both methods showed similar results when applied to compare the effect of different treatments against dentine demineralization. The only exception was the lesion depth analyzed by TMR that did not show differences between NaF varnish and control. Micro-CT and TMR also showed a positive and strong correlation when compared to measure mineral loss (r=0.78) as found by HAMBA et al. (17) (r=0.90). Despite HAMBA et al. (17) analyzed enamel; TMR and micro-CT were done in the same specimens using similar filters (Al + Cu) associated with BHC for the analysis by micro-CT, as done here. MEGANCK et al. (45) concluded that the filters combination might reduce artefacts, as well as BHC might help in obtaining homogeneity in gray scale values (46). However, the correlation for LD found by HAMBA et al. (17) was higher (r=0.91) than in our study (r=0.57), which could be justified by the types of substrate applied by them (they used enamel with natural

lesions presenting higher mineral loss than our dentine specimens demineralized under microcosm biofilm).

On the other hand, JEON et al. (47) found a low correlation between TMR and micro-CT (r=0.37) with respect to mineral loss, differently from the study of HAMBA et al. (17), which might be due to the lesser demineralized enamel lesions produced by them *in vitro* [110.8 to 905.4 % min vol. µm vs. 4,000 to 33,000 % min vol. µm from HAMBA et al. (17)]. The authors justified the low correlation due to the absence of a reliable calibration curve in the TMR analysis for early-demineralized lesions, while the micro-CT presents a correct calibration for such types of lesion. It is important to note that JEON et al. (47) did not use similar filter as HAMBA et al. (17), which might have also influenced their results.

The only study done in dentine, using micro-CT and TMR, applied an abiotic model to induce caries (demineralizing solution for 96 h) (16). Despite the authors did not statistically correlate the TMR and micro-CT data, LO et al. (16) concluded that changes of similar magnitude were detected by both methods after dentine remineralization. They suggested that micro-CT could replace TMR.

Based on the results, our conclusion is that fluoride varnishes, special TiF_4, are still the best option to reduce dentine demineralization under biofilm model. Micro-CT is a convenient and non-destructive method to measure dentine carious lesions; however, the type of dental substrate, the degree of demineralization and the measurement settings might influence its correlation with TMR.

This experiment shall be repeated *in situ* for the confirmation of the results and, thereafter, the TiF_4 varnish tested for the prevention of root carious lesions under RCT model in elderly, using micro-CT as one of the response variables.

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Conflicts of interest

The authors state no conflicts of interest.

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Table 1. Median and interquartile interval of ΔZ and LD obtained by TMR and micro-CT

Treatments	ΔZ (% min vol. µm)		LD (µm)	
	TMR	**Micro-CT**	**TMR**	**Micro-CT**
TiF_4	1170±335[a]	740±908[a]	76±27.5[a]	93±51.0[a]
NaF	2475±1300[a]	1934±462[a]	108±35.2[ab]	101±25.5[a]
CHX	4390±1825[b]	3345±1429[b]	144±44.1[c]	149±40.8[b]
Placebo	4563±1380[b]	4037±956[b]	151±27.4[c]	174±75.0[b]
Control	4235±720[b]	3384±626[b]	130±23.0[bc]	161±26.3[b]

Different letters show significant differences among the treatments (Kruskal-Wallis followed by Dunn test, $p<0.0001$, $n=16$).

2.3 ARTICLE 3

Article submitted to the Journal of Dentistry (ANNEX E)

Antimicrobial and anti-caries effects of 4% Titanium Tetrafluoride varnish under a microcosm biofilm model on dentin

Short title: *Effects of 4% TiF_4 on dentin carious lesions*

Daiana Moreli Soares dos Santos[a] (daianamoreli@gmail.com), Juliana Gonçalves Pires[a] (jugpires@gmail.com), Aline Silva Braga[a] (aline_s.braga@hotmail.com), Priscila Maria Aranda Salomão[a] (pri_barrabonita@yahoo.com.br), Marília Afonso Rabelo Buzalaf[a] (mbuzalaf@fob.usp.br), Ana Carolina Magalhães[a] (acm@usp.br)

[a]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo. Alameda Dr. Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, São Paulo, Brazil.

Corresponding author:
Ana Carolina Magalhães
Department of Biological Sciences
Bauru School of Dentistry – University of São Paulo
Al. Dr. Octávio Pinheiro Brisolla, 9-75. Bauru – SP – Brazil
Zip code: 17012-901
Phone/Fax. + 55 14 32358497
E-mail: acm@usp.br

Key-words: Antimicrobial, demineralization, dentine, fluoride, microcosm biofilm, titanium.

Declaration of interest:

None.

Abstract

OBJECTIVES: The present study aimed to evaluate the effect of titanium tetrafluoride varnish on the viability and metabolism of a microcosm biofilm as well as on development of carious lesions in dentin.

METHODS: Bovine root dentin samples were treated for 6h as following: A) 4% TiF_4 varnish (pH 1.0, 2.45% F); B) 5.42% NaF varnish (pH 5.0, 2.45% F); C) 2% chlorhexidine (CHX) gel – positive control D) placebo varnish or E) untreated – negative control (n=4 x biological triplicate). Treated dentin samples were exposed to human saliva mixed with McBain saliva (1:50) for 8h in 24-wells plates. Thereafter, McBain saliva containing 0.2% sucrose was applied on the samples for 16h (37°C, 5% CO_2). McBain saliva was daily replaced in a total time of 5 days.

RESULTS: All treatments significantly reduced the biofilm viability. However, none of them was able to reduce the CFU counting for total microorganism, total streptococci and *mutans streptococci*. NaF significantly reduced the number of Lactobacillus sp. compared to control. No treatment was able to decrease the lactic acid production neither EPS synthesis, except CHX that significantly reduced the amount of insoluble EPS. Only fluorides were able to reduce dentin demineralization compared to control, but TiF_4 had the best effect in reducing mineral loss and lesion depth.

CONCLUSIONS: Despite TiF_4 varnish had no relevant antimicrobial effect, it was able to reduce the development of dentin carious lesions under this model.

CLINICAL SIGNIFICANCE: As found for enamel, TiF_4 varnish may not have relevant antimicrobial effect but it is effective in reducing dentin demineralization.

Introduction

Titanium tetrafluoride (TiF_4) varnish has shown to be more effective in reducing enamel demineralization and in increasing enamel remineralization compared to sodium fluoride (NaF) varnish under *in vitro* and *in situ* models [1-3]. On the other hand, no study attempted to test the effect of TiF_4 on the prevention of dentin carious lesions formation, but only under erosive challenges [4-6].

Its mechanism of action occurs by the formation of an acid-resistant layer rich in titanium oxide and hydrated titanium phosphate on the tooth surface, providing mechanical protection and higher fluoride uptake compared to NaF, which in turn increase the acid resistance of dental enamel [7,8].

