TiF₄ and NaF varnishes induce low levels of apoptosis in murine and human fibroblasts through mitochondrial Bcl-2 family and death receptor signalling

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1. Introduction

Sodium fluoride (NaF) a popular fluoride salt that is applied to prevent tooth caries and erosion (Castillo, Salomão, Buzalaf, & Magalhães, 2015; Magalhães, Wiegand, Rios, Buzalaf, & Lussi, 2011; Pessan, Toumba, & Buzalaf, 2011). NaF is included in professional and home-care products since it is considered safe and has no relevant local side-effects; however, in vitro studies in fibroblasts have shown that NaF can cause damage at the cellular level (Inkiewicz-Stepniak, Santos-Martinez, Medina, & Radomski, 2014; Jeng et al., 1998; Lee et al., 2008; Otsuki, Sugiyama, Amano, Yasui, & Sakagami, 2011; Salomão et al., 2017). NaF can also induce apoptosis depending on the concentration and frequency of application (Inkiewicz-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 the undesired systemic effects on hard tissues that can be caused by excessive fluoride ingestion, such as dental or skeletal fluorosis (Ekstrand, Koch, & Petersson, 1980; Olympio et al., 2009; Pessan et al., 2005). Soft tissues, such as the liver, heart and kidney, are also susceptible to the toxic effects of fluoride in water (Zuo et al., 2018).

Since 2008, TiF₄ varnish has been tested as an alternative to NaF varnish, especially for patients with a high risk of tooth caries and/or erosion. Several in vitro and in situ studies have shown that TiF₄ varnish has a stronger protective effect against tooth demineralization than NaF varnish (Comar et al., 2012, 2015; Comar et al., 2017; Magalhães et al., 2008, 2016; Martins de Souza, Vertuan, Buzalaf, & Magalhães, 2017). A recent in situ study demonstrated that TiF₄ varnish was able to induce remineralization of artificial carious lesions regardless of the cariogenic activity, while NaF varnish 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 TiF₄ varnish on dental lesions. However, it is important to determine the cytotoxic potential of TiF₄ varnish before applying it in patients, due to its low pH. A decrease in the pH of the medium facilitates fluoride influx but depresses efflux, inducing intracellular accumulation and inhibiting cell proliferation (Kawase &
Recently, our research group showed that both NaF and TiF₄ varnish similarly reduce NIH/3T3 viability and increase cell death (Salomão et al., 2017). Therefore, there is a need to understand their mechanisms of toxicity. Apoptosis is one cell death mechanism. Some studies have shown that NaF induced apoptosis in fibroblasts via the mitochondria mediated by both the Bcl-2 family and death receptor pathways (Inkiewicz-Stepniak et al., 2014; Lee et al., 2008). Therefore, the aims of this study are to compare the level of apoptosis induced by both fluorides, as well as to elucidate the apoptotic mechanisms involved.

It is important to use a primary human cell instead of an immortalized murine lineage cell, as done in our previous study (Salomão et al., 2017), to better simulate the in vivo conditions (Inkiewicz-Stepniak et al., 2014; Jeng et al., 1998; Lee et al., 2008; Otsuki et al., 2011). Therefore, this study also investigated the apoptotic mechanisms of both fluorides on NIH/3T3 cells and primary human gingival fibroblasts (HGF).

Previous studies have allowed a long time for contact between the fluoride and the cells. This does not simulate the real oral conditions, in which saliva washes the product away and thereby reduces the concentration in the mouth over time (Inkiewicz-Stepniak et al., 2014; Salomão et al., 2017). Tweetman, Sköld-Larsson, and Modéér, (1999) showed that significant levels of fluoride in saliva do not persist 6 h after treatment with fluoride varnishes in vivo. Accordingly, Comar, Souza, Grizzo, Buzalaf, and Magalhães, (2014) demonstrated that the peak of fluoride release into saliva from the varnishes occurs in the first 3–6 h in vitro.

