UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

PRISCILA MARIA ARANDA SALOMÃO

Effect of titanium tetrafluoride varnish on fibroblasts (NIH/3T3): viability assay, morphological analysis and cell signals for apoptotic pathways

Efeito do verniz de tetrafluoreto de titânio sobre fibroblastos (NIH/3T3): ensaios de viabilidade, morfologia e sinalização celular para apoptose

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Orientadora: Profª. Drª Ana Carolina Magalhães

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ABSTRACT

Effect of titanium tetrafluoride varnish on fibroblasts (NIH/3T3): viability assay, morphological analysis and cell signals for apoptotic pathways

Current knowledge supports the application of TiF_4 varnish to protect against tooth caries and erosion; however, it is indispensable to know its cytotoxic potential and the mechanism involved on it before applying in patients. Therefore, this study aimed to evaluate 1) The cytotoxic effect of titanium tetrafluoride (TiF_4) varnish compared with sodium fluoride (NaF) varnish on murine fibroblast (NIH/3T3), varying the fluoride concentration and time of treatment and 2) The percentage of apoptosis and its mechanism (both mitochondrial mediated by the Bcl-2 family- and death receptor-pathways) in human gingival fibroblasts (HGF) and murine fibroblasts (NIH/3T3) treated with TiF_4 varnish compared to NaF varnish for 6 h. Step 1) NIH/3T3 were exposed to NaF or TiF_4 varnishes containing 0.95, 1.95 or 2.45% F, for 6, 12 or 24 h. MTT viability (n=6) and Hoescht/PI stain assays (n=3) as well as the cells morphology (HE, only for 24 h, n=3) and stiffness (AFM, only for 2.45% F, 6 or 12 h) were analyzed. Both varnishes, at 1.90 and 2.45% F, reduced cells viability by similar extent (33-86% at 6 h, 35-93% at 12 h, and 87-98% at 24 h) compared to control, regardless of the type of fluoride. TiF_4 and NaF (2.45% F) reduced cell stiffness to a similar extent, but only TiF_4 differed from control. Step 2) HGF and NIH/3T3 were exposed to NaF or TiF_4 (2.45% F) varnishes for 6 h. Cells were examined by the TUNEL method using fluorescence microscope. The caspases-3, -8 and -9 activities were assessed. The cDNA for cytocrome c, Bax, Bad, Bcl-2, VDAC-1 and Fas-L was amplified by quantitative PCR (qPCR). Bax, Bcl-2 and Fas-L were further detected by western blot. Both fluorides similarly increased the percentage of apoptosis, while they failed in activating caspases-3, -8 and -9 for both types of cells. Bax/Bcl-2 ratio, cytochrome C and VDAC-1 gene expressions were not altered by both fluoride treatments. However, NaF varnish increased the amplification of Fas-L gene for NIH/3T3 and HGF, while TiF_4 varnish induced lower Bad/Bcl-2 ratio expression compared to control for NIH/3T3, but not for HGF. No effect of the fluorides was detected in the proteins analysis. TiF_4 and NaF have similar cytotoxicity on NIH/3T3, which is dependent on the F concentration and the exposure time. Both fluorides, at the studied conditions, similarly induce a low percentage of apoptosis, with consequent modest activation of Bcl-2 and Fas-L-dependent signaling pathways.

Keywords: Apoptosis. Toxicity. Fibroblasts. Sodium fluoride. Titanium.

RESUMO

Efeito do verniz de tetrafluoreto de titânio sobre fibroblastos (NIH/3T3): ensaios de viabilidade, morfologia e sinalização celular para apoptose

Conhecimento atual suporta a aplicação de verniz de TiF_4 para proteção contra cárie e erosão dentárias; entretanto, é indispensável conhecer o seu potencial citotóxico e o mecanismo envolvido antes de aplicá-lo em pacientes. Portanto, o objetivo deste estudo foi avaliar 1) o efeito citotóxico do verniz de tetrafluoreto de Titânio (TiF_4) comparado ao fluoreto de sódio (NaF), em fibroblastos NIH/3T3, variando a concentração de fluoreto e o tempo de tratamento 2) a porcentagem de apoptose e seus mecanismos (ambos mitocondrial mediado pela família Bcl-2 e pelo receptor de morte celular) em fibroblastos gengivais humanos (FGH) e fibroblastos murinos (NIH/3T3) tratados com verniz de TiF_4 comparado com verniz de NaF por 6 h. Etapa 1) NIH/3T3 foram expostos a vernizes de NaF e TiF_4 contendo 0,95, 1,95 ou 2,45% F, por 6, 12 ou 24 h. Ensaios de viabilidade por MTT (n=6) e Hoechst 33342/iodeto de propídeo (n=3) bem como a morfologia (HE, apenas para 24 h, n = 3) e a rigidez celular (MFA, apenas para 2,45% F, 6 ou 12 h) foram realizados. Ambos os vernizes com 1,90 e 2,45% F reduziram a viabilidade das células de forma semelhante (33-86% em 6 h, 35-93% em 12 h e 87-98% em 24 h) em comparação com o controle, independentemente do tipo de fluoreto. TiF_4 e NaF (2,45%) reduziram de forma similar a rigidez celular, mas somente TiF_4 diferiu do controle no período de 6 h. Etapa 2) FGH e NIH/3T3 foram tratadas com verniz de NaF ou TiF_4 por 6h. As células foram examinadas pelo método de TUNEL, usando microscopia de fluorescência. A atividade das caspases -3, -8 e -9 foram avaliadas. O cDNA para citocromo C, Bax, Bad, Bcl-2, VDAC-1 e Fas-L foi amplificado e quantificado por PCR em tempo real (qPCR). A expressão das proteínas Bax, Bcl-2 e Fas-L foi quantificada por western blot. Ambos os fluoretos aumentaram de forma semelhante a porcentagem de apoptose, enquanto falharam na ativação de caspases-3, -8 e -9 para ambos tipos celulares. A expressão gênica da relação Bax/Bcl-2, do citocromo C e do VDAC-1 não foram alteradas por ambos fluoretos. No entanto, o verniz NaF aumentou a amplificação do gene Fas-L para ambas as células, enquanto que o verniz TiF_4 induziu menor expressão da razão Bad/Bcl-2 em comparação com o controle para NIH/3T3, mas não para FGH. Nenhum efeito foi detectado na análise de proteínas. TiF_4 e NaF apresentam citotoxicidade similar em NIH/3T3, a qual é dependente da concentração de F e do tempo de exposição. Ambos os fluoretos, nas condições estudadas, induzem uma baixa porcentagem de apoptose, com consequente modesta ativação das vias de sinalização dependentes de Bcl-2 e Fas-L.

Palavras-chave: Apoptose. Toxicidade. Fibroblastos. Fluoreto de sódio. Titânio.

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1 INTRODUCTION

1 INTRODUCTION

NaF has been widely and successfully applied for the prevention of dental caries, but not of tooth erosion (TEN CATE, 1997; WIEGAND; ATTIN, 2003; TEN CATE, 2004; MAGALHÃES et al., 2011; PESSAN; TOUMBA; BUZALAF, 2011; CASTILHO et al., 2015; MAGALHÃES et al., 2017) and its preventive potential, when applied at high concentration, is mainly due to the formation of a calcium fluoride-like layer on tooth (SAXEGAARD; RÖLLA, 1888; OGAARD; SEPPÄ; RÖLLA, 1994; VOGEL, 2011; KOESER et al., 2014) that acts as a physical barrier as well as a source of fluoride for enamel and/or dental biofilm during acid challenges. Despite its extensive clinical uses, the effect of NaF is sometimes limited for high-risk patients (especially for patients with tooth erosion), requiring a very intensive fluoridation regime (GANSS; SCHLUETER; KLIMEK, 2007; ARRUDA et al., 2012; MARINHO et al., 2013; WEYANT et al., 2013). Therefore, current studies have focused on fluoride compounds that might have greater efficacy against tooth demineralization, for instance those containing polyvalent metal ions, such as titanium tetrafluoride (TiF_4).

The potential of TiF_4 to prevent tooth demineralization has been investigated since 1997 (BÜYÜKYILMAZ; OGAARD; RØLLA, 1997). Its protective effect is related to the formation of an acid-resistant surface coating and an increased fluoride uptake (COMAR et al., 2017a). The coating is rich in hydrated hydrogen titanium phosphate and titanium oxide, which might act as a diffusion barrier (BÜYÜKYILMAZ; OGAARD; RØLLA, 1997; ACRA et al., 2012; LUSSI; CARVALHO, 2015; COMAR et al., 2017a). The efficacy of TiF_4 is highly dependent on its low pH (WIEGAND et al., 2009; HOVE et al., 2011) and the type of vehicle applied (COMAR et al., 2012). It has been shown that TiF_4 varnish is more efficient than a TiF_4 solution (MAGALHÃES et al., 2008a; MAGALHÃES et al., 2008b; COMAR et al., 2012) and NaF varnish (MAGALHÃES et al., 2008a; MAGALHÃES et al., 2008b; COMAR et al., 2012; COMAR et al., 2015; MAGALHÃES et al., 2016; COMAR et al., 2017b; MARTINES DE SOUZA et al., 2017) against tooth demineralization. Furthermore, fluoride varnish, but not the solution, is the first choice for young children, since it is low absorbed by the gastrointestinal system, and, therefore, it presents a low risk of undesired systemic effects, caused by excessive fluoride intake, such as dental or

skeletal fluorosis (EKSTRAND; KOCH; PETERSSON, 1980; PESSAN et al., 2005; OLYMPIO et al., 2009;).

With respect to the influence of the pH, previous studies have shown that TiF_4 (0.5 M F) could significantly reduce enamel erosion at its native pH (pH 1.2), but not at a pH buffered to 2.1 (HOVE et al., 2011) or 3.5 (WIEGAND et al., 2009). However, the low pH of TiF_4 might impair its clinical use, because of possible adverse side effects such as desquamation and/or astringent effect on the mucosa. A scanning electron microscopy study has demonstrated that 1% TiF_4 solution has cytotoxic effects on L929 fibroblasts (SEN; KAZEMI; SPANGBERG, 1998). Our research group has recently compared the cytotoxic effect of NaF and TiF_4 solutions on murine fibroblasts (NIH/3T3) and both fluorides (0.95 – 2.45% F) showed similar cytotoxic potential (SALOMÃO et al., 2017). However, there is no information about the cytotoxic potential of TiF_4 varnish.

Considering that the current knowledge supports the execution of clinical trials to test the protective effect of TiF_4 varnish (but not of the solution) against tooth caries and erosion, it is indispensable to know its cytotoxic potential and the mechanism involved on it before applying in patients.

One of the mechanisms involved in cells death is apoptosis, which is a genetically “programmed” cell death (ELMORE, 2007), resulting in chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (KRISKO et al., 2008). The apoptosis can occur via intrinsic or mitochondrial mediated by Bcl-2 family or via extrinsic, also known as death-receptor pathway (Fas-L). The Bcl-2 family controls the intrinsic apoptotic pathway, regulating the release of molecules from mitochondrial to cytosol, followed by caspases -9 and -3 activation (AGALAKOVA; GUSEV, 2012; SHOSHAN-BARMATZ; DE; MEIR, 2017). The extrinsic or death-receptor pathway involves the action of Fas-L on cells membrane, a ligand of death receptor able to induce a sequence of apoptotic events such as the caspases -8 and -3 activation (HENGARTNER, 2000; ELMORE, 2007; LEE et al., 2008).

Some studies have investigated the mechanism involved in NaF toxicity, suggesting that it induces apoptosis through mitochondrial pathways (higher expression of Bax - pro-apoptotic protein, and inhibition of Bcl-2 - anti-apoptotic protein) and by increasing gene expression of Fas ligand (Fas-L), a death receptor binder present on the cells membrane (LEE et al., 2008; INKIELEWICZ-STEPNIAK et al., 2014).

Considering the best effect of TiF_4 varnish on prevention of tooth demineralization and the lack of studies on its cytotoxic potential, the aims of this thesis were 1) to evaluate and compare the cytotoxic effect of NaF and TiF_4 varnishes and solutions, varying fluoride concentration (0.95, 1.95 and 2.45% F) and time of application (6, 12 and 24 h), in respect to the cells (NIH/3T3) viability (MTT assay, and Hoechst-Iodide assay), morphology (HE) and stiffness (AFM) and 2) to compare the percentage of apoptosis induced by both fluoride varnishes (TiF_4 and NaF, 2.45% F, 6 h) on NIH/3T3 and human gingival fibroblasts (HGF), as well as to elucidate the mechanism involved in its occurrence (mediated by mitochondria, regulated by the Bcl-2 family, and/or mediated by death receptor Fas/Fas-L present in the cell membrane).

2 ARTICLES

2 ARTICLES

To achieve both aims of this thesis, the articles presented were written according to *Plos One* and *Toxicology* guidelines, respectively

- ARTICLE 1 – The cytotoxic effect of TiF_4 and NaF on fibroblasts is influenced by the experimental model, fluoride concentration and exposure time

- ARTICLE 2 – Apoptosis induced by TiF_4 and NaF varnishes on murine and human fibroblasts (NIH3/T3 and HGF): mitochondrial mediated by Bcl-2 family- and death receptor-dependent apoptotic mechanisms

2.1 Article 1[1]

PLOS ONE

RESEARCH ARTICLE

The cytotoxic effect of TiF_4 and NaF on fibroblasts is influenced by the experimental model, fluoride concentration and exposure time

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Abstract

Objective

Titanium tetrafluoride (TiF_4) has shown promising effect in preventing tooth lesions. Therefore, we compared the cytotoxicity of TiF_4 with sodium fluoride (NaF) (already applied in Dentistry) considering different fluoride concentrations, pH values and experimental models.

Materials and methods

Step 1) NIH/3T3 fibroblasts were exposed to mediums containing NaF or TiF_4 (from 0.15 to 2.45% F), both at native and adjusted pH, for 6 h. Step 2) NIH/3T3 were exposed to NaF or TiF_4 varnishes with 0.95, 1.95 or 2.45% F (native pH), for 6, 12 or 24 h. We applied MTT (1^{st} and 2^{nd} steps) and Hoescht/PI stain (2^{nd} step) assays. Step 3) NIH/3T3 were exposed to NaF or TiF_4 varnish (2.45% F), at native pH, for 6 or 12 h. The cell stiffness was measured by atomic force microscopy (AFM).

Results

Step 1) All cells exposed to NaF or TiF_4 mediums died, regardless of the F concentration and pH. Step 2) Both varnishes, at 1.90 and 2.45% F, reduced cell viability by similar extents (33–86% at 6 h, 35–93% at 12 h, and 87–98% at 24 h) compared with control, regardless of the type of fluoride. Varnishes with 0.95% F did not differ from control. Step 3) TiF_4 and NaF reduced cell stiffness to a similar extent, but only TiF_4 differed from control at 6 h.

Conclusions

Based on the results of the 3 experimental steps, we conclude that TiF_4 and NaF have similar cytotoxicity. The cytotoxicity was dependent on F concentration and exposure time. This result gives support for testing the effect of TiF_4 varnish in vivo.

[1] Salomão P.M.A, Oliveira F.A., Rodrigues P.D., Al-AHJ L.P., Gasque K.C.S., Jeggle P., Buzalaf M.A.R., Oliveira R.C., Edwardson J. M., Magalhães A.C. The cytotoxic effect of TiF_4 and NaF on fibroblasts is influenced by the experimental model, fluoride concentration and exposure time. Plos One, v.12(6), p. 1-16, 2017. Available at: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0179471. Accessed on: 13 Nov. 2017.