Despite the mechanism of action of TiF_4 is well known, there is lack information about its potential as antimicrobial. A recent study of our group tested the antimicrobial and anti-caries effect of TiF_4 varnish on enamel under a microcosm biofilm model. We showed that TiF_4 had negligible antimicrobial effect, but it was able to reduce enamel carious lesions development [9]. However, nothing is known about the antimicrobial and anti-caries effect of TiF_4 varnish on dentin under a biofilm model.

Root caries lesions (RCLs) are often diagnosed in elderly due to the symptoms of hyposalivation and due to the root exposure caused by brushing or chronic periodontitis [10]. The reported global annual root caries incidence ranges from 10.1 to 40.6% [11]. Therefore, attention to prevent this type of lesion is highly recommended. Fluorides have been applied to prevent RCLs; 5% NaF varnish and 38% silver diamine fluoride prevent the emergence of new RCLs in 64 and 71%, respectively [12].

Considering that TiF_4 varnish has better anti-caries effect on enamel than NaF [9], the aim of this work was to evaluate the antimicrobial (microorganisms viability, lactic acid and extracellular polysaccharides-EPS production) and anti-caries (demineralization prevention) effects of 4% TiF_4 compared to NaF and chlorhexidine (CHX), under a microcosm biofilm model formed on root dentin. The null hypotheses were: 1) the antimicrobial effect of TiF_4 varnish is similar to those from NaF varnish and CHX gel; and 2) the anti-caries effect of TiF_4 varnish is similar to those from NaF varnish and CHX gel.

Materials and methods

Saliva collection

The local ethical committee (CEEA 58330616.7.0000.5417) firstly approved this study. Thereafter, saliva was collected from two healthy donors who met the inclusion criteria: (1) normal salivary flow (stimulated saliva flow > 1 ml/min and non stimulated saliva flow > 0.3 ml/min), (2) with a previous history of caries but no caries active (no active white-spot and/or cavitated lesions), (3) without gingivitis/periodontitis (gum bleeding or tooth mobility) and (4) without ingestion of antibiotics three months before the experiment.

Prior to the day of collection, the donors did not brush their teeth. Furthermore, they were not allowed to ingest food or drinks within the last 2h before saliva collection. The saliva was collected under stimulation by chewing a rubber material for 10 min during the morning. After collection, human saliva was pooled and diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 ml were stored at -80°C [13].

Tooth sample preparation and treatment groups

One hundred and eighty root dentin samples (4 mm × 4 mm) were prepared from bovine teeth, using a semi-precision cutting machine (Buehler, Enfield, USA). The samples were polished in a metallographic polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide disks (600-grade papers ANSI grit; Buehler) to remove grooves and to standardize the surface roughness of 0.283±0.04 μm. The average surface roughness (Ra) was assessed using contact profilometer and Mahr Surf XCR 20 software (Mahr, Göttingen, Germany). Two-thirds of the dentin surfaces were protected with nail polish to obtain control areas for the transverse microradiography (TMR) analysis. The samples were sterilized using ethylene oxide for 4h under a pressure of 0.5±0.1 kgF/cm.

Dentin samples were randomly divided in the groups according to the Ra values: A) 4% TiF_4 varnish (pH 1.0, 2.45% F); B) 5.42% NaF varnish (pH 5.0, 2.45% F); C) 2% chlorhexidine (CHX) gel – positive control (Maquira Indústria de Produtos Odontológicos Ltda, Maringá, Brazil) D) placebo varnish or E) untreated – negative control. The varnishes were produced by FGM as previously described [9].

The treatments were applied using microbrush and remained on the samples surfaces for 6h. During this period, dentin samples were stored in remineralizing solution [14]. Thereafter, the varnishes and gel were removed using cotton swab and acetone solution diluted 1:1 in water [1,2] and the nail polish was applied again before biofilm formation.

Microcosm biofilm formation

The human saliva was defrosted and mixed with McBain artificial saliva [15] in the proportion of 1:50. The McBain saliva contained 2.5 g/l of mucin from porcine stomach (type II), 2.0 g/l of bacteriological peptone, 2.0 g/l of tryptone, 1.0 g/l of yeast extract, 0.35 g/l of NaCl, 0.2 g/l of KCl, 0.2 g/l of $CaCl_2$, 0.1 g/l of cysteine hydrochloride, 0.001 g/l of hemin and 0.0002 g/l of vitamin K1, at pH 7.0. All reagents were obtained from Sigma – Aldrich (Saint Louis, USA).

The solution of human saliva and McBain saliva was added to each well containing a dentin sample (v = 1.5 ml/well) in a 24-well microplate, which was incubated at 5% CO_2 and 37°C. After 8h, the medium was removed, the dentin samples were washed twice using phosphate-buffered saline (PBS, v = 2 ml/well each time) and fresh McBain saliva now containing 0.2% sucrose was added to the wells (v = 1.5 ml/well). The microplate were incubated at 5% CO_2 and 37°C for further 16h, completing the first day of the experiment. From the second day to the 5th day, the samples were washed twice using PBS (v = 2 ml/well each time), and fresh McBain saliva containing 0.2% sucrose was added daily. The microplate was incubated at the same conditions as described above [16].

Viability analysis:

Bacterial viability

The biofilm on the dentin was stained using nucleic acid markers diluted in PBS (1 ml PBS + 1 µl SYTO9 + 1 µl propidium iodide) (v = 10 µl/well) (Kit Live & Dead® cells viability assay, Thermo Fisher Scientific, Waltham, USA) for 15 min in a dark environment. Live bacteria were stained with SYTO9, producing a green fluorescence, and dead lysed bacteria were stained with propidium iodide/SYTO9, producing a red fluorescence [17]. The biofilm was examined using confocal laser scanning microscopy - CLSM (Leica TCS SPE, Mannheim, Germany) and Leica Application Suite-Advanced Fluorescence software (LAS AF, Mannheim, Germany). Three images (275 µm^2) were captured from each sample surface and analyzed using BioImage L 2.0 software to quantify the live and dead bacteria (%).

Microorganism viability

For colony-forming unit (CFU) counting, 100 µl of the bacterial suspension obtained for the EPS analysis were diluted to 10^{-2} and spread on Petri dishes (25 µl/dish) containing four different types of agar: (1) brain heart infusion agar (BHI, Difco, Detroit, USA) for total microorganisms; (2) mitis salivarius agar (MSA, Neogen, Indaiatuba, Brazil) containing 20% sucrose and 1% potassium tellurite for total streptococci [18]; (3) SB-20 M [19] containing 15 g of bacto-casitone (Difco, Detroit, USA), 5 g of yeast extract (Kasvi, Curitiba, Brazil), 0.2 g of L-cysteine hydrochloride (Sigma, Steinheim, Germany), 0.1 g of sodium sulfite (Sigma, Steinheim, Germany), 20 g of sodium acetate (Synth, Diadema, Brazil), 200 g of coarse granular cane sugar, 15 g of agar (Kasvi, Curitiba, Brazil)

and 0.2 U ml of bacitracin (Sigma, Steinheim, Germany) in 1 l distilled water (autoclaved) for the determination of *mutans streptococci*; and (4) rogosa (MRS agar, Kasvi, Curitiba, Brazil) supplemented with 0.13% glacial acetic acid to assess the number of Lactobacillus sp. [18]. The plates were then incubated at 5% CO_2 and 37°C. After 48h, the CFU numbers were counted by two examiners and transformed to $\log_{10}$ CFU/ml [20].