Considering the current knowledge, this study tests the following null hypotheses: 1) There is no difference between NaF and TiF₄ varnishes with respect to the level of apoptosis in NIH/3T3 and HGF cells submitted to a 6 h treatment; 2) There is no difference between NaF and TiF₄ varnishes with respect to the molecular pathways involved in apoptosis of NIH/3T3 and HGF cells submitted to a 6 h 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 (approved by the Local Ethical Committee, Process n° CAEE 71,642,517.6.0000.5417), 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% CO₂. Enzymatic digestion with 0.25% trypsin (Sigma-Aldrich Co. LLC, St. Louis, USA) was used to harvest the cells for experimental analyses. 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 13 mm diameter plastic coverslip (Sarstedt, Nürnberg, Germany) in 24-well microplates at a density of 5 × 10⁵ cells per well (1.5 mL culture medium per well) for 24 h and were then treated with experimental varnishes containing 4.00% TiF₄ or 5.42% NaF (both at 2.45% F, native pH 1.0 for TiF₄ and pH 5.0 for NaF), experimental placebo varnish (similar composition of the other varnishes but without F, pH 5.0) or non-treated cells (control) for 6 h. All varnishes were prepared by FGM-Dentcare (Joinville, Brazil). The varnishes were applied as previously described for 6 h (Salomão et al., 2017). The pH of the medium was kept neutral through the experiment, regardless of the treatment.

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 8 × 10⁴ cells per well (1.5 mL culture medium per well) for 24 h and were then treated as previously described for 6 h (Salomão et al., 2017). Doxoruubicin (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 units of enzyme activity/mg of protein was calculated. For this, protein concentrations were determined by the Bradford method (BioRad, Hercules, USA).

2.4. Real time RT PCR

Cells were cultivated in 24-well microplates at a density of 8 × 10⁴ cells per well (1.5 mL culture medium per well) for 24 h and were then treated as previously described for 6 h (Salomão et al., 2017). Cells were harvested, and four wells were pooled. Then, 1 × 10⁵ cells from each treatment were submitted to mRNA extraction using the PureLink® RNA Mini Kit (Life Technologies, 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 (Mm00432042_m1 and Hs0188930_m1), Bax (Mm00432051_m1 and Hs00608203_m1), Bcl-2 (Mm00477631_m1 and Hs00181225_m1), and Fas-L genes (Mm00438864_m1 and Hs00181225_m1).

The reading was performed using the RT-PCR System ViiATM7 (Applied Biosystems, Foster, USA) under the following conditions: 95 °C for 10 min plus 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Act-b expression (Mm00475337_m1) was used as the control for NIH/3T3 cells, and RPL-13 expression (Hs00744303_s1) was used as the control for HGF cells (Applied Biosystems, Foster, USA). The relative expression was obtained using the formula 2−ΔΔCt.

2.5. Western blot

Cells were cultivated in 6-well microplates at a density of 5 × 10⁵
cells per well (7 mL culture medium per well) for 24 h and were then treated as previously described for 6 h (Salomão et al., 2017). After washing with PBS, cells were lysed with buffer solution containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl₂ 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 4 °C. Protein samples quantified by the Bradford method (50 μg/treatment) were applied to electrophoresis in a Tris-HCl 12.5% polyacrylamide gel and were subsequently transferred to a PVDF membrane. The membrane was immune-labelled with rabbit polyclonal anti-Fas ligand, 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 arbitrary density values obtained were normalized using 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 for statistical analysis. The data were submitted to a normality test (Kolmogorov-Smirnov test) and equality of variances test (Bartlett test). Values obtained from the caspase activity, RT-PCR (Bax/Bcl-2 ratio and Fas-L) and western blot assays were compared using the Kruskal-Wallis test followed by a post-hoc Dunn’s test. Values obtained from the in situ apoptosis detection by TUNEL staining and RT-PCR (Bad/Bcl-2 ratio) assays were compared using an ANOVA followed by a post-hoc Tukey test. The level of significance was set at 5%.

3. Results

3.1. In situ apoptosis detection by TUNEL staining

In NIH/3T3 cells, all varnishes significantly increased the percentage of apoptosis compared to the control. The highest percentage of apoptosis was observed for TiF₄-treated cells compared to the control and the placebo varnish; however, they did not significantly differ from the NaF-treated cells. In the HGF cells, 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 Fig. 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 (Fig. 2).

3.3. Real time RT PCR

The relative expression of the genes involved in the apoptosis pathway is shown in Figs. 3 and 4. The Bax/Bcl-2 ratio was not altered by the fluoride treatments compared to the control regardless of the type of cell. However, the NaF varnish increased the Fas-L expression level 5-fold and 14-fold compared to the control for the NIH/3T3 and HGF cells, respectively. No difference was detected between the TiF₄ and NaF-treated cells with respect to Fas-L gene expression (Fig. 3).

Cells treated with TiF₄ varnish presented a significantly lower Bad/Bcl-2 expression ratio compared to the control but not compared to the placebo and NaF varnishes in the case of the NIH/3T3 cells. For the HGF cells, TiF₄ only caused a significant difference from the placebo (Fig. 4).