Competing interests: The authors (Magalhães AC and Buzalaf MAR) have a patent called "Compósito dentário contendo tetrafluoreto de titânio na forma de verniz" (Dental compound containing titanium tetrafluoride in varnish), number PI 0705523-4, International classification: A61K 6/08; A61K 6/04 (March 1st, 2016). This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Introduction

Fluoride has been widely and successfully used for the prevention of dental caries and erosion [1–4]. The major source of fluoride is sodium fluoride (NaF), and its preventive potential at high concentrations is mainly related to the formation of a calcium fluoride-like layer on the tooth [5]. This layer behaves as a physical barrier and acts as a source of fluoride for enamel and dental biofilm during acid challenges. Despite its extensive clinical use, the effect of NaF is sometimes limited. Accordingly, a very intensive fluoridation regime may be required [6,7], especially in cases where the patient has dental erosion or a high risk for caries. Hence, recent studies have focused on fluoride compounds that might have greater efficacy against tooth demineralization, for instance those containing polyvalent metal ions, such as titanium tetrafluoride (TiF_4).

The potential of TiF_4 to prevent tooth demineralization has been investigated since 1997 [8]. Its protective effect is related to the formation of an acid-resistant surface coating and an increased fluoride uptake. The coating is rich in hydrated hydrogen titanium phosphate and titanium oxide, which might act as a diffusion barrier [8–11]. The efficacy of TiF_4 is highly dependent on the low pH of the agent and the type of vehicle. Previous studies have shown that TiF_4 could significantly reduce enamel erosion at its native pH (pH 1.2), but not at a pH buffered to 2.1 [12] or 3.5 [13]. On the other hand, TiF_4 varnish (resin-based material for a slow fluoride release) seems to be more efficient than a TiF_4 solution against tooth demineralization [14,15].

Although there is laboratory evidence about the effectiveness of TiF_4 in the management of dental erosion [1] and caries [14] compared to NaF, its effect needs to be confirmed clinically. However, the low pH of TiF_4 might impair its clinical use, because of possible adverse side effects such as an astringent effect on the mucosa. A scanning electron microscopy study has demonstrated that 1% TiF_4 solution has cytotoxic effects on L929 fibroblasts [16]. However, to our knowledge, no semi-quantitative tests have yet evaluated the effect of TiF_4 on the viability and morphology of fibroblasts.

Here we aimed to evaluate the toxicity of TiF_4, in comparison with NaF, on fibroblasts using three different experimental approaches. First, fluoride salts were added directly into the medium, simulating a mouthrinse, and cytotoxicity was assessed by MTT assay. Second, fluoride was applied as a varnish in contact with the medium and cytotoxicity was assessed by MTT and Hoechst 33342/propidium iodide (HO/PI) stain assays. Third, fluoride was applied as a varnish in contact with the medium and cytotoxicity was assessed via measurement of cell stiffness using atomic force microscopy (AFM). In addition, the dependence of the effects of fluoride on pH, fluoride concentration and exposure time was also taken into account.

Materials and methods

Cell culture

NIH/3T3 fibroblasts (ATCC® CRL1658™) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co. LLC, St. Louis, USA) supplemented with antibiotics (100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) and 10% (v/v) fetal bovine serum (FBS; GIBCO Laboratories, Life Technologies, Inc., New York, USA), at 37˚C in a humidified atmosphere of 5% CO_2. Enzymatic digestion with 0.25% trypsin (Sigma-Aldrich Co. LLC, St. Louis, USA) was used to harvest cells for experimental analysis.

Step 1. Effect of fluoride solutions on cell viability

Cells were plated in 96-well microplates at a density of 5×10^3 cells per well (200 μl culture medium per well, six wells per group) and treated with DMEM (containing antibiotics and

serum) containing different TiF_4 or NaF concentrations (at native and adjusted pH values). Table 1 shows the experimental groups. The native pH values were 9.5 and 1.0 for DMEM containing the highest NaF and TiF_4 concentrations, respectively. The pH of NaF-DMEM and TiF_4-DMEM solutions was adjusted to 4.5 by addition of 1.28 M phosphoric acid and 0.78 M sodium citrate, respectively [13]. A positive control consisted of medium with no fluoride salt. Cell viability was assayed by MTT assay (see below) after 6 h of treatment (in biological triplicate).

Step 2. Effect of fluoride-containing varnishes on cell viability

Cells were plated in 24-well microplates at a density of 10^4 (for the MTT assay) and 5×10^4 (for the other assays) per well (1.5 ml culture medium per well) and treated with varnishes containing TiF_4 or NaF (0.95%, 1.90% and 2.45% F, at native pH) for 6, 12 and 24 h. The varnish was applied to a protrusion (30 mg varnish per well) that was immersed in the medium, allowing fluoride release at a distance of 5 mm from the cells. A positive control consisted of medium with no fluoride. The control medium contained either 1% fetal bovine serum (FBS for the MTT assay) or 10% FBS (for all assays). A negative control consisted of medium containing sodium dodecyl sulfate (only for the MTT assay). The fluoride content of the media was measured by using an ion-specific electrode (Orion Research, Model 9409) and a miniature calomel electrode (Accumet, #13-620-79), both coupled to a potentiometer (Orion Research, Model EA 940), following hexamethyldisiloxane-facilitated diffusion [17,18]. Cell viability was tested by MTT assay or by HO/PI staining, and the cells' morphology was assessed using hematoxylin and eosin (H+E) staining (in biological triplicate).

Steps 1 and 2. MTT cytotoxicity assay

The medium above the cells was removed and the wells were washed with phosphate-buffered saline (PBS). Culture medium containing MTT (0.5 mg ml^{-1}; Sigma-Aldrich Co. LLC, St. Louis, USA) was added and the cells were incubated with it for 4 h at 37°C in an atmosphere of 5% CO_2. The formazan crystals produced were dissolved in dimelthyl sulfoxide (DMSO; Synth Labsynth Prods. Ltda, Diadema, Brazil). After 30 min, absorbance at 540 nm was measured using a scanning spectrophotometer (Fluorstar Optima—BMG Labtech, Ortenberg, Germany) [19]. Percentage viability was calculated, considering the positive control as 100%.

Table 1. Step 1. Experimental treatment groups including different fluoride salts, fluoride concentrations and pH.

Fluoride salt	Concentration	pH
NaF	5.42% (2.45% F)	
	2.71% (1.23% F)	9.5 (native pH)
	1.35% (0.61% F)	Or
	0.68% (0.31% F)	4.5
	0.34% (0.15% F)	
TiF_4	4.00% (2.45% F)	
	2.00% (1.23% F)	1.0 (native pH)
	1.00% (0.61% F)	Or
	0.50% (0.31% F)	4.5
	0.25% (0.15% F)	

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Step 2. Hoechst 33342/ PI (HO/PI) staining

Differential staining with specific fluorochromes was used to distinguish living and dead cells. Cells were trypsinized and suspended in 100 μl PBS and 100 μl of a solution containing 25% PI (1 mg ml^{-1} in water), 50% fluorescein diacetate (1.5 mg ml^{-1} in DMSO), 10% HO (1 mg ml^{-1} in water) (Sigma-Aldrich Co. LLC, St. Louis, USA) and 15% PBS. Ten microlíters of the samples were placed in glass slabs and covered with coverslip. The area of analysis was 0.030 mm^2. Cells were classified as either viable (spherical blue nucleus stained by HO, green cytoplasm stained by fluorescein diacetate, excited at 360 nm), non-viable cells (blue nucleus with apoptotic bodies stained by HO, green cytoplasm) or necrotic (red enlarged nucleus with spherical vesicles stained by PI, excited at 538 nm) using confocal microscopy (63x Leica TCS_SPE). The non-viable and necrotic cells were considered to be dead. The percentages of living and dead cells were quantified [20,21].

Step 2. Assay of cell morphology

Cells (only after 24h treatment) were fixed with 4% formaldehyde for 10 min and stained with H+E. Cells were examined using an inverted optical microscope (10x, Leica DM IRBE). Cell morphology was classified as follows:

(-) No reaction: no evidence of morphological changes.

(+) Mild reaction: some cells with small morphological changes.

(++) Moderate: detachment of some cells, presence of collapsed or rounded cells, few changes in cell number.

(+++) Severe: cellular fragments, severe reduction in cell number, collapsed and rounded cells with surface blebs.

(++++) Very severe: cell lysis, cell contour loss, severe reduction in cell number, few cells preserved, almost all cells collapsed or totally destroyed, most cells shriveled and wrinkled.

Step 3. Effect of fluoride-containing varnishes on cell stiffness

Cells were plated in Petri dishes (60 x 15 mm), at a density of 3×10^5 (7 ml medium per dish) and treated with varnishes containing TiF_4 or NaF (2.45% F, at native pH), or placebo varnish, for 6 or 12 h. The varnish was applied to four protrusions attached to the cover of the Petri dish (30 mg per protrusion). The protrusions became immersed in the medium, allowing fluoride release at a distance of 5 mm from the cells. For positive controls, varnish was omitted. Experiments were carried out in biological triplicate (n = 3 dishes for each condition).

After washing with PBS, cells were exposed to HEPES-buffered saline solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4). The stiffness of individual cells (10 curves for each cell) was measured using a Bioscope atomic force microscope (Bruker, Santa Barbara, USA) mounted on Axiovert 135M microscope (Zeiss, Germany) and controlled by a NanoScope IIIa controller. Silicon nitride AFM cantilevers (spring constant 0.01 N/m) with spherical tips (Novascan, Iowa, USA) were used as mechanosensors. A force-distance curve was plotted, relating the bending of the cantilever (or the applied force) to its position relative to the cell. The stiffness of the cell was deduced from the linear slope of this curve (~400 nm). Analyses were carried out using Atomic J Software version 1.6.0 [22], to determine Young's modulus values.

All steps. Statistical analysis

The software GraphPad InStat version 2.0 for Windows (Graph Pad Software, San Diego, USA) was used. The MTT, HO/PI and F release data passed the normality test (Kolmogorov-Smirnov test), but not the equality of variances test (Bartlett test, $P < 0.05$). Values for percentage cell viability (MTT and HO/PI) and fluoride release were compared using Kruskal-Wallis test followed by Dunn's test. Cell stiffness values were compared using ANOVA followed by the post-hoc Bonferroni test. Percentage of viability (MTT) and fluoride release were correlated using Pearson's test. The level of significance was set at 5%.

Results

Step 1

After 6 h, all fluoride treatments reduced cell viability by 100% compared with the positive control ($P < 0.0001$), with no significant differences between the fluoride salts, concentrations, or pH values (Table 2).

Step 2

The fluoride concentrations in the medium as a result of release from the varnishes ranged between 3 and 140 ppm. After 6 and 24 h, the various groups behaved similarly; specifically, TiF_4 (2.45% F) and NaF (1.90% and 2.45% F) varnishes significantly released more fluoride than the control and placebo. On the other hand, the varnishes with the lowest fluoride concentrations did not differ from the control and placebo (Table 3, $P < 0.0001$). After 12 h, the results among the various groups were similar to those over other periods; however, in this case TiF_4 (1.90% F) released more fluoride than placebo/control, while NaF (1.90% F) did not. Differences between the experimental times were found for all groups, except for the control, NaF (0.95% F) and placebo.

As judged by the results of the MTT assay, after 6 and 12 h, TiF_4 (2.45% F) and NaF (1.90% and 2.45% F) varnishes significantly reduced the percentage cell viability compared to positive control (10% FBS), similarly to the negative control. No significant difference was found

Table 2. Step 1. Median (interquartile interval) of the % of cellular viability assessed by MTT after treatment with F solutions.

Groups	%	Groups	%
Control	87.1 (60.8)		
5.42% NaF pH 9.5	0.0*(0.0)	4% TiF_4 pH 1.0	0.0 (2.6)
5.42% NaF pH 4.5**	0.0 (10.3)	4% TiF_4 pH 4.5**	3.6 (8.3)
2.71% NaF pH 9.5	0.0* (0.0)	2% TiF_4 pH 1.0	0.0 (1.0)
2.71% NaF pH 7.5**	0.0 (0.0)	2% TiF_4 pH 4.5**	0.0 (0.0)
1.35% NaF pH 9.0	0.0* (0.0)	1% TiF_4 pH 1.5	0.0 (0.5)
1.35% NaF pH 8.0**	0.0* (0.0)	1% TiF_4 pH 4.5**	25.5 (9.3)
0.68% NaF pH 9.0	0.0* (0.0)	0.5% TiF_4 pH 3.5	3.6 (7.7)
0.68% NaF pH 8.5**	0.0* (0.0)	0.5% TiF_4 pH 5.0**	4.1 (9.8)
0.34% NaF pH 8.5	0.0* (0.0)	0.25% TiF_4 pH 5.0	5.2 (13.9)
0.34% NaF pH 9.0**	0.0* (0.0)	0.25% TiF4 pH 5.5**	0.0 (0.5)

* shows groups that presented median values statistically different from the median values of the control (Kruskal-Wallis and Dunn tests, P < 0.0001).

** final pH values obtained by using a pH paper indicator, after the serial dilution of the most concentrated fluoride medium.

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Table 3. Step 2. Median (interquartile interval) of the released fluoride (ppm) from the varnishes to the medium.

Groups	6h	12h	24h
Positive Control	0.01 (0.00)^{bA}	0.01 (0.01)^{bA}	0.01 (0.01)^{cA}
4.00% TiF_4 (2.45% F)	16.30 (2.18)^{aA}	67.11 (55.06)^{aB}	31.24 (12.82)^{abAB}
3.10% TiF_4 (1.90% F)	8.12 (5.66)^{abA}	53.5 (1.75)^{aB}	27.59 (2.18)^{abcAB}
1.50% TiF_4 (0.95% F)	9.25 (1.57)^{abA}	15.81 (8.74)^{abAB}	19.37 (10.30)^{abcB}
5.42% NaF (2.45% F)	16.56 (3.84)^{aA}	90.50 (51.47)^{aB}	95.79 (54.22)^{abB}
4.20% NaF (1.90% F)	17.49 (5.14)^{aA}	38.15 (13.70)^{abAB}	139.61 (14.50)^{aB}
2.10% NaF (0.95% F)	8.52 (5.11)^{abA}	2.95 (1.21)^{abA}	4.19 (4.77)^{bcA}
Placebo	0.01 (0.01)^{bA}	0.01 (0.00)^{bA}	0.01 (0.02)^{cA}

Values in the same column with distinct superscript lowercase letter show significant differences among the treatment groups. Values in the same line with distinct superscript uppercase letters show significant differences among the experimental times (Kruskal-Wallis test followed by Dunn's test, P < 0.0001).

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between positive control and 1% FBS control. TiF_4 (0.95% and 1.90% F), NaF (0.95% F) and placebo varnishes were not cytotoxic at these times (Fig 1A and 1B, $P < 0.0001$). After 24 h, the results among the groups were similar to previous periods, but in this case TiF_4 (1.90% F) varnish did have a cytotoxic effect (Fig 1C, $P < 0.0001$).

No significant differences were found between the fluoride salts, except at 24 h for 1.90% F, in which TiF_4 was more cytotoxic than NaF. With respect to the experimental periods, the fluoride toxicity was significantly increased after 24 h compared to 6 h and 12 h (except for NaF, 0.95% F); however, toxicities at 6 h and 12 h did not differ from each other.