Metabolism analysis:

Lactic acid production

After 5 days of biofilm formation, dentin samples were incubated in buffered peptone water (BPW) (Synth, Diadema, Brazil) supplemented with 0.2% sucrose (v = 1 ml sample) for 3h anaerobically to allow the biofilm to produce lactic acid. The anaerobic conditions were obtained using the Whitley A35 Anaerobic Workstation (Don Whitley Scientific, Shipley, UK), with the environment maintained at 80% N_2, 10% CO_2, 10% H_2 and 37°C. Lactate concentrations were evidenced via the enzymatic method (lactic dehydrogenase method, Boehringer, Mannheim, Germany) in the BPW solution according to the manufacturer's instructions. Then, the absorbance was measured at 340 nm using a microplate reader (Fluorstar Optima – BMG Labtech, Ortenberg, Germany). The values were expressed as mmol lactate/ l (BPW).

Extracellular polysaccharides (EPS) production

Dentin samples containing biofilm were transferred to pre-weighted microtubes containing 1 ml of saline solution (0.89% NaCl) and sonicated for 15 s at 20 W (Unique, Indaiatuba, Brazil). The cleaned dentin samples were removed, the tubes were weighed again and the biofilm weight was calculated from the weight differences. For soluble EPS, 900 µl of the saline solution obtained above were centrifuged at 10,000 g and 4°C for 10 min. The supernatants were transferred to other microtubes. The microtubes with the sediments were stored for the insoluble EPS analysis. Three volumes of 95% ice-cold ethanol were added into microtubes containing supernatant (soluble EPS) and stored at -20°C for 30 min. After precipitation, the microtubes were centrifuged at 10,000 g and 4°C for 10 min, and the supernatant was completely removed. The pellets were resuspended in 1 M NaOH (v = 200 µl), and the total carbohydrates were measured using the phenol-sulfuric acid colorimetric assay. A glucose (mg) curve was generated. The absorbance was measured at 490 nm using a microplate reader (Fluorstar Optima – BMG Labtech, Ortenberg, Germany).

For the insoluble EPS analysis, the sediments previously obtained were resuspended in 400 µl of 1 M NaOH, vortexed for 15 s and agitated using a shaker table for 15 min at room temperature. The samples were then centrifuged at 10,000 g and 4°C for 10 min. The supernatants were transferred to new microtubes (insoluble EPS), and three volumes of 95% ice-cold ethanol were added. The microtubes were stored at -20°C for 30 min. After precipitation, the microtubes were centrifuged at

10,000 g and 4°C for 10 min, and the supernatant was completely removed. The remaining pellet in the microtube was resuspended in 1 M NaOH (v = 200 µl), and the total carbohydrates were measured using the phenol-sulfuric acid colorimetric assay as described above [21]. The values were expressed as µg EPS/g (biofilm).

Demineralization analysis: Transverse microradiography (TMR)

After cleaning, all dentin samples (except those from the lactic acid assay) were transversally sectioned and polished to obtain slices with 100-120 µm thickness. The dentin slices were fixed in a sample-holder together with an aluminum calibration step wedge with 14 steps. A microradiograph was taken using an X-ray generator (Softex, Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates were developed for 4 min, rinsed in deionized water, fixed for 7 min in a dark environment, then rinsed in running water for 10 min and air dried (all procedures were performed at 20°C). The developed plate was analyzed using a transmitted light microscope fitted with a 20× objective (Zeiss, Oberkochen, Baden-Württemberg, Germany), a charge-coupled device camera (CCD, Canon, Tokyo, Japan) and a computer. Two images per sample were obtained using data acquisition (version 2012) and interpreted using calculation (version 2006) software from Inspektor Research System (Amsterdam, Netherlands). The mineral content was calculated based on the work of Angmar et al. [22], assuming 50 vol% of mineral content for the sound dentin and that the lesion depth ends when dentin presents 95% of the mineral content (about 47.5%). The integrated mineral loss (ΔZ, vol% µm), lesion depth (LD, µm) and the average mineral loss over the lesion depth (R, vol%) were calculated.

Statistical analysis

All experiments (1 – viability by CLSM, 2 – CFU counting and EPS, 3 – lactic acid assays) were performed in triplicate with four data points for each replicate. Dentin samples from the experiments 1 and 2 were used for TMR analysis (n= 24). Data were statistically analyzed using software GraphPad InStat for Windows (GraphPad Software, San Diego, USA). The normal distribution and homogeneity were checked using the Kolmogorov–Smirnov test and Bartlett test, respectively. The percentages of live and dead microorganisms and the TMR data were compared using analysis of variance (ANOVA) followed by Tukey-Kramer test. For the lactic acid production we applied ANOVA only. For the CFU counting (Lactobacillus sp. and total microorganisms) and insoluble EPS production, Kruskal-Wallis test followed by Dunn test were applied. For CFU counting (total streptococci and *mutans streptococci*) and soluble EPS production we applied Kruskal-Wallis test only. The level of significance was set at 5%.

Results

All treatments significantly reduced the biofilm viability; i.e. the percentage of live (green) microorganisms (Figure 1, $p<0.0001$). CHX had the best effect on the reduction of biofilm viability, differing from the fluorides significantly, which in turn did not differ from each other. Figure 2 shows representative CLSM images of the microcosm biofilms, evidencing the different antimicrobial effects of the treatments.

Despite the numbers of total microorganisms were lower for some treatments groups, none of them were able to significantly reduce the CFU counting for total microorganism, and neither for total streptococci and *mutans streptococci* compared to placebo and control. The exception was NaF, which significantly reduced the number of Lactobacillus sp. compared to control ($p=0.0154$, Table 1).

No treatment was able to decrease the lactic acid production (Table 2) neither extracellular polysaccharides synthesis (Table 3), except CHX that significantly reduced the insoluble EPS amount ($p=0.0012$).

All fluorides were able to significantly reduce dentin demineralization (ΔZ, LD and R values), but TiF_4 had the best effect in reducing mineral loss and lesion depth. CHX had no effect in reducing dentin demineralization compared to placebo and control (Table 4, $p<0.0001$). Figure 3 shows representative TMR images of dentin lesions from each group.

Discussion

A recent study of our group has shown no antimicrobial effect of TiF_4 varnish, but a significant ability to reduce carious lesions development in enamel under a microcosm biofilm model [9]; however, there is lack information about the effect of this experimental varnish on dentin lesions. Considering the increase prevalence of RCLs in elderly [10,11,23], all efforts to find a good alternative to avoid the development of those lesions are extremely relevant. Accordingly, our study showed no antimicrobial effect of TiF_4, allowing accepting the null hypothesis 1, since TiF_4 did not differ from NaF and CHX. On the other hand, we rejected the null hypothesis 2 since TiF_4 varnish had the best anti-caries effect under this experimental model.

