Archives of Oral Biology 91 (2018) 57–62

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Cytotoxicity of novel fluoride solutions and their influence on mineral loss from enamel exposed to a *Streptococcus mutans* biofilm

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ARTICLE INFO

Keywords:
Fluoride
Nanotechnology
Dental caries
S. mutans biofilm

ABSTRACT

Objective: This study evaluated the cytotoxicity, antimicrobial activity and *in vitro* influence of new fluoridated nanocomplexes on dental demineralization.
Design: The nanocomplexes hydroxypropyl-β-cyclodextrin with 1% titanium tetrafluoride (TiF_4) and γ-cyclodextrin with TiF_4 were compared to a positive control (TiF_4), a blank control (without treatment) and negative controls (hydroxypropyl-β-cyclodextrin, γ-cyclodextrin, deionized water), following 12- and 72-hour complexation periods. The cytotoxicity was assessed using the neutral red dye uptake assay at T1–15 min, T2–30 min and T3–24 h. A minimum bactericidal concentration (MBC) against *Streptococcus mutans* (ATCC 25175) was performed. Enamel blocks were exposed to an *S. mutans* biofilm, and the percentage of surface microhardness loss was obtained. Biocompatibility and microhardness data were analysed using ANOVA/Tukey tests ($p < 0.05$).
Results: At T1, the cell viability results of the nanocomplexes were similar to that of the blank control. At T2 and T3, the 72 h nanocomplexes demonstrated cell viability results similar to that of the blank, while the 12 h solutions showed results different from that of the blank ($p < 0.05$). All fluoridated nanocompounds inhibited *S. mutans* (MBC = 0.25%), while the MBC of TiF_4 alone was 0.13%. All fluoridated compounds presented a percentage of surface microhardness loss lower than that of deionized water ($p < 0.05$).
Conclusions: The new fluoridated nanocomplexes did not induce critical cytotoxic effects during the experimental periods, whilst they did show bactericidal potential against *S. mutans* and inhibited enamel mineral loss.

1. Introduction

Titanium tetrafluoride (TiF_4) is a preventive agent against enamel dissolution, both in caries lesions (Mundorff, Little, & Bibby, 1972; Shrestha, Mundorff, & Bibby, 1972; Reed & Bibby, 1976; Büyükyilmaz et al., 1994; Comar et al., 2012; Alexandria et al., 2017) and in dental erosion lesions (Levy, Rios, Buzalaf, & Magalhães, 2014; Souza, Lima, Comar, Buzalaf, & Magalhães, 2014). This agent also presents remineralizing properties over the dental enamel substrate (Comar et al., 2012; Vieira et al., 2017), positively changes tubule occlusion of eroded/abraded human dentin *in situ* (Mantilla et al., 2017), and can be incorporated into primer (two-step self-etching adhesive system), presenting great stability (Torres et al., 2017). Despite these beneficial characteristics, TiF_4 has not been used extensively in dental practice, due to its hydrolysis (Buslaev, Dyer, & Ragsdale, 1967) and very low pH (Exterkate & ten Cate, 2007). This has, in turn, impaired both its potential home use by patients, and its handling in the clinical environment (Exterkate & ten Cate, 2007). In an attempt to overcome such

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https://doi.org/10.1016/j.archoralbio.2018.04.008
Received 29 November 2017; Received in revised form 24 March 2018; Accepted 12 April 2018
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drawbacks, nanocomplex solutions, formed with TiF_4 and different configurations of cyclodextrins, have been developed.

Cyclodextrins are a family of oligosaccharides with hydrophobic inner and hydrophilic outer characteristics, which allow the formation of noncovalent host–guest inclusion complexes with organic and hydrophobic compounds (Loftsson & Brewster, 1996; Lay, Ni, Yu, & Shen, 2016). In biotechnology science, cyclodextrins have been widely used as complexing agents, due to their properties of stability, durability, reliability, increasing the aqueous solubility and bioavailability of drugs, reducing undesirable tastes and smells, and their economic cost of production (Tiwari, Tiwari, & Rai, 2010).