The percentage cell viability (by MTT assay) and fluoride release from the varnishes showed a moderate but significant inverse correlation (r: -0.66, 95% CI: -0.84 to -0.35, $P = 0.0004$). Hence, cellular viability showed a tendency to decrease as the fluoride concentration of the varnish rose.

According to the HO/PI assay, after 6 h, there were no significant differences in the percentage of living cells among the various treatments (Fig 2A). After 12 h, the treatments causing a significant reduction in the percentage of living cells were TiF_4 (2.45% F) and NaF (1.90% and 2.45% F) compared with the positive control (Fig 2B). After 24 h, the results were similar to 12 h, but in this case TiF_4 (1.90% F) varnish also showed cytotoxicity (Fig 2C). The varnishes with lowest fluoride concentrations (for both TiF_4 and NaF, 0.95% F) and placebo varnishes were not cytotoxic. Representative images for the 24 h treatments are shown in Fig 3. No significant differences were found between the effects of fluoride salts according to the HO/PI assay. With respect to the experimental periods, fluoride toxicity was significantly increased after 12 h compared with 6 h for NaF (1.90% and 2.45% F), and after 24 h compared with 12 h for TiF_4 (1.90% and 2.45% F). For the other treatments, there was no significant difference between the periods.

The observed alterations in cell morphology confirmed the results for cell viability. After 24 h, both TiF_4 and NaF varnishes (1.90% and 2.45% F) drastically reduced the number of cells and caused morphological alterations in the cells that remained, while the lowest concentrated fluoride varnishes (TiF_4 and NaF, 0.95% F) caused moderate morphological alterations with only slight changes in cell number. No differences were seen between the fluoride salts (Fig 4).

Step 3

Over a 6 h period, TiF_4 varnish (2.45% F) significantly reduced cell stiffness compared with the control and placebo, while the stiffness of cells exposed to NaF varnish (2.45% F) did not

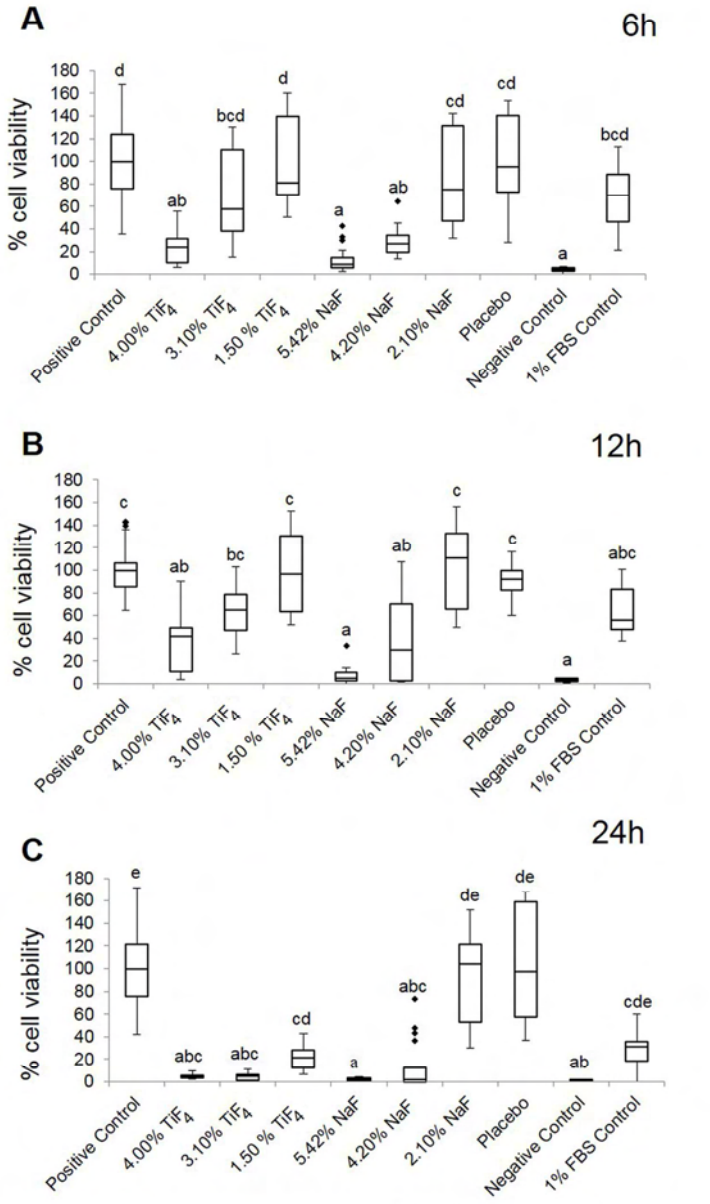

Fig 1. Step 2. Box plots of the percentage cell viability, according to MTT assay, for the various experimental varnishes' treatments after (A) 6 h, (B) 12 h and (C) 24 h. 4.00% TiF_4 and 5.42% NaF

(2.45% F); 3.10% TiF_4 and 4.20% NaF (1.90% F); 1.50% TiF_4 and 2.10% NaF (0.95% F). Distinct lower-case letters show significant differences among the treatment groups (n = 6 for each group; Kruskal-Wallis test followed by Dunn's test, $P < 0.0001$).

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differ from the control and placebo. There was no significant difference between the effects of TiF_4 and NaF varnishes (Fig 5A). Over a 12 h period, TiF_4 and NaF varnishes significantly reduced cell stiffness compared with the control and placebo (Fig 5B). There was no significant difference between the effects of TiF_4 and NaF varnishes.

Discussion

Cytotoxicity assays are important tools in dentistry and should be performed before a novel material comes into clinical use [11,23]. NIH/3T3 has been widely applied in cell culture for several decades, including tests of cell viability and toxicity of biomaterials used in Dentistry as it can be used as a substitute for human gingival fibroblasts [24,25].

TiF_4 reduces enamel demineralization, and improves remineralization of dental caries lesions [14,15,26,27] and tooth erosion progression *in vitro* and *in situ* [1,12,13,28,29]. It thus seems to be a promising agent for the control of both dental lesions and cytotoxicity studies are warranted to establish its safety. NaF, the most common fluoride compound used in dentistry worldwide, was included in this study to provide a comparison with TiF_4. It has been shown previously to be cytotoxic depending on the cell type, fluoride concentration, and duration of the treatment [9,30–33].

Despite some studies have shown cytotoxicity of NaF, it has been considered safe especially when included in varnish. Varnish has some advantages compared to mouthrinse or gel, due to its adherence to the tooth surface, allowing a longer contact time between fluoride and enamel (from 6h to 12h). A previous study has shown that NaF and TiF_4 (2.45% F) varnishes partially release fluoride along to 12h in contact with water and artificial saliva (in a range of 15–25 ppm F) [34], amount of fluoride lower than found in DMEM in this study. Varnish is known to have low toxicity due to the low quantity used during application and to the low absorption in intestine and concentration in plasma. It is also well tolerated and accepted by the patients [35–38].

Our study aimed to compare the cytotoxicity potential of NaF and TiF_4, both at high and similar fluoride concentrations as those found in products indicated for professional application. Experiments using solutions containing different fluoride concentrations were aimed at simulating the application of TiF_4 as a mouthrinse. The concentrations were chosen according to previous studies [14,15], which were then reduced (up to 16-fold) to mimic the natural clearance by saliva. We tested solutions at the native pH found in the medium after the addition of the fluoride salt, as well as solutions in which the pH was adjusted to 4.5. Adjustment of pH, as previously reported [13], is important to allow comparison between both fluoride salts under the same experimental conditions, and also for understanding the roles of pH and fluoride concentration in cytotoxicity. Fluoride can cross cell membranes as HF, in response to a pH gradient. Thus, the lower the extracellular pH, the higher the amount of fluoride that enters into the cell [39]. A pH value of 4.5 was chosen since it is the lowest pH value acceptable in fluoride containing products for topical use [40].

It has been reported that the toxicity of TiF_4 might be related to its low pH [16]. When applied as solutions, TiF_4 and NaF had comparable cytotoxic effects. Our data disagree with a previous report that 1% TiF_4 (pH 1.35) at native pH is more toxic than NaF (pH 8.45), which was mainly attributed to the lower pH of the first [16]. In our study, the MTT assay did not show the same trend, which might be due to the fact that all treatments drastically reduced cell

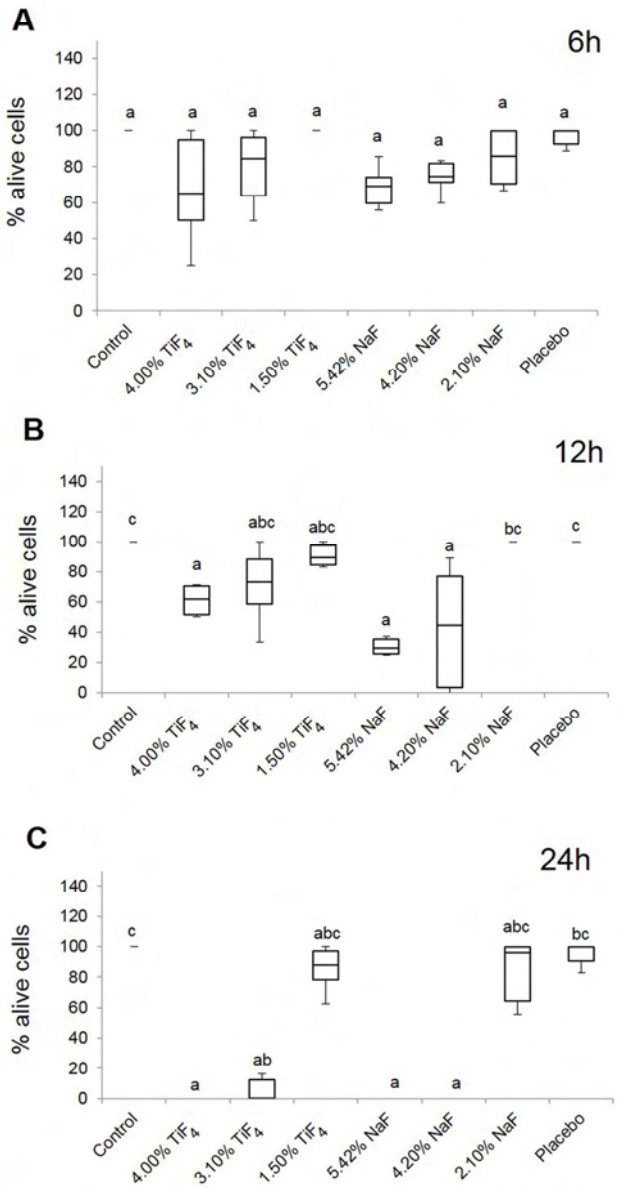

Fig 2. Step 2. Box plots of the percentage cell viability, according to HO/PI staining, for the various experimental varnishes' treatments after (A) 6 h, (B) 12 h and (C) 24 h. 4.00% TiF_4 and 5.42% NaF

(2.45% F); 3.10% TiF_4 and 4.20% NaF (1.90% F); 1.50% TiF_4 and 2.10% NaF (0.95% F). Distinct lowercase letters show significant differences among the treatment groups (n = 3 for each group; Kruskal-Wallis test followed by Dunn's test, $P < 0.0001$). The number of cells found in positive control was: 8.67±1.37 (6h), 9.50 ±2.17 (12h) and 11.83±1.83 (24h).

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viability. In other words, the toxic effect of pH was masked by the presence of high fluoride concentration. It is important to highlight that it is also possible that sodium citrate, used to increase the pH of the TiF_4 solution, could have a cytotoxic effect similar to those caused by the low pH itself. Thus, it seems that the increase in the pH of TiF_4 solutions might not benefit the patient, at least not through the use of sodium citrate.

The differences between our TiF_4 data and previous results [16] could be due to the experimental design. Sen et al. [16] applied fluoride to dentin slices, on which the cells were grown, rather than adding it directly to the medium as we did. The fluoride treatment could have interfered with cell attachment to the slices. Furthermore, Sen et al. [16] used scanning electron microscopy to detect cytotoxic effects. They also used a different cell line, although L929 and NIH/3T3 cells have a similar proliferative rate [41] and viability [42], and so would be expected to respond to fluoride treatment in a similar fashion.

We propose the incorporation of TiF_4 into a varnish for professional application because varnishes have been shown to be better than an equivalent solution for the prevention of dental caries and erosion [14,43]. In our study, the TiF_4 and NaF varnishes partially released their fluoride content in DMEM, and we found a negative correlation between fluoride release and viability. In respect to the analyzed variables, the two highest fluoride concentrations tested here were cytotoxic, reducing viability and causing morphological changes, while the lowest fluoride concentration was not. Additionally, a 24 h treatment led to lower cell viability and survival than 6 h and 12 h treatments. On the other hand, TiF_4 and NaF varnishes were similarly cytotoxic.

Inkielewicz-Stepniak et al. [23] showed a reduction in viability of fibroblasts treated with 1.5 mM but not 0.5 and 1 mM NaF, after 24 h of treatment. Similarly, Tsutsui et al.[33] found that cytotoxicity increased linearly with increasing NaF doses (2.3 to 9.5 mM) and time of exposure (1–24 h). On the other hand, Lee et al. [44] found that doses higher than 20 mM NaF were necessary to reduce cell viability. Our data, considering a range of 0.16 to 7.3 mM F in the medium, are more in accordance with the first two of the above-mentioned three studies.

Vieira et al. [45] have shown that the effect of TiF_4 against enamel erosion is greater when a highly concentrated solution is applied. Comar et al. [46] also showed that more fluoride is deposited on tooth enamel as the fluoride concentration in TiF_4 varnishes rises. Generally, dental varnishes contain a range of 2.26% to 6% F [47], and the varnishes remain in contact with the tooth for 6–12 h [47,48]. Hence, the relatively high cytotoxicity that we found at 24 h does not reflect the real-life situation; rather, the 6 and 12 h data support the possibility of testing TiF_4 varnish in further clinical trials, since it was not shown to be more toxic than NaF varnish, which is already widely used clinically.

Lee et al.[49] and Subbiah et al.[50] have suggested that AFM indentation analysis can be used as a new method for evaluating cytotoxicity. In our study, measurement of cell stiffness by AFM confirmed the similarity between NaF and TiF_4 toxicities. We analyzed stiffness over a depth of 400 nm, which reveals mechanical alterations related to the cytoskeletal proteins beneath the plasma membrane [51]. Considering that the AFM indentation technique is able to reveal the effects of different treatments on cell elasticity in response to cytoskeleton disruption [52,53], we speculate that the fluoride varnishes could cause partial disorganization of the cytoskeleton, which should be studied in detail further.