In order to obtain the information about a possible antimicrobial effect of TiF_4 varnish, we applied microcosm biofilm model, which is the closest *in vitro* biofilm model to the *in vivo* situation [24]. It was produced from the inoculum of human saliva, which contains hundreds of microorganisms species [25]. Furthermore, our model allowed checking not only the effect on the biofilm, but also on the tooth.

On dentin, all treatments (fluorides and CHX) were able to significantly reduce the number of viable microorganisms in biofilm compared to control (CLSM results). However, only CHX had a significant effect on enamel [9]. It is likely that more Ti and F remained on dentin compared to enamel surface, which might be due to the organic content of dentin that could allow a better retention and

effect of the treatments. This hypothesis should be proved in the future. Other important point to take into account is that the experiment with enamel was performed for 14 days, which could be contributed to the missing effect of fluorides.

On the other hand, the antimicrobial effect was not evident by using CFU counting, in agreement with the enamel results [9]. Despite the numbers of total microorganisms were lower for some treatments groups, none of them were able to significantly reduce the CFU counting compared to control. This result is contradictory compared to CLSM images and could be due to the CFU method. For CFU counting, the microorganisms are allowed to grow for further 48h in a new and favorable environment, without any treatment, which could have favored their recovery. Other important point to be further analyzed is the microbiome profile of this type of biofilm, since other species not tested in this study might be part of the biofilm [26,27]. Compared to enamel [9], the CFU numbers were generally lower in the biofilm formed on dentin, which could be justified by the period of experiment.

An interesting result was that NaF significantly reduced the number of Lactobacillus sp., similarly to other studies [28-31]. Fluoride may penetrate into the bacteria and inhibit enzymes (such as enolase), reducing energy supply and damaging the cell [29,30,32,33]. Lactobacillus sp. has shown to better grow in multispecies biofilm, turning the most prevalent bacteria under such models [34]. On the other hand, *mutans streptococci* have acquired resistance to fluoride due to its constant use in the last decades, leading these bacteria to create mechanisms of adaptation [35], justifying the findings of the present study.

Which respect to the biofilm metabolism, none of the treatments was able to reduce the lactic acid and EPS production, except CHX on insoluble EPS. Our results were different from those found in previous works, however, the authors applied CHX as rinse daily or immediately after microcosm biofilm on enamel or hydroxyapatite or on the biofilm produced *in vivo* [36-38]. It was previously observed that CHX reduced lactic acid production after a short period of time only, not being effective after a long periods [36,39]. CHX, applied as 0.2% rinse twice a day during 10 days on dental plaque, reduced acid production after two days but after 1 month without its use the acid production returned to the baseline values [36]. The use of 40% chlorhexidine varnish every two days for one week was able to reduce lactic acid production only after two weeks, from the third week to ninth CHX did not show more effect on acid production compared to the baseline [39]. Martins et al. [40] found reduction of soluble and insoluble EPS production after daily treatment with CHX using monospecies biofilm, but nothing is known about microcosm biofilm. It is likely that CHX, applied only once before microcosm biofilm formation, has no enough substantivity to interfere inside the cells, but only on their membrane, reducing viability and the production of insoluble EPS, but not of lactic acid.

With respect to fluoride, previous works have found reduction in lactic acid and EPS production under microcosm biofilm [37], monospecies biofilm [41] and multispecies biofilm [42,43]. However, fluoride was daily applied as solution or toothpaste and it was applied during or

immediately after biofilm formation. Maybe fluoride do not have a good effect if applied only once before the microcosm biofilm formation, which one is more aggressive.

Differently from the results of the microbiological assays, fluorides were able to reduce the development of carious lesions in dentin, in agreement with enamel [9]. The anti-caries mechanism is in fact not related to the biofilm, but to the interaction of the fluorides with the tooth surface, producing layers rich in CaF_2 (in case of NaF) and in TiO_2, $Ti_3(PO4)_4.H_2O$ and CaF_2 (in case of TiF_4), which are acid resistant, reducing demineralization. The glaze like-layer produced by TiF_4 is known to be more acid resistant than those produced by NaF [8], justifying the best effect of TiF_4 in the present study.

Despite some antimicrobial effect was found for CHX, it had no anti-caries effect on dentin in agreement with Göstemeyer et al. [44] under monospecies biofilm. The anti-caries effect was found only when CHX was applied daily in microcosm biofilm [38]. A systematic review highlighted that the anti-caries effect of CHX is inconclusive [45].

The result of the present study is very promising when thinking about the prevalence of RCLs in elderly. The protective effect of TiF_4 on dentin needs to be further confirmed under *in situ* models as done by Comar et al. [3]. The authors found that 4% TiF_4 varnish was the only treatment able to improve enamel remineralization regardless of the cariogenic activity, while NaF varnish failed in preventing further demineralization under high cariogenic activity [3].

Conclusions

In conclusion, TiF_4 varnish had no relevant antimicrobial effect, but it was able to reduce the development of carious lesions in dentin under this model.

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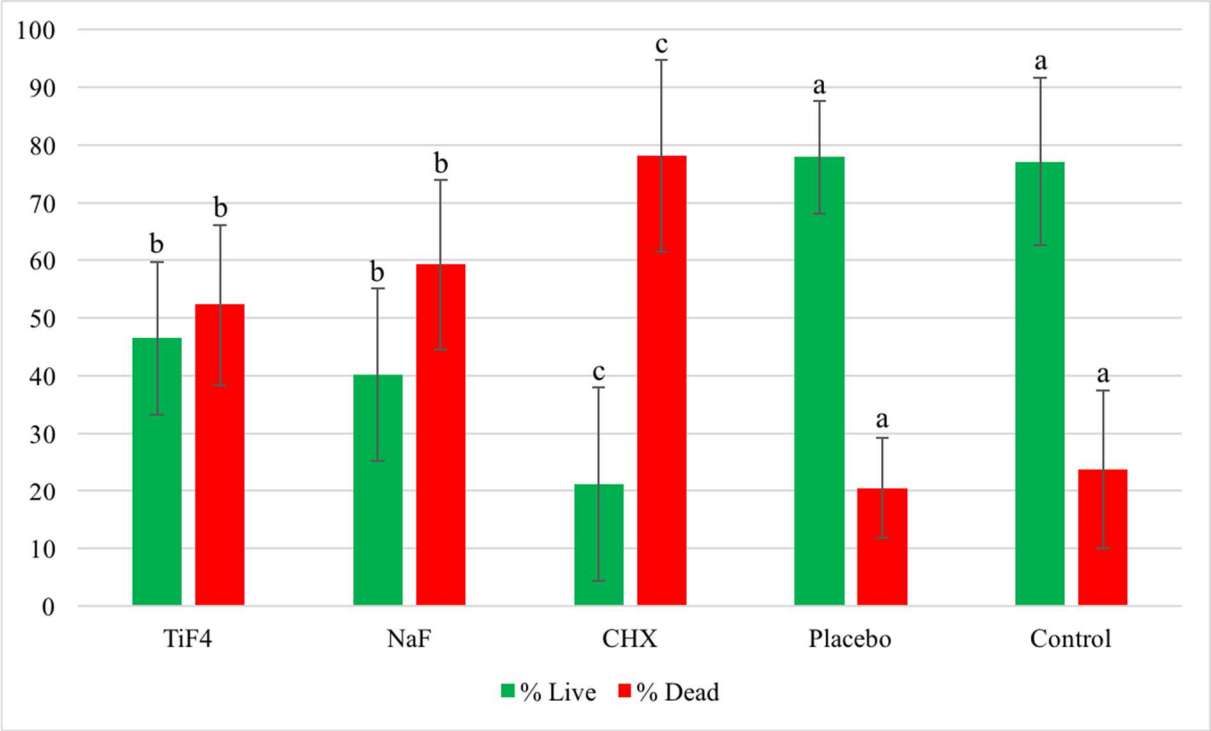