3.4. Western blot

The relative expression levels of apoptosis pathway proteins are shown in Figs. 5 and 6. The Bax/Bcl-2 ratio and Fas-L expression 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 TiF₄ (at 2.45%F) had similar cytotoxic effects on NIH/3T3 cells, decreasing cell viability by ∼80% (MTT assay) after 6 h of treatment (Salomão et al., 2017). The present study has shown that the level of cell death by apoptosis 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 cell death (Barbier, Arreola-Mendoza, & Del Razo, 2010; Kerr, Wyllie, & Currie, 1972). Necrosis has been observed in different cells treated with high fluoride concentrations due to an increase of oxidative stress (Barbier et al., 2010; Ghosh, Das, Manna, & Sil, 2008). Satoh et al. (2005) also suggest that NaF may induce non-apoptotic cell death, such as necrosis or autophagy, in fibroblasts.

The TUNEL assay was applied in the present study to detect apoptosis. In this method, a DNA strand break is detected enzymatically using modified nucleotides to label free 3′-OH termini that are generated upon DNA fragmentation in apoptotic cells (Gavrieli, Sherman, & Ben-Sasson, 1992). However, the sensitivity of the TUNEL assay is questionable since it is unclear how many DNA strand breaks are necessary for detection. Another limitation of the method is its inability to differentiate breaks caused by apoptosis from those that happen during DNA repair and gene transcription (Kockx, Muhring, Knaapen, & de Meyer, 1998; Schaper, Elsässer, & Kostin, 1999; Watanabe et al., 2002).

To overcome this limitation, a second method (caspase activity detection) was applied to confirm the occurrence of apoptosis (Watanabe et al., 2002).

In our study, no differences were found between NaF and TiF₄ with respect to the level of apoptosis induction, which allowed us to accept the first null hypothesis. This result is in agreement with a previous publication of our group (Salomão et al., 2017), which showed no difference between both fluorides with respect to loss of viability in NIH/3T3 cells, despite both varnishes presenting different pH values. However, the medium pH was not changed by TiF₄ varnish application, which may explain the different result presented here versus those from the study of Sen, Kazemi, and Spångberg, (1998).

Despite the low level of apoptosis found in the present study, we tried to understand the mechanism involved in its occurrence (mitochondrial signalling via 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).

In a manner that was 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 performed a 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 on devices that allowed fluoride to be released to the medium containing the cells at a distance of 5 mm (Salomão et al., 2017). Furthermore, to attribute the observed effect to the fluorides, a placebo varnish group was included. All varnishes (fluoridated or placebo) had a similar composition to isolate the effects of the type of fluoride.

Another strong point of our study was the inclusion of 2 types of cells, which showed slight differences in their responses to 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 & Dufour, 2012). In the present study, HGF cells were more susceptible to the effect of the fluorides than NIH/3T3 cells, regardless of the type of salt. Generally, a higher percentage of apoptosis was found for HGF cells after 6 h of treatment compared to NIH/3T3 cells, which may be explained by the higher rate of proliferation of the murine lineage and consequent higher resistance to cytotoxic agents.

According to previous data, the amount of F released from both varnishes in DMEM is approximately 0.9 mM in the 6-h period (Salomão et al., 2017). Lee et al. (2008) showed that 20 mM of fluoride (NaF) induced apoptosis in ~18% of HGF cells during the same length of treatment, 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 show that the fluorides are able to change miRNA expression (for some genes) but not the synthesis of proteins (based on our western blots) or enzymatic activity (based on our caspase activity assays). These results might be due to the low fluoride content released by the varnishes into the medium and the short duration of application. Another possible explanation is the induction of cell death by a non-apoptotic pathway, as previously mentioned. We speculate that higher fluoride concentrations and/or longer periods of treatment might induce changes in

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**Fig. 3.** Box plots of the Bax/Bcl-2 ratio and Fas-L relative expressions for both cells. Distinct letters show significant differences among the treatments. Statistical values were determined using the Kruskal-Wallis followed by Dunn’s test (Bax/Bcl-2 ratio: p > 0.05 for both cells; Fas-L: p < 0.0001 for both cells).

**Fig. 4.** Mean and standard deviation values for Bad/Bcl-2 ratio for both cells. Distinct letters show significant differences among the treatments (Statistical values were determined by ANOVA followed by Tukey test. Bad/Bcl-2 ratio: p < 0.05 for both cells).
protein synthesis and enzymatic activity. However, testing such conditions is not realistic when we consider what happens in vivo.