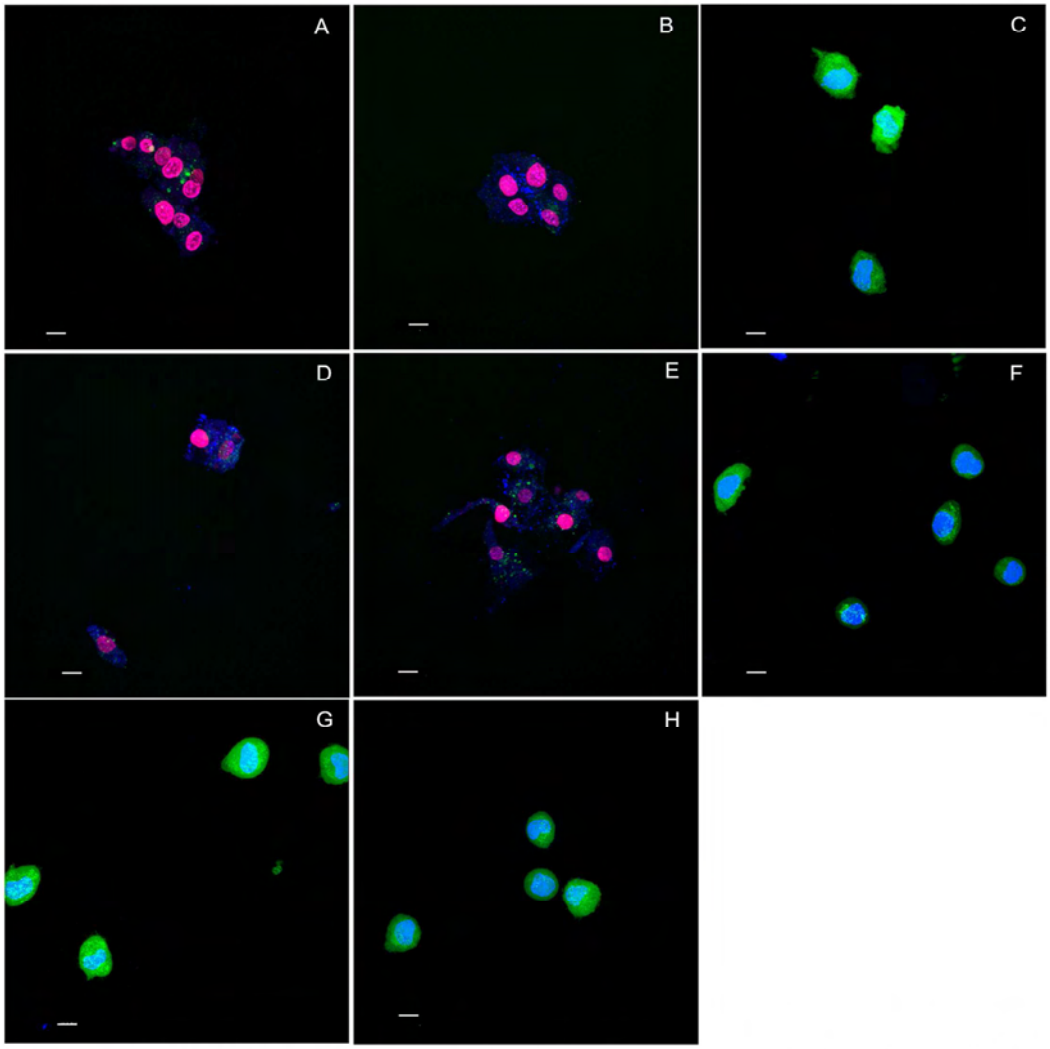

Fig 3. Step 2. Representative confocal images of the 24 h varnishes' treatments. (A) 4.00% TiF_4, (B) 3.10% TiF_4, (C) 1.50% TiF_4, (D) 5.42% NaF, (E) 4.20% NaF, (F) 2.10% NaF, (G) Control, (H) Placebo. 4.00% TiF_4 and 5.42% NaF (2.45% F); 3.10% TiF_4 and 4.20% NaF (1.90% F); 1.50% TiF_4 and 2.10% NaF (0.95% F). Scale bar, 10 μm.

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The mechanism underlying the cytotoxic effects of fluoride has been extensively investigated. Jeng et al. [11] showed that NaF at concentrations above 4 mM reduces protein synthesis and cytosolic ATP levels, and interferes with mitochondrial function, as also shown in our

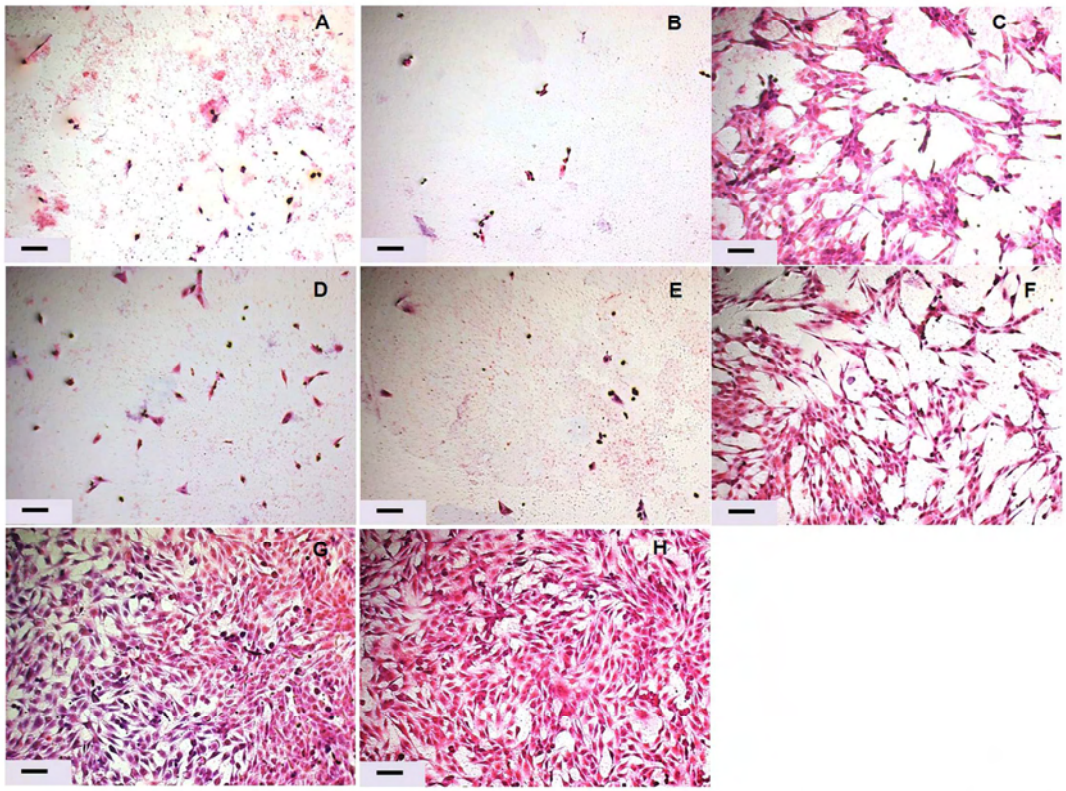

Fig 4. Step 2. Representative H+E images of the 24 h varnishes' treatments. (A) 4.00% TiF_4, (B) 3.10% TiF_4, (C) 1.50% TiF_4, (D) 5.42% NaF, (E) 4.20% NaF, (F) 2.10% NaF, (G) Control, (H) Placebo. 4.00% TiF_4 and 5.42% NaF (2.45% F); 3.10% TiF_4 and 4.20% NaF (1.90% F); 1.50% TiF_4 and 2.10% NaF (0.95% F). Scale bar, 100 μm.

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study by the MTT assay. Lee et al. [44] showed that 20 mM fluoride induced chromatin condensation and DNA fragmentation in human gingival fibroblasts, which would lead to apoptosis. In this previous study, NaF also increased mitochondrial release of cytochrome C into the cytosol, enhanced caspase-9, -8 and -3 activities, increased the cleavage of poly (ADP-ribose) polymerase (PARP), and up-regulated the voltage-dependent anion channel (VDAC). Finally, NaF also up-regulated the Fas-ligand (Fas-L), a ligand of death receptor; Bcl-2, a member of the anti-apoptotic Bcl-2 family, was down-regulated [44], in agreement with a recent study of Inkielewicz-Stepniak et al. [23]. Otsuki et al. [54] demonstrated that NaF enhanced the expression and dephosphorylation of Bcl-2-associated death promoter (Bad) protein. Bad protein forms a complex with carbonic anhydrase II (CAII), and NaF stimulates the detachment of CAII from the Bad-CAII complex and its replacement in a Bad-Bcl-2 complex, inducing apoptosis. Taken together, the studies mentioned above suggest that NaF induces apoptosis through both death receptor- and mitochondria-mediated pathways regulated by the Bcl-2

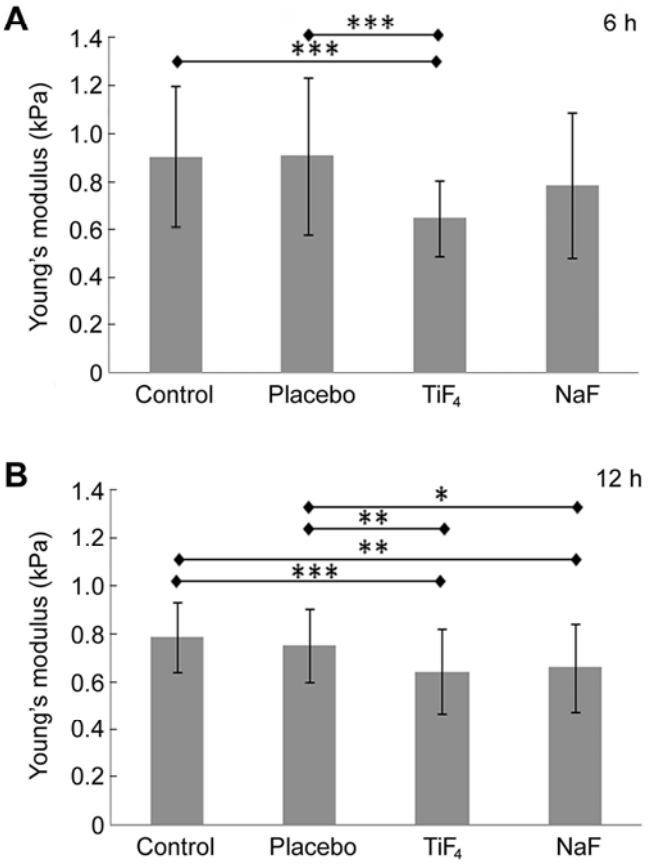

Fig 5. Step 3. Mean and standard deviation values for cell stiffness (Young's modulus) for samples subjected to different varnishes' treatments for either 6 h (A) or 12 h (B). Asterisks show significant differences between groups (ANOVA, followed by the post-hoc Bonferroni test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). For (A), Control and 5.42% NaF (2.45% F), n = 45; Placebo, n = 46; 4.00% TiF_4 (2.45% F), n = 47. For (B), Control, n = 45; Placebo, n = 49; 4.00% TiF_4 (2.45% F), n = 47; 5.42% (2.45% F) NaF, n = 46.

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family. The cytotoxic mechanism of TiF_4 is so far unexplored as yet, although it is likely that similar processes are involved.

Conclusions

Based on the results of the 3 experimental steps, we have shown that TiF_4 and NaF have similar cytotoxic effects on fibroblast viability, stiffness and morphology. The cytotoxic effects mainly depend on the fluoride concentration and exposure time. This result gives support for testing the effect of TiF_4 varnish in vivo.

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Conceptualization: ACM PMAS RCO JME.

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Methodology: ACM PMAS RCO JME.

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Resources: ACM RCO JME.

Supervision: ACM JME.

Visualization: PMAS.

Writing – original draft: PMAS ACM.

Writing – review & editing: PMAS FAO PDR LPA KCSG PJ MARB RCO JME ACM.

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2.2 Article 2 - Apoptosis induced by TiF_4 and NaF varnishes on murine and human fibroblasts (NIH3/T3 and HGF): mitochondrial mediated by Bcl-2 family- and death receptor-dependent apoptotic mechanisms

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Abstract

This study evaluated the percentage of apoptosis and its mechanism in human gingival fibroblasts (HGF) and murine fibroblasts (NIH/3T3) treated with titanium tetrafluoride (TiF_4) varnish compared to sodium fluoride (NaF) varnish. HGF and NIH/3T3 were cultured in DMEM supplemented with 10% fetal bovine serum at 37ºC in a humidified atmosphere of 5% CO_2. The cells were treated with TiF_4 or NaF (both 2.45%F) or placebo varnish for 6h. Cells were examined by the TUNEL method using fluorescence microscope. The caspase-3, -8 and -9 activities were assessed. The cDNA for cytocrome c, Bax, Bad, Bcl-2, VDAC-1 and Fas-L was amplified by quantitative PCR (qPCR). Bax, Bcl-2 and Fas-L were further detected by western blot. Both fluorides similarly increased the percentage of apoptosis, while they failed to activate caspases-3, -8 and -9 for both types of cells. Bax/Bcl-2 ratio, cytochrome C and VDAC-1 gene expressions were not altered by either fluoride treatment regardless of the type of cell. NaF varnish increased the amplification of Fas-L gene for NIH/3T3 and HGF, while TiF_4 varnish induced lower Bad/Bcl-2 ratio expression compared to control for NIH/3T3 but not for HGF. No effect of the fluorides was detected in the proteins analysis. In conclusion, NaF and TiF_4, at the studied conditions, similarly induce a low percentage of apoptosis with consequent modest activation of Bcl-2 and Fas-L-dependent signaling pathways. Generally, HGF is more susceptible to the fluoride effects than NIH/3T3.

Keywords: Apoptosis; Fas-L; Human gingival fibroblast; NIH/3T3 fibroblast; Sodium fluoride; Titanium tetrafluoride.

1. Introduction

Sodium fluoride (NaF) has been the most popular fluoride applied to prevent dental caries and erosion (Castilho et al. 2015; Magalhães et al. 2011; Pessan et al. 2011). NaF has been included in professional and home-care dental products since is considered safe and has no relevant local side-effects, although *in vitro* studies have shown that NaF can cause damage at the cellular level in fibroblasts (Inkielewicz-Stepniak et al. 2014; Jeng et al. 1998; Lee et al. 2008; Otsuki et al. 2011; Salomão et al. 2017). It can also induce apoptosis depending on the concentration and frequency of application (Inkielewicz-Stepniak et al. 2014; Lee et al. 2008). Among professional products, NaF varnish is the first choice for young children, as it is only slightly absorbed by the gastrointestinal system, and, therefore, it presents a low risk of undesired systemic effects, caused by excessive fluoride intake, such as dental or skeletal fluorosis (Ekstrand et al. 1980; Olympio et al. 2009; Pessan et al. 2005).

Since 2008, TiF_4 varnish has been tested as an alternative to NaF varnish, especially for patients with high risk of dental caries and erosion. Several *in vitro* and *in situ* studies have shown that TiF_4 varnish has a higher protective effect against tooth demineralization than NaF varnish (Comar et al. 2012; Comar et al. 2015; Comar et al. 2017; Magalhães et al. 2008; Magalhães et al. 2016; Martines de Souza et al. 2017). A recent *in situ* study showed that TiF_4 varnish was able to induce remineralization of artificial caries lesions regardless of the cariogenic activity, while NaF failed to prevent further demineralization under high cariogenic challenges (Comar et al. 2017). Therefore, current knowledge supports the execution of clinical trials to test the protective effect of TiF_4; however, it is indispensable to determine its cytotoxic potential before applying it in patients.

Recently, our research group showed that both NaF and TiF_4 similarly reduced NIH/3T3 viability and increased cell death (Salomão et al. 2017). However, it is important to understand their mechanism of toxicity. Apoptosis is one of the mechanisms involved in cells death. Some studies have shown that NaF induced apoptosis in fibroblasts via both mitochondria mediated by the Bcl-2 family- and death receptor- pathways (Inkielewicz-Stepniak et al. 2014; Lee et al. 2008). Therefore, the aims of this study are to compare the percentage of apoptosis induced by both fluorides, as well as to elucidate the mechanism involved in its occurrence.