Figure 1. Mean and standard deviation of the percentage of live and dead bacteria. Different letters show significant differences among the treatments. ANOVA and Tukey-Kramer test ($p<0.0001$)

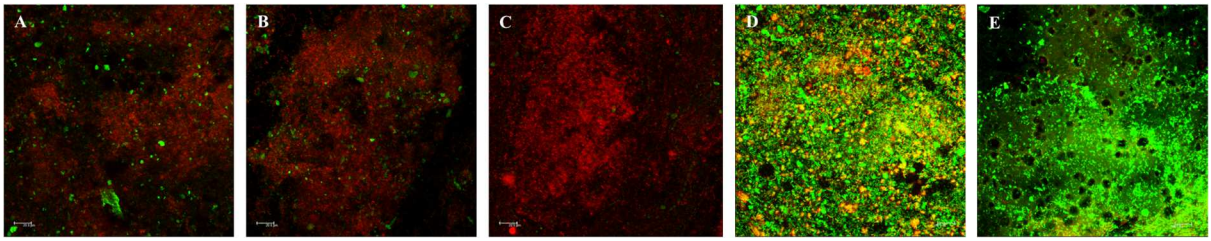

Figure 2. Representative CLSM images of the microcosm biofilms under different treatments: A) TiF_4 B) NaF C) CHX D) Placebo E) Control

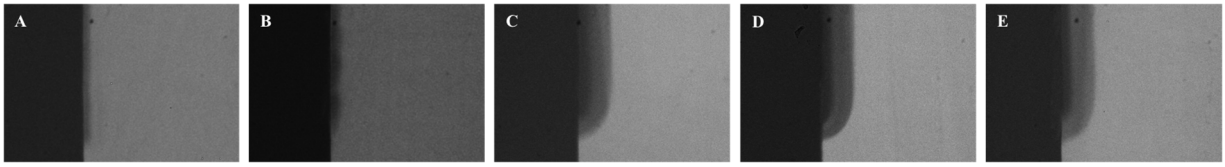

Figure 3. Representative TMR images of artificial dentin carious lesions under different treatments: A) TiF_4 B) NaF C) CHX D) Placebo E) Control

Table 1. Median (interquartile interval) of colony forming unit (CFU) counting (log_{10} CFU/ml) for total microorganisms, total streptococci, *mutans streptococci* and Lactobacillus sp.

Treatments	Total microorganisms	Total streptococci	*Mutans streptococci*	Lactobacillus sp.
TiF_4	5.72 (0.32)[a]	4.90 (0.37)[a]	5.40 (0.44)[a]	5.38 (0.32)[ab]
NaF	4.91 (0.55)[a]	4.94 (0.66)[a]	4.33 (0.52)[a]	3.95 (0.24)[a]
CHX	5.22 (0.46)[a]	5.21 (0.34)[a]	4.34 (0.82)[a]	4.71 (1.84)[ab]
Placebo	5.57 (0.73)[a]	4.88 (0.50)[a]	4.98 (0.74)[a]	4.27 (0.27)[ab]
Control	5.67 (0.16)[a]	4.83 (0.25)[a]	4.97 (0.35)[a]	5.50 (0.48)[b]

Similar letters in the same column show no significant differences among the treatments and different letters in the same column show significant differences among the treatments. Total microorganisms and Lactobacillus sp. (Kruskal-Wallis and Dunn test, p=0.0154 and p=0.0104, respectively); total streptococci and *mutans streptococci* (Kruskal-Wallis, p=0.6651 and p=0.1009, respectively).

Table 2. Mean and standard deviation of the amount of lactic acid produced by the microcosm biofilms under different treatments

Treatments	**Lactic acid production (mmol/l)**
TiF_4	0.41 ± 0.21^a
NaF	0.64 ± 0.31^a
CHX	0.89 ± 0.53^a
Placebo	0.74 ± 0.42^a
Control	0.45 ± 0.29^a

Similar letters show no significant differences among the treatments. ANOVA (p=0.0658).

Table 3. Median (interquartile interval) of soluble and insoluble EPS produced by the microcosm biofilms under different treatments

Treatments	Soluble EPS (µg/g)	Insoluble EPS (µg/g)
TiF_4	5.73 (4.78)[a]	3.32 (1.97)[ab]
NaF	3.38 (5.11)[a]	3.77 (3.77)[ab]
CHX	7.70 (14.17)[a]	1.51 (1.56)[a]
Placebo	10.49 (12.41)[a]	4.84 (3.13)[b]
Control	8.73 (4.99)[a]	4.40 (3.20)[b]

Similar letters in the same column show no significant differences among the treatments and different letters in the same column show significant differences among the treatments. Soluble EPS (Kruskal-Wallis, p=0.0732) and insoluble EPS (Kruskal-Wallis and Dunn test, p=0.0012).

Table 4. Mean and standard deviation of the integrated mineral loss (ΔZ, vol%. μm), lesion depth (LD, μm) and the average mineral loss (R, vol%) presented by the demineralized dentin samples

Treatments	ΔZ (vol%.μm)	LD (μm)	R (vol%)
TiF_4	1131±506[a]	68.8±27.2[a]	16.03±2.51[a]
NaF	2060±541[b]	93.3±16.9[b]	20.49±3.08[b]
CHX	3682±726[c]	120.9±17.2[c]	31.31±2.33[c]
Placebo	3700±861[c]	126.7±22.6[c]	29.33±2.92[c]
Control	3827±810[c]	126.3±17.5[c]	28.76±4.06[c]

Different letters in the same column show significant differences among the treatments. ANOVA and Tukey-Kramer test ($p<0.0001$).

3 DISCUSSION

3 DISCUSSION

Microcosm biofilm is the closest *in vitro* biofilm model to the *in vivo* situation (TANG et al., 2003), which can be produced using the inoculum from human saliva or dental biofilm, which contain lot of microorganisms' species (SISSONS, 1997). Despite the benefits of the microcosm biofilm (TANG et al., 2003; SALLI; OUWEHAND, 2015), most of the studies have applied this model to produce enamel carious lesions only (PRATTEN; WILSON, 1999; AZEVEDO et al., 2014; MASKE et al., 2016), highlighting the need of further studies on dentin. Researchers often choose one model to supply nutrients to the microorganisms for the microcosm biofilm formation, mostly though the use of multiple well plates (an example of static model) (SALLI; OUWEHAND, 2015; SIM; DASHPER; REYNOLDS, 2016; MASKE et al., 2017). No attention has been given to compare the impact of the type of model for providing nutrients (dynamic or static model) on the microcosm biofilm viability and on carious lesion formation in dentin.