Caspases are cysteine proteases that are activated in apoptotic cells, and they are classified as either initiators or effectors. Their activities are initiated by stimulus generated either in the cell membrane or inside the cell and is followed by a cascade of events that finally induce DNA fragmentation (Agalakova & Gusev, 2012; Hengartner, 2000). Despite the fact that F (especially NaF) has been shown to inhibit protease activity, especially that of MMPs and cysteine cathepsins in different tissues (DenBesten et al., 2002), our study showed no effect of fluorides on the tested caspases.

The lack of effect of the fluorides on caspase activity 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 0–20 mM NaF for 4 h. Otsuki et al. (2011) also failed to observe caspase-3 activation in HGF after 6 h of treatment with 10 mM NaF. On the other hand, Lee et al. (2008) showed caspases activity after a 6 h-treatment of HGF with 20 mM NaF, a concentration that is much higher than those released by our varnishes into the medium.

We investigated the intrinsic pathway, also called the mitochondrial pathway mediated by the 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 the mitochondria to the cytosol, followed by caspase -9 and -3 activation (Agalakova & Gusev, 2012; Shoshan-Barmatz, De, & Meir, 2017). Bax and Bad, both pro-apoptotic members of the Bcl-2 family, and the anti-apoptotic Bcl-2 were analysed in the present study by RT-PCR.

Fig. 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. Statistical values were determined using the Kruskal-Wallis test (p > 0.05).

Fig. 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. Statistical values were determined using the Kruskal-Wallis test (p > 0.05).
The extrinsic or death-receptor pathway involves the action of Fas-L on the cell membrane, a death receptor ligand that is able to induce a sequence of apoptotic events such as caspase-8 and -3 activation (Elmore, 2007; Hengartner, 2000; Lee et al., 2008). In the present study, we analysed Fas-L gene expression.

With respect to RT-PCR results, no differences were found between NaF and TiF₄, allowing us to accept the 2nd null hypothesis; in other words, we could not find any differences in gene expression involved in the apoptosis process (except for the Fas-L gene). It is important to keep in mind that post-translational modification was not analysed in the present study, such as (de)phosphorylation or proteolytic cleavage of Bad, which has been discussed as a possible death signal of the core apoptotic machinery at the mitochondria (Danial & Korsmeyer, 2004; Danial, 2009; Youle & Strasser, 2008). Correia et al. (2015) also show that the anti-apoptotic functions of Bcl-2 are governed not only by changes in expression but also by phosphorylation. We did not study this process deeply, since our results (Bax/Bcl-2 and caspases activity) do not support our previous hypothesis that apoptosis would be the main mechanism involved in the toxicity of fluoride varnishes.

An interesting result was 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 upregulation of Fas-L expression in HGF after 6 h of treatment with 5–20 mM NaF. However, we could not detect higher protein synthesis in NaF-treated cells by western blot. It is likely that NaF was able to increase the mRNA expression; however, the 6-h treatment might not be enough time to significantly change protein synthesis.

Our results are also in agreement with Inkilewicz-Stepniak et al. (2014), who found no difference in Bax and Bcl-2 expression for cells treated with 0.5 and 1 mM NaF compared to the cells from the control. However, TiF₄ induced a lower Bad/Bcl-2 gene expression ratio in NIH/3T3 cells, which could be considered to be an anti-apoptotic effect. However, this finding was not confirmed in HGF cells or by western blot, so it may not be clinically relevant.

Lee et al. (2008) detected a down-regulation of Bcl-2 (≥ 10 mM NaF), an anti-apoptotic protein, but they could not find an alteration in Bax, a pro-apoptotic factor, regardless of the fluoride concentration. The only possible explanation for the lack of such effects in the present study is the low amount of fluoride released by the varnishes into the medium during the 6 h of contact.

On the other hand, the present results are very promising considering the clinical application of the experimental TiF₄ varnish to prevent tooth cavities and erosion in the future. Based on the results of the present study, we expect a low incidence of local side effects (such as muco-pdesquamation) by its application. Taken together, the results of the present study and of all works in the field of tooth caries and erosion protection (Comar et al., 2012, 2015; Comar et al., 2017; Magalhães et al., 2008, 2016; Martines de Souza et al., 2017) support the conduction of clinical trials.

5. Conclusion

NaF and TiF₄, in the studied conditions, similarly induce a low level of apoptosis, with consequent modest activation of Bcl-2 and Fas-L-dependent signalling pathways. Generally, HGF cells are more susceptible to the fluoride effects than NIH/3T3 cells.

Conflict of interest

None.

Ethical approval

Ethics Committee from Bauru School of Dentistry/USP – Brazil (CAAE 71,642,517.6.0000.5417).

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