On the other hand, it would be very important to apply a primary human cell instead of an immortalized murine linage cell, as done in our previous study (Salomão et al. 2017), to better represent the *in vivo* conditions (Inkielewicz-Stepniak et al. 2014; Jeng et al. 1998; Lee et al. 2008; Otsuki et al. 2011). Therefore, the study investigated the apoptosis mechanisms of both fluorides on NIH/3T3 and primary human gingival fibroblasts (HGF).

Previous studies have allowed a long contact time between the fluoride and the cells, which does not simulate the real conditions of saliva washing the product and thereby reducing its concentration in the mouth over time (Inkielewicz-Stepniak et al. 2014; Salomão et al. 2017). Twetman et al. (1999) showed that significant levels of fluoride in saliva did not exceed 6h after treatment with fluoride varnishes *in vivo*. Furthermore, Comar et al. (2014) demonstrated that the peak of fluoride release into saliva from the varnishes occurred in the first 3-6h *in vitro*.

Considering the current knowledge, this study tests the following null hypothesis: 1) There is no difference between NaF and TiF_4 varnish with respect to the percentage of apoptosis of NIH/3T3 and HGF submitted to 6h treatment; 2) There is

no difference between NaF and TiF_4 varnish with respect to the molecular pathways involved in apoptosis of NIH/3T3 and HGF submitted to 6h treatment.

2. Material and Methods

2.1. Cell culture

NIH/3T3 fibroblasts (ATCC® CRL1658™) and primary human gingival fibroblasts (HGF) previously obtained from the healthy gingival tissue of patients receiving treatment at Bauru School of Dentistry (approved by the Ethical Committee of Bauru School of Dentistry-University of São Paulo Process n° CAAE 71642517.6.0000.5417) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co. LLC, St. Louis, USA) supplemented with antibiotics (100 IU ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin) and 10% (v/v) fetal bovine serum (FBS; GIBCO Laboratories, Life Technologies, Inc., New York, USA) at 37°C in a humidified atmosphere of 5% CO_2. Enzymatic digestion with 0.25% trypsin (Sigma-Aldrich Co. LLC, St. Louis, USA) was used to harvest the cells for the experimental analysis. All experiments were carried out in experimental triplicate (n=3 wells or pool of wells) and in three independent experiments (biological triplicate).

2.2. In situ apoptosis detection by TUNEL staining

Cells were cultivated on a 13mm diameter plastic coverslip (Sarstedt, Nümbrecht, Germany) in 24-well microplates at a density of $5x10^4$ per well (1.5 ml culture medium per well) for 24 h and were then treated with experimental varnishes containing TiF_4 or NaF (2.45% F, native pH 1.0 for TiF_4 and pH 5.0 for NaF), experimental placebo varnish (no F, pH 5.0) or non-treated cells (control) for 6 h. The

varnishes were applied as previously described (Salomão et al. 2017). After washing with PBS, the cells were examined by the TUNEL method using ApopTag® Plus Fluorescein *In Situ* Apoptosis Detection Kit (EDM Millipore Corporation, Hayward, USA) according to the manufacturer's instructions. The samples were visualized using a fluorescent microscope (40x, Leica DM IRBE, Wetzlar, Germany). Five images of each coverslip were captured and a mean of the percentage of apoptosis was calculated.

2.3. Caspase activation assay

Cells were cultivated in 24-well microplates at a density of $8x10^4$ per well (1.5 ml culture medium per well) for 24 h and were then treated as previously described for 6 h. Doxorubicin (15 µg/mL) was used as a positive control for apoptosis. Cells were harvested, and eight wells were pooled for each treatment, to obtain the required number of cells for the experiment. Caspase activity was assessed using a caspase-3, -8 and -9 Colorimetric Activity Assay Kit (EDM Millipore Corporation, Hayward, USA) according to the manufacturer's instructions. The final product (pNA) was measured using a microplate reader (Synergy H1, BioTek®, Winooski, USA) at 405 nm. The unit of enzyme activity/mg of protein was calculated. For this, protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, USA).

2.4. Real Time RT PCR

Cells were cultivated in 24-well microplates at a density of $8x10^4$ per well (1.5 ml culture medium per well) for 24 h and were then treated as previously described for 6 h. Cells were harvested, and four wells were pooled. Then, $1x10^5$ cells from each treatment were submitted to mRNA extraction using PureLink® RNA Mini Kit (Life Lechnologies, Carlsbad, USA), and the isolated RNA was quantified using a

NanoDrop™ 1000 (Thermo Fisher Scientific, Waltham, USA). The cDNA was obtained using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, USA) according to the manufacturer's instructions. The cDNA samples were incubated with Taqman® Gene Expression Master Mix and Taqman® Gene Expression assay (Applied Biosystems, Foster, USA) for Bad, Bax, Bcl-2, Cytochrome C, VDAC-1 and Fas-L genes. The reading was done using the Real-Time RT-PCR-System ViiATM7 (Applied Biosystems, Foster, USA). The Act-b expression was used as the control for NIH/3T3, and RPL-13 expression was used as the control for HGF (Applied Biosystems, Foster, USA). The relative expression was obtained using the formula $2^{-(\Delta\Delta Ct)}$.

2.5. Western Blot

Cells were cultivated in 6-well microplates at a density of $5x10^5$ per well (7 ml culture medium per well) for 24 h and were then treated as previously described for 6h. After washing with PBS, cells were lysed with buffer solution containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$ and 0.2% Nonidet P-40 supplemented with protease inhibitors (Sigma-Aldrich Co. LLC, St. Louis, USA) and phosphatase inhibitor (0.2 M Sodium orthovanadate). The lysates were pooled (3 wells), sonicated (10 s) and centrifuged at 10.000 rpm for 10 min at 4ºC. Protein samples quantified by Bradford method (50 µg/treatment) were applied to electrophoresis in Tris-HCl 12.5% polyacrylamide gel and were, subsequently, transferred to a PVDF membrane. The membrane was immune-labeled with rabbit polyclonal anti-Fas ligand or rabbit monoclonal anti-Bcl-2 or anti-Bax primary antibodies (ABCAM INC., Cambridge, MA, USA) followed by secondary anti-Rabbit IgG conjugated to HRP (Horseradish Peroxidase, ABCAM INC., Cambridge, MA, USA) and Amersham™ ECL™ Prime

Western Blotting Detection Reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The relative densities of the bands were determined by densitometry analysis using Image J software (National Institute of Health, NIH image, Bethesda, USA). The density arbitrary values obtained were normalized for alpha-Tubulin (ABCAM INC., Cambridge, MA, USA), as an internal control.

2.6. Statistical analysis

The software GraphPad InStat version 2.0 for Windows (Graph Pad Software, San Diego, USA) was used. The data were submitted to normality test (Kolmogorov-Smirnov test) and equality of variances test (Bartlett test). Values obtained from the caspases activity, RT-PCR (Bax/Bcl-2 ratio, cytochrome C and Fas-L) and western blot assays were compared using Kruskal-Wallis test followed by the post-hoc Dunn's test. Values obtained from the *in situ* apoptosis detection by TUNEL staining and RT-PCR (Bad/Bcl-2 ratio and VDAC-1) assays were compared using ANOVA followed by the post-hoc Tukey test. The level of significance was set at 5%.

3. Results

3.1. In situ apoptosis detection by TUNEL staining

For NIH/3T3, all varnishes significantly increased the percentage of apoptosis compared to the control. The highest percentage of apoptosis was seen for TiF_4-treated cells compared to the control and the placebo varnish; however, there was no significant difference compared to the NaF-treated cells. For the HGF, both fluoride varnishes similarly increased the percentage of apoptosis compared to both the placebo varnish and the control (Table 1). Representative images are shown in Figure 1.

3.2. Caspase activation assay

Both fluoride varnish treatments did not increase the activity of caspases-3, -8 and -9 compared to the control or the placebo varnish for both types of cells (Figure 2).

3.3. Real Time RT PCR

The relative expression of the genes involved in the apoptosis pathway is shown in Figures 3 and 4. The Bax/Bcl-2 ratio and cytochrome C expressions were not altered by the fluoride treatments compared to the control regardless of the type of cell. However, NaF varnish increased by 5-fold and 14-fold the expression of Fas-L compared to the control for the NIH/3T3 and HGF, respectively. There was no difference between the TiF_4 and NaF-treated cells with respect to Fas-L gene expression (Figure 3).

Cells treated with TiF_4 varnish presented significantly lower Bad/Bcl-2 ratio expression compared to the control but not compared to the placebo and NaF varnishes in the case of the NIH/3T3. For the HGF, TiF_4 only significantly differed from the placebo. VDAC-1 expression was not altered by the fluorides; however, the placebo increased its relative expression compared to the control for both cells (Figures 3 and 4).

3.4. Western Blot

The relative expression of the proteins involved in the apoptosis pathway is shown in Figures 5 and 6. The Bax/Bcl-2 ratio and Fas-L expressions were not altered by the fluoride treatments regardless of the type of cell.

4. Discussion

Previous work from our group has shown that both NaF and TiF_4 (at 2.45%F) had similar cytotoxic effects on NIH/3T3, decreasing the cell viability by ~ 80% (MTT assay) after 6 h of treatment (Salomão et al. 2017). The present study has shown that the amount of cell death by the apoptosis mechanism is very low (<5%), suggesting that F might only induce viability loss (seen in the MTT assay) and/or that other mechanisms of cell death might be involved in the cytotoxicity of fluorides such as necrosis, a violent form of cells death (Barbier et al. 2010; Kerr et al. 1972). Necrosis has been observed in different cells treated with high fluoride concentrations via increase of oxidative stress (Barbier et al. 2010; Ghosh et al. 2008). Satoh et al. (2005) also suggest that NaF may induce non-apoptotic cell death, such as necrosis or autophagy, in fibroblasts.

TUNEL assay was applied in the present study to detect apoptosis. In this method, the DNA strands break is detected enzymatically by labeling the free 3'-OH termini with modified nucleotides that are generated upon DNA fragmentations in apoptotic cells (Gavrieli et al. 1992). However, its sensibility is questionable, since it is unclear how many DNA strand breaks are necessary for the detection. Other limitation of the method is its inability to differentiate breaks by apoptosis from those that happens during DNA repair and gene transcription (Kockx et al. 1998; Schaper et al. 1999; Watanabe et al. 2002). To overcome this limitation, a second method (caspases activity detection) was applied to confirm the occurrence of apoptosis (Watanabe et al. 2002).

In our study, no differences were found between NaF and TiF_4 with respect to the percentage of apoptosis induction, which allowed us to accept the first null hypothesis. This result is in agreement with previous publication of our group (Salomão

et al. 2017), which showed no difference between both fluorides with respect to NIH/3T3 viability loss.

Despite the low percentage of apoptosis found in the present study, we tried to understand the mechanism involved in its occurrence (mitochondria mediated by the Bcl-2 family- and/or death receptor- pathways), following the idea of previous works on the cytotoxicity mechanism of NaF (Inkielewicz-Stepniak et al. 2014; Lee et al. 2008).

Different from our previous study (Salomão et al. 2017), we decided to test a unique concentration of fluoride equivalent to the amount of fluoride found in commercial varnishes (such as Colgate Duraphat®). We also followed the time of 6 h application according to the manufacturer's recommendations and the results of previous works (Comar et al. 2014; Twetman et al. 1999). The varnishes were not applied in direct contact with the cells, but they were applied to devices that allowed fluoride to be released to the medium containing the cells at a distance of 5 mm (Salomão et al. 2017).

Another strong point of our study was the inclusion of 2 types of cells, which showed slight differences in their response to the effect of the tested fluorides. Immortalized cells (NIH/3T3) are genetically manipulated, which might influence their response to cytotoxic agents. To overcome this issue, primary cells should be used, whenever possible, to confirm the findings (Huang et al. 1999; Kaur and Dufour 2012). In the present study, HGF was more susceptible to the effect of the fluorides than NIH/3T3, regardless of the type of salt. Generally, higher percentage of apoptosis were found for HGF after 6h of treatment compared to NIH/3T3, which may be explained by the higher velocity of proliferation of the murine linage and consequent higher resistance to cytotoxic agents.

According to previous data, the F release from both varnishes in DMEM is about 0.9 mM in the 6-hour period (Salomão et al. 2017). Lee et al. (2008) showed that 20 mM of fluoride (NaF) induced 18% of HGF apoptosis in the 6-hour period, whereas Satoh et al. (2005) did not see DNA fragmentation in fibroblasts treated for 6 h with 0 to 20 mM NaF. Therefore, our results are in accordance with the literature.

With respect to the mechanism involved in apoptosis, our results showed that the fluorides were able to change mRNA expression (for some genes) but not the synthesis of proteins (western blot) or enzymatic activity (caspases assays). These results might be due to the low content of fluoride released by the varnishes into the medium and the short duration of application. Another possible hypothesis to justify the results is the induction of cell death by non-apoptotic pathway, as previous mentioned. We speculate that higher fluoride concentrations and/or longer periods of treatment would induce changes in the protein synthesis and enzymatic activity. However, they are not clinically relevant when we consider what happens *in vivo*.

Caspases are cysteine proteins activated in apoptotic cells that are divided into initiators and effectors. Their activities are initiated by stimulus generated in the cells' membrane or inside the cells, followed by a cascade of events, which finally induce DNA fragmentation (Agalakova and Gusev 2012; Hengartner 2000).

The lack of an effect of the fluorides on the caspases' activities are in accordance with Satoh et al. (2005), who did not find an alteration in the activities of caspases -3, -8 and -9 in HGF treated with 0 to 20 mM NaF for 4h. Otsuki et al. (2011) also failed to activate caspases -3 in HGF after 6h of treatment with 10 mM NaF. On the other hand, Lee et al. (2008) showed activity of caspases after a 6h-treatment of HGF with 20 mM NaF.

We investigated the intrinsic pathway, called also mitochondrial pathway mediated by Bcl-2 family, and the extrinsic or death-receptor pathway involved in the apoptotic effect of fluorides. The Bcl-2 family controls the intrinsic apoptotic pathway, regulating the release of molecules from mitochondria to cytosol, followed by caspases -9 and -3 activation (Agalakova and Gusev 2012; Shoshan-Barmatz et al. 2017). Bax and Bad, both pro-apoptotic members of the Bcl-2 family and the anti-apoptotic Bcl-2 were analyzed in the present study by RT-PCR. Furthermore, we included VDAC-1 and cytochrome C gene expression analysis. Although VDAC-1 plays an important role in the apoptosis cells, releasing cytochrome C from mitochondria into cytosol (Agalakova and Gusev 2012; Lee et al. 2008; Shoshan-Barmatz et al. 2017), it is also a multifunctional channel of mitochondria, which control the flux of other metabolites (Shoshan-Barmatz et al. 2017).

The extrinsic or death-receptor pathway involves the action of Fas-L on the cells' membrane, a ligand of death receptor able to induce a sequence of apoptotic events such as the caspases -8 and -3 activation (Elmore 2007; Hengartner 2000; Lee et al. 2008). In the present study, we analyzed the Fas-L gene expression.

With respect to RT-PCR results, no differences were found between NaF and TiF_4, allowing us to accept the 2nd null hypothesis. However, some interesting results were found when fluorides were compared to the control. NaF induced a higher expression of Fas-L for both types of cells compared to the control, suggesting that its mechanism of apoptosis could be extrinsic. Lee et al. (2008) also observed an up regulation of Fas-L expression in HGF after 6h of treatment with 5-20 mM NaF. However, we could not find higher proteins synthesis in NaF-treated cells using western blot. It is likely that NaF was able to increase the mRNA expression; however, the 6h treatment might be not enough time to significantly change the protein synthesis.