In the first paper of this dissertation, the microcosm biofilm produced under semi-dynamic model presented superior viability compared to those produced under the static model, which might be due to the continuous flow of nutrients 10 h per day, allowing more microbial growth, and/or washing the metabolites produced by the microorganisms that could be dangerous to themselves. On the other hand, this model produced less aggressive dentin carious lesions, probably due to the washing of the acids produced by the microorganisms. The continuous flow was simulated only during 10 h, since some periods of flow absence simulate the nighttime, where salivary flow is close to zero (PROCTOR, 2016). No atmosphere control was provided during 10 h of continuous flow, due to the limitation of our artificial mouth system, which might have favored the growth of aerobic and facultative bacteria, which in turn could justify the production of less demineralized lesions.

Other interesting result was the higher number of typical subsurface lesions produced by the static compared to the semi-dynamic model. In agreement, Owens et al. (2017) showed that semi-dynamic biofilm model induced less evident subsurface layer, while Arthur et al. (2013) found well-defined subsurface lesions for the static model. The continuous flow in semi-dynamic model may have washed

away free minerals from biofilm, reducing their availability to precipitate on the dentin surface.

Therefore, the 1st null hypothesis can be rejected; both models are able to differently produce viable cariogenic biofilm and dentin carious lesions. Further studies shall give attention to the microbiome profile of biofilms produced by both methods.

For the measurement of dental carious lesions, TMR is considered the gold standard method, but it presents some disadvantages (mostly related to the samples preparation) that have prompted the researchers to look for new methods such as micro-CT. Micro-CT can be applied *in vivo* (LO; ZHI; ITTHAGARUN, 2010; HAMBA et al., 2012; RIBEIRO et al., 2015) since is a non-destructive method and offers the complete information about the full volume of the sample. On the other hand, micro-CT requires long measurements of the data with good quality and its software is not suitable for some analysis comparable to the current TMR standard (LO; ZHI; ITTHAGARUN, 2010).

In the second paper of this dissertation, TMR and micro-CT were correlated, showing similar results when applied to compare the effect of different treatments (fluoride and CHX) against dentin demineralization. The only exception was the lesion depth (LD) analyzed by TMR that could not differentiate NaF varnish from the control, which might be justified by the low sample number and the high standard deviation. Micro-CT and TMR also showed a positive and strong correlation when compared to measure mineral loss in accordance with the study of Hamba et al. (2012). Despite Hamba et al. (2012) analyzed enamel; they applied the same samples for both analyses (micro-CT and TMR), using similar filters associated with beam hardening correction (BHC), as done in our micro-CT analysis. Filters combination may reduce artifacts (MEGANCK et al., 2009), as well as BHC may help in obtaining homogeneity in gray scale values (NEVES et al., 2010). However, the correlation of LD data found by Hamba et al. (2012) was different from the present study, which could be justified by the type of dental substrate as well as the parameter applied to define the end of the lesion.

Jeon et al. (2007) found a low correlation between TMR and micro-CT with respect to mineral loss, differently from the study of Hamba et al. (2012), which might

be justified by the lesser demineralized enamel lesions produced by them *in vitro*. The authors justified the low correlation due to the absence of a reliable calibration curve in the TMR analysis for early-demineralized lesions, while the micro-CT presents a correct calibration for such type of lesion. Furthermore, the authors did not use similar filter as Hamba et al. (2012), which might have also influenced their results.

The only study done in dentin, using micro-CT and TMR, applied an abiotic model to induce carious lesions (LO; ZHI; ITTHAGARUN, 2010). Despite the authors did not statistically correlate the TMR and micro-CT data, Lo, Zhi and Itthagarun (2010) concluded that changes of similar magnitude were detected by both methods after dentin remineralization, making micro-CT suitable to measure dentin carious lesions.

Therefore, the 2nd null hypothesis can be accepted, since micro-CT and TMR has similar ability to measure dentin mineral loss and lesion depth.

Considering the increase prevalence RCLs in elderly (GRIFFIN et al., 2004; SAUNDERS; MEYEROWITZ, 2005; HAYES; BURKE; ALLEN, 2017), all attempt to find a good alternative to prevent the development of those lesions is extremely important. Fluoride (NaF) varnish has shown good effect on the prevention of RCLs (MAGALHÃES, 2017). Accordingly, our research group recently demonstrated that TiF_4 varnish had significant ability to reduce carious lesions development in enamel under a microcosm biofilm model, but it did not have relevant antimicrobial effect (SOUZA et al., 2018). However, there is lack information about the effect of the experimental TiF_4 varnish on dentin lesions under biofilm model.

In the third paper of this dissertation, all treatments (fluorides and CHX) were able to significantly reduce the number of viable microorganisms in microcosm biofilm formed on dentin compared to control (CLSM results). On the other hand, only CHX had a significant effect on enamel (SOUZA et al., 2018). It is likely that more Ti and F remained on dentin surface compared to enamel surface, due to the organic content of the former one. Other important point to be considered is that the experiment with enamel was performed during 14 days, which could have contributed to the missing effect of fluorides on microorganisms' viability.

However, the antimicrobial effect on dentin biofilm was not evident by using CFU counting, in agreement with the enamel results (SOUZA et al., 2018). No treatment was able to significantly reduce the CFU counting (for total microorganism, total streptococci, *mutans streptococci* and Lactobacillus sp.) compared to control. This result is contradictory when compared to CLSM images and could be due to the CFU method, where the microorganisms grow for further 48 h in a new and favorable environment, which could have allowed their recovery.

An interesting result was that NaF significantly reduced the number of Lactobacillus sp., similarly to other studies (XIE; LI; ZHOU, 2008; ARTHUR et al., 2013; MEI et al., 2013; EVANS et al., 2015). Fluoride may penetrate into the bacteria and inhibit enzymes related to the metabolism, damaging the cell (MARQUIS; CLOCK; MOTA-MEIRA, 2003; KOO, 2008; ARTHUR et al., 2013; MEI et al., 2013). Lactobacillus sp. has shown to better grow in multispecies biofilm compared to other microorganisms, being the most prevalent bacteria under such models (YU et al., 2017). On the other hand, *mutans streptococci* have acquired resistance to fluoride due to its constant use in the last decades, leading the bacteria to create mechanisms of adaptation (LIAO et al., 2017), justifying the lack effect of fluorides on this specie.