Our results are also in agreement with Inkielewicz-Stepniak et al. (2014), who found no difference in the Bax and Bcl-2 expression for cells treated with 0.5 and 1 mM NaF compared to the cells from the control. However, TiF_4 induced a lower gene expression Bad/Bcl-2 ratio on NIH/3T3, which could be considered an anti-apoptotic effect. However, this finding was not confirmed in HGF and not by western blot, which could be not clinically relevant.

Lee et al. (2008) found other alterations in western blot such as the increase in the expression of VDAC-1 (≥ 5 mM NaF) and the level of Cytochrome C release (≥ 10 mM NaF). Furthermore, these authors also found a down-regulation of Bcl-2 (≥ 10 mM NaF), an anti-apoptotic protein, but they could not find alteration in Bax, a pro-apoptotic member, regardless of fluoride concentration. The only possible explanation for the lack of such effects in the present study is the low fluoride amount released from the varnishes into the medium.

On the other hand, the present result is very promising considering the future clinical use of the experimental TiF_4 varnish to prevent dental caries and erosion development. Based on the results of the present study, we expect a low incidence of local side effects (such as mucosa desquamation) by its application. Taken together, the results of the present study and all works done in the field of tooth caries and erosion protection (Comar et al. 2012; Comar et al. 2015; Comar et al. 2017; Magalhães et al. 2008; Magalhães et al. 2016; Martines de Souza et al. 2017) support the conduction of clinical trials.

4.1. Conclusions

NaF and TiF_4, at the studied conditions, similarly induce a low percentage of apoptosis, with consequent modest activation of Bcl-2 and Fas-L-dependent signaling pathways. Generally, HGF is more susceptible to the fluoride effects than NIH/3T3.

Acknowledgments

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Competing interests: The authors declare no conflicts of interest. The author Magalhães AC has a patent called "*Compósito dentário contendo tetrafluoreto de titânio na forma de verniz*" (Dental compound containing titanium tetrafluoride in varnish), number PI 0705523-4, International classification: A61K 6/08; A61K 6/04 (March 1st, 2016).

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Table caption

Table 1. Mean and standard deviation values of the percentage-% of apoptosis assessed by TUNEL staining for NIH/3T3 and HGF after 6 h treatment.

Figure captions

Figure 1. Representative images (TUNEL assay, 40x) of the cells after 6 h of treatment: (A) Control, (B) Placebo, (C) 4.00% TiF_4, (D) 5.42% NaF for NIH/3T3 and (E) Control, (F) Placebo, (G) 4.00% TiF_4, (H) 5.42% NaF for HGF. DAPI stained all cell nuclei. TUNEL-positive cells showed fluorescein staining (see arrow). Scale bar, 20 μm.

Figure 2. Box plots of the activity of Caspases-3, -8 and -9 for both cells. Doxorubicin (15 μg/mL) was applied as the positive control for apoptosis. Distinct letters show significant differences among the treatments. Kruskal-Wallis test followed by Dunn's test (Caspase-3: $p=0.0002$ for NIH/3T3 and $p=0.0012$ for HGF. Caspase-8: $p<0.0001$ for NIH/3T3 and $p=0.0002$ for HGF. Caspase-9: $p<0.0001$ for both NIH/3T3 and HGF).

Figure 3. Box plots of the Bax/Bcl-2 ratio, Cytochrome C and Fas-L relative expressions for both cells. Distinct letters show significant differences among the treatments. Kruskal-Wallis followed by Dunn's test (Bax/Bcl-2 ratio: $p>0.05$ for both cells; cytochrome C: $p>0.05$ for NIH/3T3 and $p<0.001$ HGF; Fas-L: $p<0.0001$ for both cells).

Figure 4. Mean and standard deviation values for Bad/Bcl-2 ratio and VDAC-1 relative expressions for both cells. Distinct letters show significant differences among the treatments (ANOVA followed by Tukey test. Bad/Bcl-2 ratio: $p<0.05$ for both cells; VDAC-1: $p<0.05$ for NIH/3T3 and $p<0.001$ for HGF).

Figure 5. A. Representative image of the western blot for NIH/3T3 cells. B. Box plots of the Bax/Bcl-2 ratio and Fas-L normalized densitometry values for NIH/3T3 cells. Similar letters show no significant differences among the treatments. Kruskal-Wallis test ($p>0.05$).

Figure 6. A. Representative image of the western blot for HGF cells. B. Box plots of the Bax/Bcl-2 ratio and Fas-L normalized densitometry values for HGF cells. Similar letters show no significant differences among the treatments. Kruskal-Wallis test ($p>0.05$).

Table 1

Groups/ cells	NIH/3T3	HGF
Control	0.00±0.00[a]	0.00±0.00[a]
Placebo	0.13±0.23[b]	0.21±0.50[a]
TiF_4	1.00±0.73[c]	3.16±1.89[b]
NaF	0.63±0.27[bc]	4.41±2.29[b]

Footnote: Values in the same column with distinct superscript lowercase letters show significant differences (ANOVA followed by Tukey test. % apoptosis $p=0.0019$ and $p<0.0001$ for NIH/3T3 and HGF, respectively)

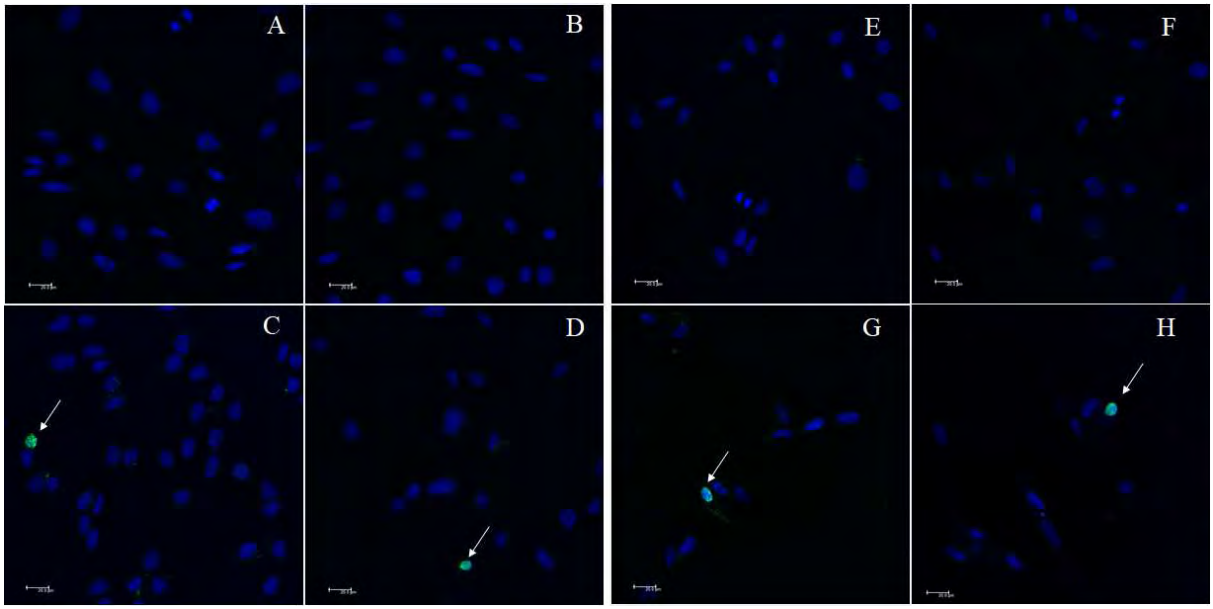


Figure 1

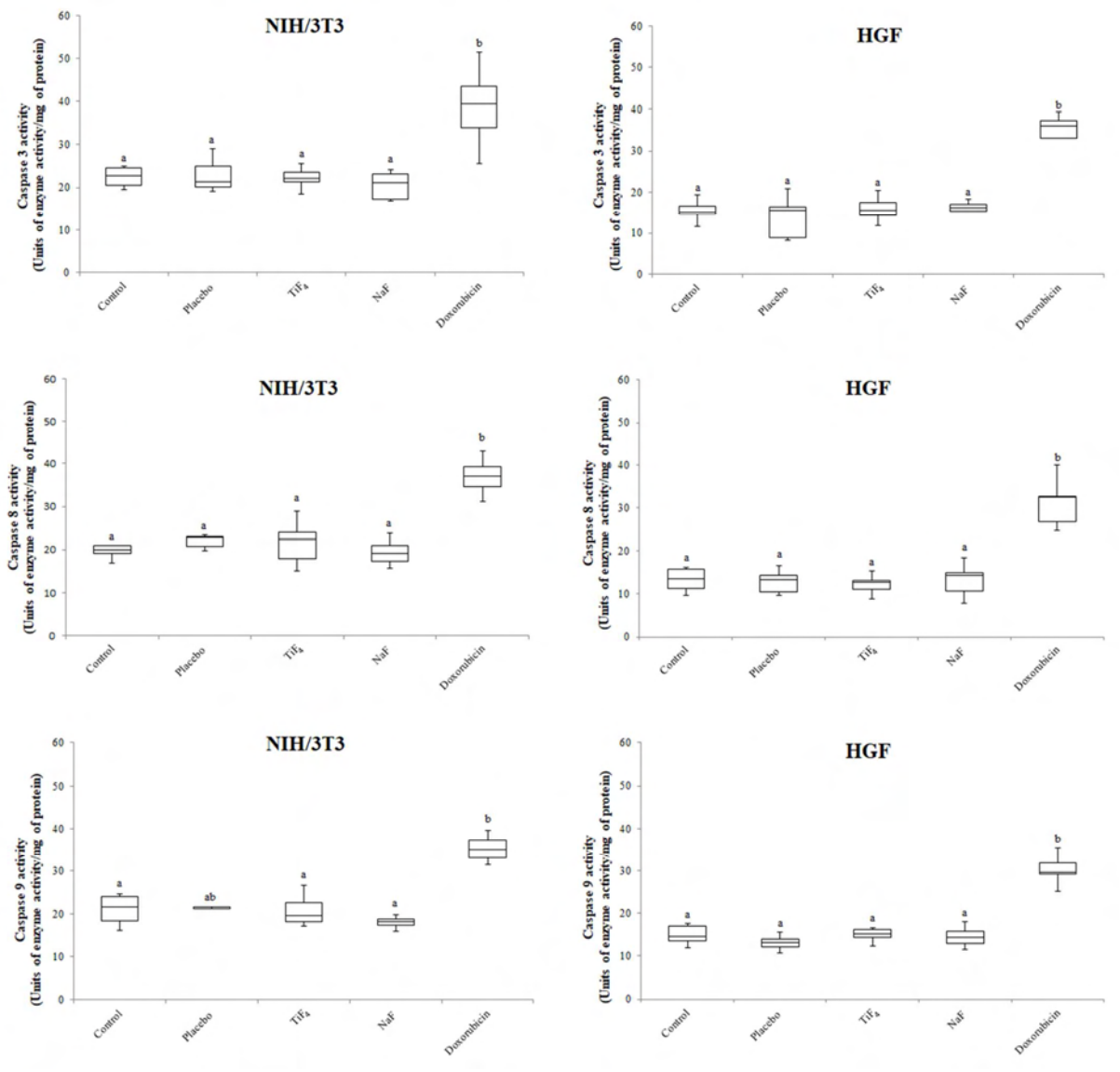

NIH/3T3
HGF
Caspase 3 activity (Units of enzyme activity/mg of protein)
Caspase 8 activity (Units of enzyme activity/mg of protein)
Caspase 9 activity (Units of enzyme activity/mg of protein)
Control
Placebo
TiF4
NaF
Doxorubicin

Figure 2

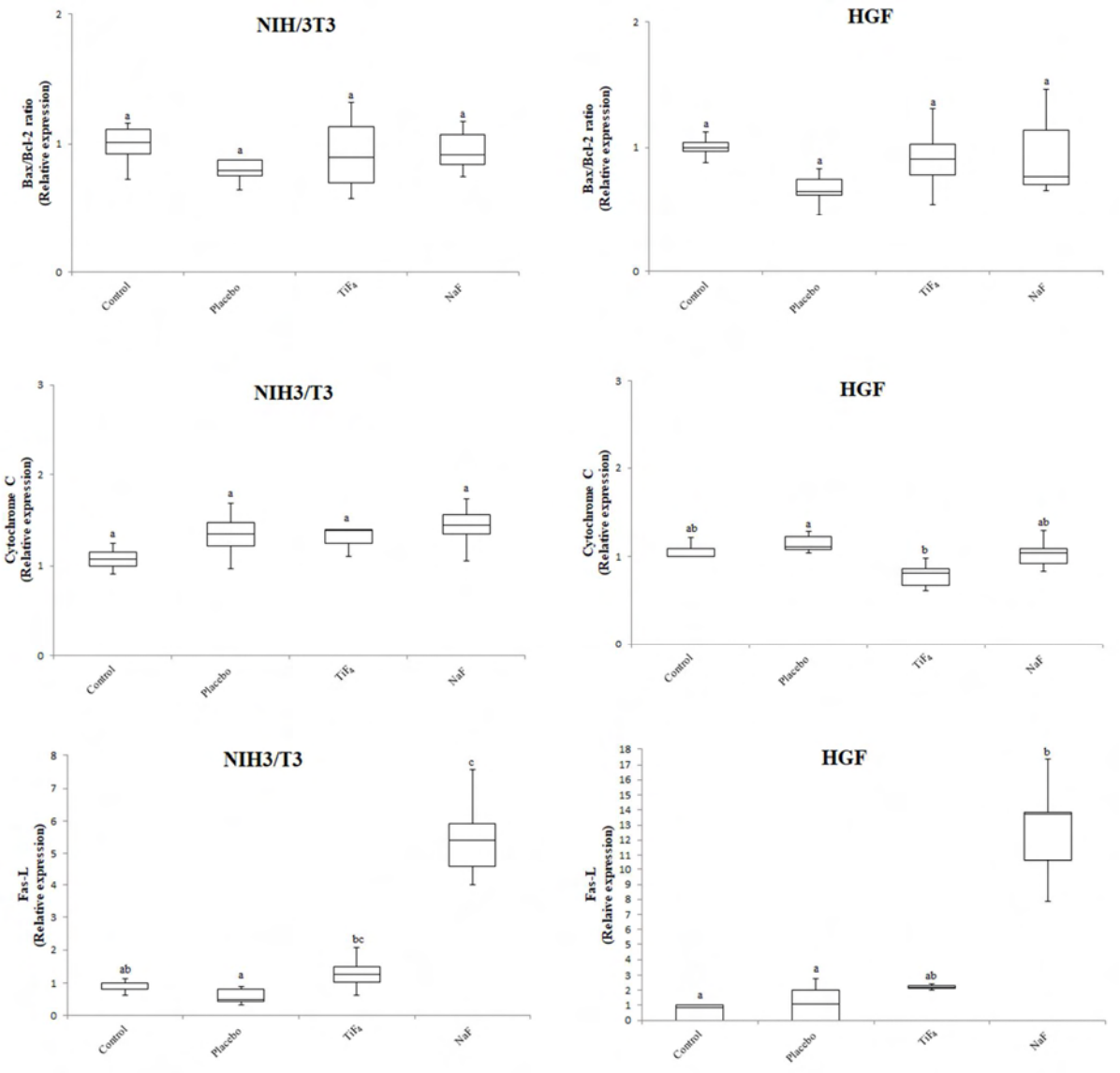

NIH/3T3
HGF
Bax/Bcl-2 ratio (Relative expression)
NIH3/T3
HGF
Cytochrome C (Relative expression)
NIH3/T3
HGF
Fas-L (Relative expression)
Control
Placebo
TiF4
NaF