With respect to biofilm metabolism, no treatment was able to reduce the lactic acid and EPS production, except CHX on insoluble EPS in dentin biofilm. Our results were different from those found in previous works about CHX (GEORGIOS; VASSILIKI; SOTIRIOS, 2015; FERNANDEZ Y MOSTAJO et al., 2017; BRAGA; PIRES; MAGALHÃES, 2018). However, CHX has been daily applied as rinse (GEORGIOS; VASSILIKI; SOTIRIOS, 2015; BRAGA; PIRES; MAGALHÃES, 2018) or applied immediately after microcosm biofilm formation (FERNANDEZ Y MOSTAJO et al., 2017) on enamel (BRAGA; PIRES; MAGALHÃES, 2018) or hydroxyapatite samples (FERNANDEZ Y MOSTAJO et al., 2017) or it has been tested under *in vivo* model (GEORGIOS; VASSILIKI; SOTIRIOS, 2015). It was previously observed that CHX is able to reduce lactic acid production after a short period of time as well, but it is not effective after long periods *in vivo* (GERARDU et al., 2007; GEORGIOS; VASSILIKI; SOTIRIOS, 2015).

Martins et al. (2018) found reduction of soluble and insoluble EPS production after daily treatment with CHX using monospecies biofilm, but nothing is known about the effect of a unique application under microcosm biofilm. It is likely that CHX, applied only once before microcosm biofilm formation, has no enough substantivity to interfere inside the cells, but only on their membrane affecting EPS production only. Further studies shall better elucidate why CHX acts on insoluble EPS, but not on soluble EPS under the present experimental model.

With respect to fluoride, previous works have found reduction in lactic acid and EPS production under microcosm biofilm (FERNANDEZ Y MOSTAJO et al., 2017), monospecies biofilm (DENG; VAN LOVEREN; TEN CATE, 2005) and multispecies biofilm (CHENG et al., 2017; THURNHEER; BELIBASAKIS, 2018). However, fluoride was daily applied as rinse (DENG; VAN LOVEREN; TEN CATE, 2005; FERNANDEZ Y MOSTAJO et al., 2017; THURNHEER; BELIBASAKIS, 2018) or toothpaste (CHENG et al., 2017) or it was applied during (DENG; VAN LOVEREN; TEN CATE, 2005; CHENG et al., 2017; THURNHEER; BELIBASAKIS, 2018) or immediately after biofilm formation (FERNANDEZ Y MOSTAJO et al., 2017). As for CHX, fluoride might have not satisfatory metabolic effect on bacteria if applied only once before microcosm biofilm formation.

On the other hand, fluorides were able to reduce the development of carious lesions in dentin, in agreement with enamel study (SOUZA et al., 2018). The anti-caries mechanism of fluorides is not related to any relevant antimicrobial action, but to their interaction with the tooth surface before biofilm formation, producing an acid resistant layer able to reduce mineral loss. The glaze like-layer produced by TiF_4 [TiO_2, $Ti_3(PO_4)_4.H_2O$ and CaF_2] is known to be more acid resistant than those (CaF_2 globules) produced by NaF (COMAR et al., 2017a) and our 3rd study was able to show such differences using TMR, which was not so evident in the 2nd study due to smaller sample number. Despite the result was not statistically significant in the 2nd article, TiF_4 was numerically better than NaF (TiF_4: 72% reduction of ΔZ versus NaF: 42%).

Finally, the last study showed no relevant antimicrobial effect of TiF_4, allowing us to accept the null hypothesis 3a. On the other hand, we rejected the null hypothesis 3b, since TiF_4 varnish had the best anti-caries effect under this

experimental model. Further *in situ* studies are required to confirm the effect of TiF_4 varnish on the control of RCLs, using both TMR and micro-CT analysis as response variable. The inclusion of micro-CT analysis shall help planning future clinical trials on this field with elderly presenting RCLs.

4 CONCLUSION

4 CONCLUSION

Study 1: The nutrient's supply model may have influence on the microcosm biofilm viability and on the profile of dentin carious lesions;

Study 2: Micro-CT may be a suitable non-destructive method to measure dentin demineralization;

Study 3: TiF_4 varnish has no relevant antimicrobial effect, however, it is the best option to reduce the development of dentin carious lesions under microcosm biofilm model.

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APPENDIXES

APPENDIX A – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN DISSERTATION

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION

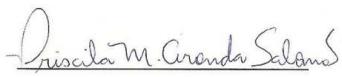
We hereby declare that we are aware that the article **"Comparison between static and semi-dynamic models for microcosm biofilm formation on dentin"**, included in **Dissertation** of the student **Daiana Moreli Soares dos Santos**, was not used and will not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 23/08/2018.

Daiana Moreli Soares dos Santos

Juliana Gonçalves Pires

Aline Silva Braga



Priscila Maria Aranda Salomão

Ana Carolina Magalhães

APPENDIX B – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN DISSERTATION

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION

We hereby declare that we are aware that the article **"Micro-CT versus TMR analysis of dentine treated with different fluorides and demineralized under a microcosm biofilm model"**, included in **Dissertation** of the student **Daiana Moreli Soares dos Santos**, was not used and will not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 23/08/2018.

Daiana Moreli Soares dos Santos

Aline Silva Braga

Marta Rizk

Annette Wiegand

Ana Carolina Magalhães

APPENDIX C – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN DISSERTATION

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION

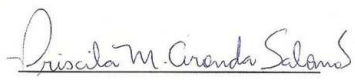
We hereby declare that we are aware that the article **"Antimicrobial and anti-caries effects of 4% Titanium Tetrafluoride varnish under a microcosm biofilm model on dentin"**, included in **Dissertation** of the student **Daiana Moreli Soares dos Santos**, was not used and will not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, 23/08/2018.

Daiana Moreli Soares dos Santos

Juliana Gonçalves Pires

Aline Silva Braga



Priscila Maria Aranda Salomão

Marília Afonso Rabelo Buzalaf

Ana Carolina Magalhães

ANNEXES

ANNEX A – APPROVAL OF THE ETHICS COMMITTEE IN RESEARCH ONHUMAN BEINGS

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação do efeito antimicrobiano do verniz de tetrafluoreto de titânio a 4% utilizando modelo de biofilme microcosmo cariogênico em dentina

Pesquisador: Daiana Moreli Soares dos Santos

Área Temática:

Versão: 4

CAAE: 58330616.7.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.940.206

Apresentação do Projeto:

Idem ao Parecer Consubstanciado nº 1.929.043 de 15/02/2017.

Objetivo da Pesquisa:

Idem ao Parecer Consubstanciado nº 1.929.043 de 15/02/2017.

Avaliação dos Riscos e Benefícios:

Idem ao Parecer Consubstanciado nº 1.929.043 de 15/02/2017.

Comentários e Considerações sobre a Pesquisa:

Idem ao Parecer Consubstanciado nº 1.929.043 de 15/02/2017.

Considerações sobre os Termos de apresentação obrigatória:

Idem ao Parecer Consubstanciado nº 1.929.043 de 15/02/2017.

Conclusões ou Pendências e Lista de Inadequações:

Referido projeto de pesquisa fora considerado com pendência em reunião deste CEP realizada no dia 08/02/2017, pela ausência do comprovante do registro da pesquisa junto à CEEPA (Comissão de Ética no Ensino e Pesquisa em Animais), pelo fato de utilização de dentes bovinos no estudo.