Figure 3

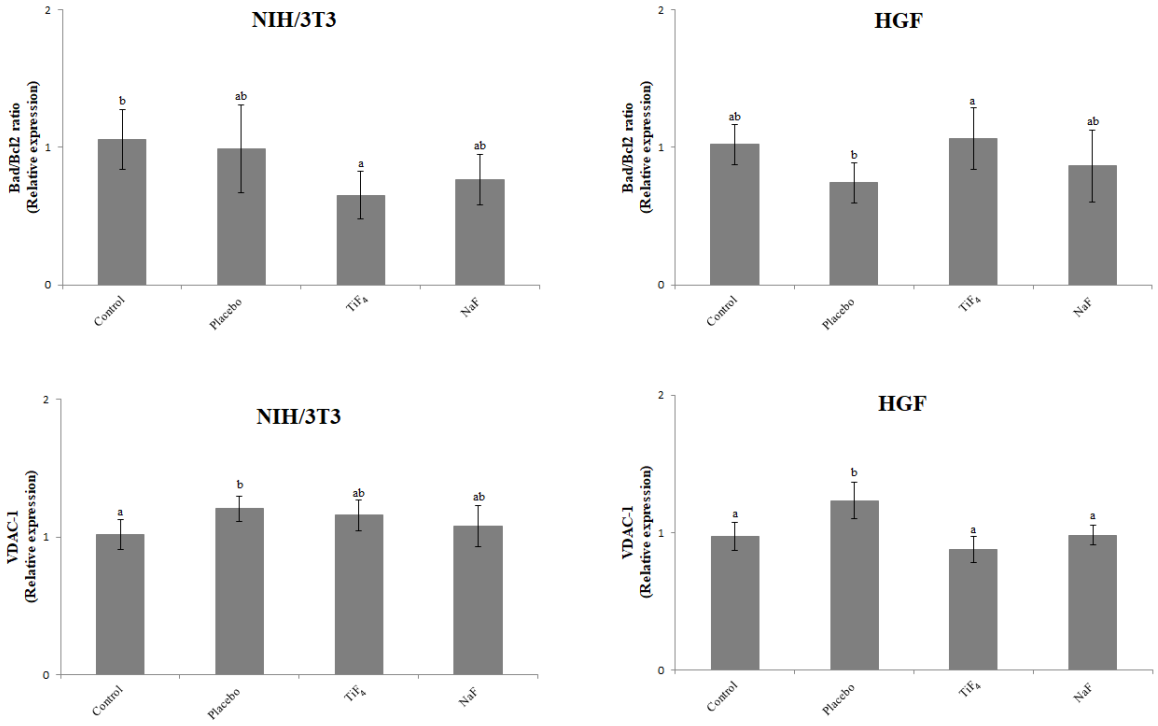

NIH/3T3
Bad/Bcl2 ratio (Relative expression)
b
ab
a
ab
Control
Placebo
TiF4
NaF
HGF
Bad/Bcl2 ratio (Relative expression)
ab
b
a
ab
Control
Placebo
TiF4
NaF
NIH/3T3
VDAC-1 (Relative expression)
a
b
ab
ab
Control
Placebo
TiF4
NaF
HGF
VDAC-1 (Relative expression)
a
b
a
a
Control
Placebo
TiF4
NaF

Figure 4

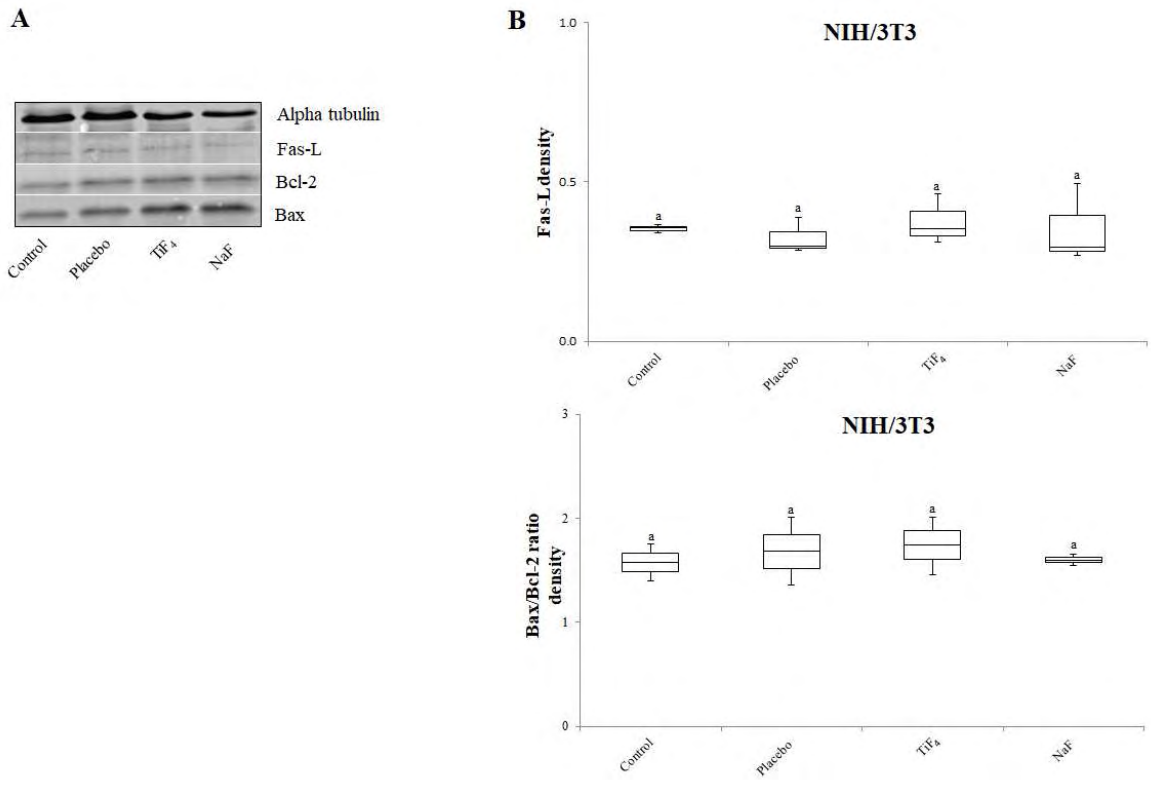
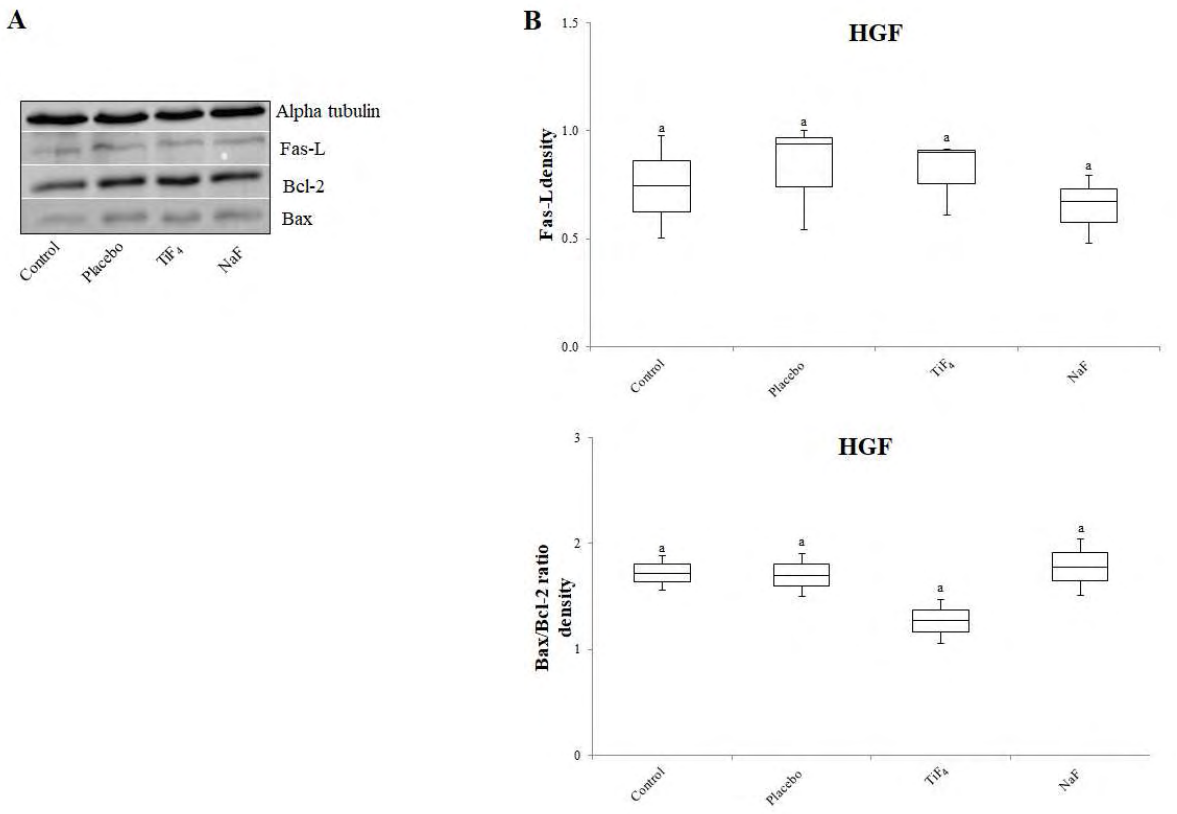

A
Alpha tubulin
Fas-L
Bcl-2
Bax
Control
Placebo
TiF4
NaF
B
NIH/3T3
Fas-L density
1.0
0.5
0.0
a
Control
Placebo
TiF4
NaF
NIH/3T3
Bax/Bcl-2 ratio density
3
2
1
0

Figure 5


A
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Bax/Bcl-2 ratio density
3
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Figure 6

3 DISCUSSION

3 DISCUSSION

Cytotoxicity assays are important tools in dentistry and should be performed before a novel material comes into the clinical use (JENG et al., 1998; INKIELEWICZ-STEPNIAK et al., 2014). Fibroblasts culture (from animals or humans) has been widely applied to test toxicity of biomaterials in Dentistry (LEE et al., 2008; INKIELEWICZ-STEPNIAK et al., 2014; KANSU et al., 2014; NISHIDA et al., 2016).

As stated in the Introduction, the experimental TiF_4 varnish is able to prevent and treat dental caries and erosive lesions better than the its solution and the conventional NaF varnish (MAGALHÃES et al., 2008a; MAGALHÃES et al., 2008b; COMAR et al., 2012; COMAR et al., 2015; LUSSI; CARVALHO, 2015; MAGALHÃES et al., 2016; COMAR et al., 2017b; MARTINES DE SOUZA et al., 2017). Therefore, *in* vitro and *in* vivo (animals) cytotoxicity studies are warranted to establish its safety for humans. NaF, on the other hand, was included in this study as a positive control since it is the fluoride salt mostly applied in the clinical therapy. Despite some studies have shown cytotoxicity of NaF (LEE et al., 2008; OTSUKI et al., 2011; INKIELEWICZ-STEPNIAK et al., 2014; SALOMÃO et al., 2017), it is still considered clinically safe.

We should keep in mind that other factors may have influence in the fluoride-induced cytotoxicity such as the type of cell, fluoride concentration and duration of the treatment (TSUTSUI et al., 1984; MATSUI et al., 2007; KARUBE et al., 2009; OTSUKI et al., 2011; ACRA et al., 2012; SALOMÃO et al., 2017).

The first paper of this thesis tested both fluoride solution and varnish on NIH/3T3, showing a very high cytotoxic potential of the solutions compared to the varnishes, regardless of the fluoride salt, which might be explained by the fact that not all fluoride from the varnishes were released into the medium (0.9 mM for 2.45% F after 6 h). We found a negative correlation between the fluoride release and the cell viability.

In both papers, the varnishes were not in direct contact with the cells, but they were applied on devices that allowed fluoride to be released to the medium containing the cells at a distance of 5 mm (SALOMÃO et al., 2017). With respect to the results of the 1st paper, it is very clear that the concentration of fluoride and the time of application have a significant impact on the cytotoxic potential of the fluorides

rather than the type of fluoride salt, inducing reduction of cell viability as well as changes in cell morphological and stiffness.

In accordance with our results, Inkielewicz-Stepniak et al. (2014) also showed a reduction in the viability of fibroblasts treated with 1.5 mM but not with 0.5 and 1 mM NaF, after 24 h of treatment. Similarly, Tsutsui et al. (1984) found that the cytotoxicity linearly increased with higher NaF doses (2.3 to 9.5 mM) and time of exposure (1-24 h). On the other hand, Lee et al. (2008) found that doses higher than 20 mM NaF were necessary to reduce cell viability.

Based on the result of the 1st paper, the focus of the 2nd paper was only the fluoride varnishes. Other important reason to study more deeply the cytotoxic potential of the varnishes only is their promising effect on the prevention of dental caries and erosion (MAGALHÃES et al., 2008a; COMAR et al., 2012; COMAR et al., 2015; LUSSI; CARVALHO, 2015; MAGALHÃES et al., 2016; COMAR et al., 2017b; MARTINES DE SOUZA et al., 2017).

For the 2nd paper, we decided to study only one fluoride concentration (2.45% F), equivalent to the amount of fluoride found in commercial varnishes (such as Colgate Duraphat®). We also followed the time of 6 h application according to manufactures' recommendations and to the results of previous works (TWETMAN; SKÖLD-LARSSON; MODÉER, 1999; COMAR et al., 2014). Twetman, Sköld-Larsson and Modéer (1999) showed that significant levels of fluoride in saliva did not exceed 6 h after treatment with fluoride varnishes *in vivo*. Furthermore, Comar et al. (2014) demonstrated that the peak of fluoride release from the experimental varnishes occurs in the first 3-6h *in vitro*.

The 2nd paper showed that the amount of cell death by the apoptosis mechanism is very low (<5%), suggesting that F might induced only viability loss (> 80% in the MTT assay applied in the 1st paper) after 6 h of treatment and/or that other mechanisms of cell death might be involved in the cytotoxicity of fluorides such as necrosis, a violent form of cells death (KERR; WYLLIE; CURRIE, 1972; BARBIER; ARREOLA-MENDOZA; DEL RAZO, 2010). Necrosis has been observed in different cells treated with high fluoride concentrations via increase of oxidative stress (GHOSH et al. 2008; BARBIER; ARREOLA-MENDOZA; DEL RAZO, 2010). Satoh et al. (2005) also suggest that NaF may induce non-apoptotic cell death, such as necrosis or autophagy, in fibroblasts.

Regardless of the most prevalent mechanism of cell death, our study showed no differences between NaF and TiF_4 with respect to the percentage of apoptosis induction.

Despite the low percentage of apoptosis found in the present study, we tried to understand the mechanism involved in its occurrence (mitochondrial mediated by the Bcl-2 family- and/or death receptor- pathways), following the idea of previous works on the cytotoxicity mechanism of NaF (LEE et al., 2008; INKIELEWICZ-STEPNIAK et al., 2014).

One strong point in the 2nd paper was the inclusion of two types of cells, which showed slight differences in their response to the effect of the tested fluorides. Immortalized cells (NIH/3T3) are genetically manipulated, which might influence their response to cytotoxic agents. To overcome this issue, primary cells should be used, whenever is possible, to confirm the findings (HUANG et al., 1999; KAUR; DUFOUR, 2012). In the present study, HGF was more susceptible to the effect of the fluoride varnishes than NIH/3T3, regardless of the type of salt. Generally, higher percentage of apoptosis was found for HGF after 6 h of treatment in comparison to NIH/3T3, which may be explained by the higher velocity of proliferation of the murine linage and consequent high resistance to cytotoxic agents.

The F release from both varnishes in DMEM was about 0.9 mM in the 6-hour period. Lee et al. (2008) showed that 20 mM of fluoride (NaF) induced 18% of HGF apoptosis in the 6-h period, whereas Satoh et al. (2005) did not see DNA fragmentation in fibroblasts treated for 6 h with 0 to 20 mM NaF. Therefore, our results are in accordance with the literature.

With respect to the mechanism involved in apoptosis, our results showed that fluorides were able to change mRNA expression (for some genes), but not the proteins synthesis (western blot) or enzymatic activity (caspases assays). These results might be due to the low content of fluoride released by the varnishes into the medium and/or the short duration of application. We speculate that higher fluoride concentrations and/or longer periods of the treatment would induce changes in the protein synthesis and enzymatic activity. However, they are not clinically relevant when we consider what happens *in vivo*.

Caspases are cysteine proteins activated in apoptotic cells that are divided into initiators and effectors. Their activities are initiated by stimulus generated at the

cells membrane or inside the cells, followed by a cascade of events, which finally induce DNA fragmentation (HENGARTNER, 2000; AGALAKOVA; GUSEV, 2012).

The lack effect of fluorides on the caspases activities are in accordance with Satoh et al. (2005), who did not show alteration in caspases -3, -8 and -9 activities in HGF treated with 0 to 20 mM NaF for 4h. Otsuki et al. (2011) also failed in activating caspase-3 in HGF cells after 6 h of treatment with 10 mM NaF. On the other hand, Lee et al. (2008) showed activity of caspases after 6 h treatment of HGF with 20 mM NaF.

We investigated the intrinsic pathway, called mitochondrial mediated by Bcl-2 family, and extrinsic or death-receptor pathway involved in the apoptotic effect of fluorides. The Bcl-2 family controls the intrinsic apoptotic pathway, regulating the release of molecules from mitochondria to cytosol, followed by caspases -9 and -3 activation (AGALAKOVA; GUSEV, 2012; SHOSHAN-BARMATZ; DE; MEIR, 2017). Bax and Bad, both pro-apoptotic members of Bcl-2 family, and the anti-apoptotic Bcl-2 were analyzed in the present study by RT-PCR. Furthermore, we included VDAC-1 and cytochrome C gene expression analysis. Although VDAC-1 plays an important role in apoptosis cells, releasing cytochrome C from mitochondria into cytosol (LEE et al., 2008; AGALAKOVA; GUSEV, 2012; SHOSHAN-BARMATZ; DE; MEIR, 2017), it is considered a multifunctional channel of mitochondria, which control the flux of different metabolites not necessarily involved in apoptosis (SHOSHAN-BARMATZ; DE; MEIR, 2017).

The extrinsic or death-receptor pathway involves the action of Fas-L on cells membrane, a ligand of death receptor able to induce a sequence of apoptotic events such as the caspases -8 and -3 activation (HENGARTNER, 2000; ELMORE, 2007; LEE et al., 2008). In the present study, we analyzed the Fas-L gene expression.

With respect to RT-PCR results, no differences were found between NaF and TiF_4. However, some interesting results were found when fluorides were compared to control. NaF induced a higher expression of Fas-L for both type of cells compared to the control, suggesting that its mechanism of apoptosis could be extrinsic. Lee et al. (2008) also observed an up regulation of Fas-L expression in HGF after 6 h of treatment with 5-20 mM NaF. However, we could not find higher proteins synthesis in NaF-treated cells by using western blot. It is likely that in our study NaF was able to increase the mRNA expression, however, the 6h-treatment might have not be enough time to significantly change the protein synthesis.

Our results are also in agreement with Inkielewicz-Stepniak et al. (2014), who found no difference in the Bax and Bcl-2 expression for 0.5 and 1 mM NaF compared to the control. However, TiF_4 induced a lower gene expression of Bad/Bcl-2 ratio on NIH/3T3, which could be considered an “anti-apoptotic” effect. However, this finding was not confirmed in HGF and not by western blot, which could be not clinically relevant.

Lee et al. (2008) found other alterations in western blot such as the increase in the expression of VDAC-1 (≥ 5 mM NaF) and the level of cytochrome C release (≥ 10 mM NaF). Furthermore, these authors also found a down-regulation of Bcl-2 (≥ 10 mM NaF), an anti-apoptotic protein, but they could not find alteration in Bax expression, a pro-apoptotic member, regardless of fluoride concentration. The only reason that could explain the lack of such effects in the present study is the low fluoride amount released from the varnishes into the medium.

On the other hand, the present result is very promising considering the future clinical use of the experimental TiF_4 varnish to prevent dental lesions development. Based on the results of the present study, we expect low incidence of local side effects (such as mucosa desquamation) by its application. Taken together the results of the present study and all works done on the field of tooth caries and erosion protection (MAGALHÃES et al., 2008a; MAGALHÃES et al., 2008b; COMAR et al., 2012; COMAR et al., 2015; MAGALHÃES et al., 2016; COMAR et al., 2017b; MARTINES DE SOUZA et al., 2017), there is enough support for the conduction of clinical trials.

4 CONCLUSIONS

4 CONCLUSIONS

1) TiF_4 and NaF have similar cytotoxic effects on NIH/3T3 viability, stiffness and morphology. The cytotoxic effects are dependent on the fluoride concentration and exposure time.

2) NaF and TiF_4, at the studied conditions, similarly induce a low percentage of apoptosis on NIH/3T3 and HGF, with consequent modest activation of Bcl-2 and Fas-L-dependent signaling pathways. Generally, HGF is more susceptible to the fluoride effects than NIH/3T3.

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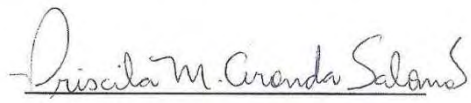
APPENDIX

APPENDIX A – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

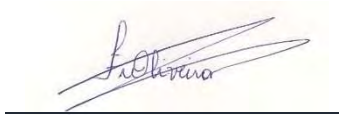
We hereby declare that we are aware of the article **The cytotoxic effect of TiF_4 and NaF on fibroblasts is influenced by the experimental model, fluoride concentration and exposure time** will be included in Thesis of the student Priscila Maria Aranda Salomão was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, November 13th, 2017.



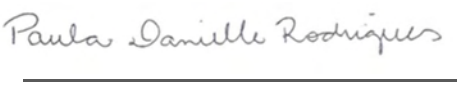
Priscila Maria Aranda Salomão

Author



Flávia Amadeu de Oliveira

Author



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Luana Polioni Al-Ahj

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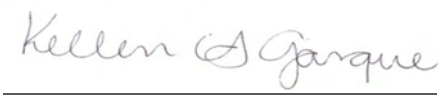

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Author

Pia Jeggle

Author

Marília Afonso Rabelo Buzalaf

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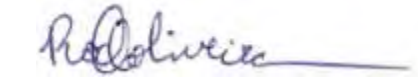
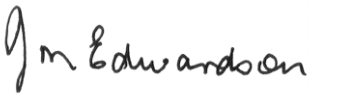


Rodrigo Cardoso de Oliveira

Author



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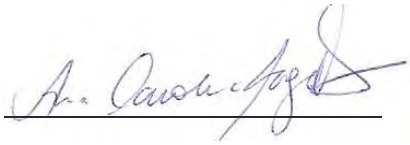


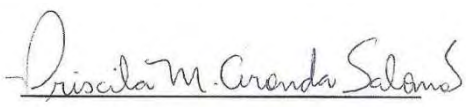
Ana Carolina Magalhães

Author

APPENDIX B – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We hereby declare that we are aware of the article **Apoptosis induced by TiF_4 and NaF varnishes on murine and human fibroblasts (NIH/3T3 and HGF): mitochondrial mediated by Bcl-2 family- and death receptor-dependent apoptotic mechanisms** will be included in Thesis of the student Priscila Maria Aranda Salomão was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, November 17th, 2017.



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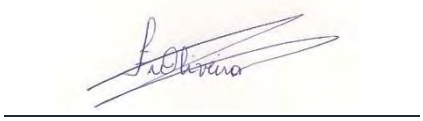

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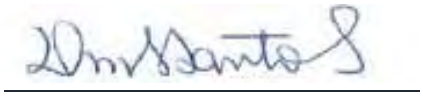

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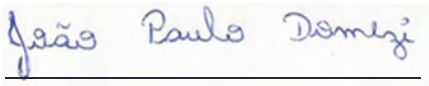

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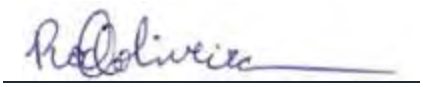

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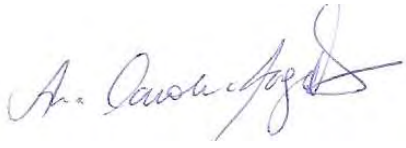
Author



Ana Carolina Magalhães
Author

ANNEXES

ANNEXES

ANNEX A – Ethics committee approval

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EFEITO DO VERNIZ DE TETRAFLUORETO DE TITÂNIO SOBRE FIBROBLASTOS GENGIVAIS HUMANOS NA SINALIZAÇÃO CELULAR PARA APOPTOSE

Pesquisador: Priscila Maria Aranda Salomão

Área Temática:

Versão: 2

CAAE: 71642517.6.0000.5417

Instituição Proponente: Faculdade de Odontologia de Bauru

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 2.270.214

Apresentação do Projeto:

Fibrobrastos Gengivais Humanos serão avaliados com relação à indução de apoptose e aos sinais celulares envolvidos na apoptose, após o tratamento com os vernizes fluoretados (TiF4 4,00% e Verniz NaF 5,42%; Placebo, sem tratamento e Doxorrubicina). Para detecção da apoptose, será utilizado um kit por marcação de TUNEL (Chemicon, Millipore), na qual os fibroblastos serão imuno-marcados, visualizados e quantificados

utilizando microscópio de fluorescência. Os sinais celulares envolvidos na apoptose serão avaliados pela atividade das caspases, PCR e Western Blot. A atividade das caspases 9, 8 e 3 será avaliada com a utilização de kits de ensaio (Chemicon, Millipore) e a absorbância será avaliada em leitor de microplaca. A expressão gênica será avaliada através do ensaio de qPCR. Para tal, utilizaremos kits comerciais para a extração e transcrição

reversa, e primers para a detecção e quantificação do transcrito para citocromo C, PARP, Bad, Bax, Bcl-2, VDAC, FasL e RPL13 (Life Technologies, CA e Applied Biosystems) no aparelho Via 7 (Applied Biosystems). A expressão proteica será avaliada por Western Blot, utilizando anticorpos primários (ABCAM) que serão escolhidos após os resultados do qPCR. Na sequência incubaremos com anticorpos secundários específicos e as bandas marcadas pelos anticorpos serão detectadas utilizando-se um kit de detecção de quimioluminescência (GE Healthcare). Os dados serão

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estatisticamente analisados considerando um nível de significância de 5%.

Objetivo da Pesquisa:

O objetivo do projeto será avaliar os sinais celulares envolvidos na apoptose de fibroblastos gengivais humanos (FGH) submetidos ao tratamento com verniz de tetrafluoreto de titânio (TiF4) comparado ao fluoreto de sódio (NaF).

Avaliação dos Riscos e Benefícios:

Utilizaremos material biológico armazenado (Fibroblastos Gengivais Humanos), doados através de cessão (carta de cessão anexada na Plataforma Brasil), portanto não haverá envolvimento direto de participantes da pesquisa.

Como não haverá envolvimento direto de participantes da pesquisa, nenhum benefício direto está previsto.

No entanto, sabemos que o conhecimento sobre o potential tóxico de novos produtos é essencial para o delineamento de estudos clínicos. Portanto, este conhecimento poderá nortear estudos clínicos que a longo prazo poderão beneficiar os indivíduos que necessitarem de tratamento com verniz de TiF4 para controle da cárie e erosão dentárias.

Comentários e Considerações sobre a Pesquisa:

A pesquisa é relevante e trará benefícios substanciais para a área de conhecimento em que está inserida.

Considerações sobre os Termos de apresentação obrigatória:

Os termos apresentados são adequados para a pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Após os esclarecimentos prestados e apresentação do TCLE assinado pelo doador de tecido gengival, considero o projeto aprovado.

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 06.09.2017, 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.

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Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_937229.pdf	31/08/2017 13:49:52		Aceito
Outros	Oficio_Pendencia_Priscila_Salomao.pdf	31/08/2017 13:36:08	Priscila Maria Aranda Salomão	Aceito
Outros	TCLE_em_acordo_Resolucao_CNSno_441_2011.pdf	31/08/2017 13:34:04	Priscila Maria Aranda Salomão	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Priscila_Salomao.docx	31/08/2017 13:28:46	Priscila Maria Aranda Salomão	Aceito
Folha de Rosto	Folha_rosto_final.pdf	19/07/2017 13:42:02	Priscila Maria Aranda Salomão	Aceito
Outros	termo_outorga_bolsa_FAPESP.pdf	04/07/2017 14:26:28	Priscila Maria Aranda Salomão	Aceito
Outros	termo_outorga_auxilio_FAPESP.pdf	04/07/2017 14:20:16	Priscila Maria Aranda Salomão	Aceito
Outros	Questionario_tecnico_do_pesquisador.pdf	04/07/2017 13:59:46	Priscila Maria Aranda Salomão	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Justificativa_ausencia_TCLE.pdf	04/07/2017 13:43:09	Priscila Maria Aranda Salomão	Aceito
Declaração de Instituição e Infraestrutura	CIP.pdf	04/07/2017 13:42:13	Priscila Maria Aranda Salomão	Aceito
Declaração de Instituição e Infraestrutura	Departamento_Ciencias_Biologicas.pdf	04/07/2017 13:39:02	Priscila Maria Aranda Salomão	Aceito
Declaração de Pesquisadores	Declaracao_de_pesquisadores.pdf	04/07/2017 13:36:59	Priscila Maria Aranda Salomão	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	cessao.JPG	20/06/2017 14:20:35	Priscila Maria Aranda Salomão	Aceito
Orçamento	Orcamento.docx	13/06/2017 16:35:10	Priscila Maria Aranda Salomão	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

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BAURU, 12 de Setembro de 2017

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

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ANNEX B – Manuscript submission letter confirmation from Toxicology

De: Toxicology <eesserver@eesmail.elsevier.com>
Data: seg, 27 de nov de 2017 às 02:03
Assunto: Toxicology: Submission Confirmation
Para: <acm@fob.usp.br>

Ms. No.: TOX-17-801
Title: Apoptosis induced by TiF4 and NaF varnishes on murine and human fibroblasts (NIH/3T3 and HGF): mitochondrial mediated by Bcl-2 family- and death receptor-dependent apoptotic mechanisms
Corresponding Author: Professor Ana Carolina Magalhães
Authors: Priscila Salomão; Flávia Oliveira; Daiana Santos; João Domezi; Thiago Dionísio; Rodrigo Oliveira;
Article Type: Full Length Article

Dear Professor Magalhães,

The above-mentioned manuscript has been received for consideration for publication in Toxicology.

Your manuscript number is: TOX-17-801

You will be able to check on the progress of the review by logging on to the Elsevier Editorial System for Toxicology as an author:

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Kind regards,

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