A pesquisadora responsável anexou tal comprovante e sou de parecer favorável à aprovação da pesquisa.

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Continuação do Parecer: 1.940.206

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO ad referendum por este CEP, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_746241.pdf	20/02/2017 16:14:03		Aceito
Outros	Aprovacao_comite_animal.pdf	20/02/2017 16:12:53	Daiana Moreli Soares dos Santos	Aceito
Outros	Oficio_pendencia_3.pdf	20/02/2017 16:11:13	Daiana Moreli Soares dos Santos	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_modificado_2.pdf	18/11/2016 08:25:45	Daiana Moreli Soares dos Santos	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_modificado.pdf	19/09/2016 22:57:13	Daiana Moreli Soares dos Santos	Aceito
Folha de Rosto	folha_de_rosto.pdf	28/07/2016 08:21:52	Daiana Moreli Soares dos Santos	Aceito
Outros	declaracao.pdf	19/07/2016 17:47:16	Daiana Moreli Soares dos Santos	Aceito
Declaração de Pesquisadores	declaracao_de_compromisso.pdf	19/07/2016 17:37:53	Daiana Moreli Soares dos Santos	Aceito
Outros	questionario_tecnico.pdf	19/07/2016 17:35:49	Daiana Moreli Soares dos Santos	Aceito
Declaração de Instituição e Infraestrutura	carta_encaminhamento.pdf	19/07/2016 17:09:03	Daiana Moreli Soares dos Santos	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Continuação do Parecer: 1.940.206

BAURU, 23 de Fevereiro de 2017

Assinado por:
Ana Lúcia Pompéia Fraga de Almeida
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

ANNEX B – APPROVAL OF THE ETHICS COMMITTEE ON THE USE OF ANIMALS

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

Uso exclusivo da CEEPA/FOB/USP
Registro número: **006/2016**
Recebido em: 20/12/2016
Maristela Petenuci Ferrari
Secretária da CEEPA – SRTE 53052

REGISTRO DE PESQUISA E/OU ENSINO, COM UTILIZAÇÃO DE CADÁVERES DE ANIMAIS, OU PARTE DELES

Finalidade:	Pesquisa
Período:	Abril/2016 à Abril/2018
Título da pesquisa:	Avaliação do efeito antimicrobiano do verniz de tetrafluoreto de titânio a 4% utilizando modelo de biofilme microcosmo cariogênico em dentina
Pesquisador Responsável:	Profa. Dra. Ana Carolina Magalhães
Pesquisador Executor:	Daiana Moreli Soares dos Santos
Colaboradores:	Priscila Maria Aranda Salomão
Dados Nota Fiscal/ Termo de Doação:	Frigol S/A (Doação)
Quantidade de Dentes Bovinos:	200 (utilizados na pesquisa = 90 dentes/5 grupos)

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
e-mail: ceepa@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br

ANNEX C – ACCEPTANCE OF THE ARTICLE IN JOURNAL OF APPLIED ORAL SCIENCE

Gmail **Daiana Moreli <daianamoreli@gmail.com>**

Journal of Applied Oral Science - Decision on Manuscript ID JAOS-2018-0163.R1

Vanessa Lara <onbehalfof@manuscriptcentral.com> 13 de julho de 2018 23:34
Responder a: vanessa@fob.usp.br
Para: daianamoreli@gmail.com, jugpires@gmail.com, aline_s.braga@hotmail.com, pri_barrabonita@yahoo.com.br, acm@usp.br

13-Jul-2018

Dear Dr. Magalhães,

It is a pleasure to accept your manuscript entitled "Comparison between static and semi-dynamic models for microcosm biofilm formation on dentin" in its current form for publication in the Journal of Applied Oral Science. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Journal of Applied Oral Science, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Vanessa Lara
Co-Editor-in-Chief, Journal of Applied Oral Science
vanessa@fob.usp.br

ANNEX D – SUBMISSION OF THE ARTICLE TO EUROPEAN JOURNAL OF ORAL SCIENCES

Gmail **Daiana Moreli <daianamoreli@gmail.com>**

Fwd: European Journal of Oral Sciences - EOS-9904-OA-18

Ana Carolina Magalhães <acm@usp.br> 24 de agosto de 2018 10:10
Para: Daiana Moreli <daianamoreli@gmail.com>

--------- Mensagem encaminhada ---------
De: **European Journal of Oral Sciences** <onbehalfof@manuscriptcentral.com>
Data: ter, 7 de ago de 2018 às 11:35
Assunto: European Journal of Oral Sciences - EOS-9904-OA-18
Para: <acm@usp.br>

07-Aug-2018

Dear Dr. Magalhães:

Thank you for submitting your manuscript entitled "Micro-CT versus TMR analysis of dentine treated with different fluorides and demineralized under a microcosm biofilm model" to the European Journal of Oral Sciences. It has been successfully submitted online and is presently being given full consideration.

Your manuscript ID number is EOS-9904-OA-18.

Please refer to the above manuscript ID in all future correspondence or when contacting the Editorial Office for questions. If there are any changes in your mailing address or e-mail address, please log in to ScholarOne Manuscripts at https://mc.manuscriptcentral.com/eos and edit your user information as appropriate.

We will contact you again as soon as we have the necessary information for an editorial decision. You can also view the status of your manuscript at any time by checking your Author Center after logging in to https://mc.manuscriptcentral.com/eos.

Sincerely,

Editorial Office
European Journal of Oral Sciences
--
Prof. Dr. Ana Carolina Magalhães
Discipline of Biochemistry/ Department of Biological Sciences
Alameda Octávio Pinheiro Brisolla 9-75
Bauru-SP-Brazil 17012-191
55 14 32358497

ANNEX E – SUBMISSION OF THE ARTICLE TO JOURNAL OF DENTISTRY

Gmail **Daiana Moreli <daianamoreli@gmail.com>**

Fwd: A manuscript number has been assigned

Ana Carolina Magalhães <acm@fob.usp.br> 24 de agosto de 2018 10:10
Para: Daiana Moreli <daianamoreli@gmail.com>

--------- Mensagem encaminhada ---------
De: **Journal of Dentistry** <eesserver@eesmail.elsevier.com>
Data: qui, 19 de jul de 2018 às 07:27
Assunto: A manuscript number has been assigned
Para: <acm@usp.br>, <acm@fob.usp.br>

Dear Dr. Magalhaes,

Your submission entitled "Antimicrobial and anti-caries effects of 4% Titanium Tetrafluoride varnish under a microcosm biofilm model on dentin" has been assigned the following manuscript number: JJOD-D-18-00613.

You will be able to check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is https://ees.elsevier.com/jjod/.

Thank you for submitting your work to this journal.

Kind regards,

Journal of Dentistry

--

Prof. Dr. Ana Carolina Magalhães
Biochemistry, Department of Biological Sciences
Bauru School of Dentistry-USP
Alameda Octávio Pinheiro Brisolla 9-75
17012-191, Bauru-SP