Two attempts in the oral research area have been employed in order to improve TiF_4 solutions properties and characteristics, using cyclodextrin-based drug delivery systems (Nassur et al., 2013; Vieira et al., 2017); however, the cytotoxic and bactericidal effects, as well as the influence of these novel solutions on enamel mineral loss, have not yet been investigated. Assessment of the effects of cyclodextrin-fluoride nanocomplex solutions on the cellular viability response is critical for further clinical application of TiF_4 formulations involving nanocarriers. Therefore, the aim of this study was to evaluate the cytotoxicity, antimicrobial activity and *in vitro* influence of new fluoridated nanocomplexes (hydroxypropyl-β-cyclodextrin or γ-cyclodextrin with 1% TiF_4, after two distinct complexation periods—12 and 72 h) on dental demineralization.

2. Materials and methods

2.1. Hydroxypropyl-β-cyclodextrin:TiF_4 and γ-cyclodextrin:TiF_4 nanocomplexes solutions

Cyclodextrins/TiF_4 nanocomplexes were prepared according to the following protocol: 0.1974 g of TiF_4 (Sigma Aldrich®, St Louis, Missouri, USA), and 2.0112 g of hydroxypropyl-β-cyclodextrin (Sigma Aldrich®, St Louis, Missouri, USA) or 2.0064 g of γ-cyclodextrin (Sigma Aldrich®, St Louis, Missouri, USA) were mixed with 20 mL of distilled water, using a magnetic stirrer, for 12 or 72 h. Next, the solutions were frozen in liquid nitrogen and lyophilized. The particle size was calibrated using a 40-mesh sieve, and the inclusion yield was calculated by UV spectroscopy. These nanosystems were characterized by X-ray powder diffraction, Fourier transform infrared spectroscopy and differential scanning calorimetry, as has been previously described (Nassur et al., 2013).

2.2. Cell culture

L-929, a fibroblast cell line (American Type Culture Collection – ATCC, Rockville, Maryland, USA) was maintained in Eagle's minimum essential medium (Cultilab, Campinas, Brazil) with 0.03 $mg\,mL^{-1}$ of glutamine, 50 μg.mL of garamicine, 2.5 $\mu g.mL^{-1}$ of fungizone, 0.25% sodium bicarbonate solution, 10 mM of HEPES (Sigma, St. Louis, Missouri, USA), and 10% bovine fetal serum as the growth medium, and incubated at 37 °C for 48 h (Dos Santos, Pithon, Da Silva Mendes, Romanos, & De Oliveira Ruellas, 2009). The cells were seeded in a 96-well plate (Corning®, Tewksbury, Massachusetts, USA) at a density of 1×10^5 per well. The culture was incubated at 37 °C ± 1 °C, 90% ± 10% humidity, and 5.0% ± 1.0% CO_2/air for 48 h, until a monolayer, with a confluence greater than 80%, was achieved.

2.3. Sample preparation for cytotoxicity analysis

Sixteen bovine enamel blocks (4 × 4 mm) were obtained from sterile bovine incisors by cutting the buccal surfaces with a diamond disk mounted in a low-speed cutting machine (Isomet, Lake Bluff, Illinois, USA). Next, they were ground sequentially, using 600 and 1200 grit Al_2O_3 paper in a semi-automatic polisher (model PLF, Fortel, SP, Brazil). They were then assigned (n = 2) to testing solutions or to a control group: 1% hydroxypropyl-β-cyclodextrin, 1% γ-cyclodextrin, 1% TiF_4, and hydroxypropyl-β-cyclodextrin:TiF_4 for 12 h; hydroxypropyl-β-cyclodextrin:TiF_4 for 72 h; γ-cyclodextrin:TiF_4 for 12 h; and γ-cyclodextrin:TiF_4 for 72 h. Each solution was centrifuged for 5 min, and 20 μL was then immediately applied to the assigned enamel block surfaces for 1 min. The blocks containing the solutions were sterilized using UV light (TROX do Brasil Ltda, FLV, 422, Curitiba, Brasil) for 30 min. Next, the blocks and the applied solution were immersed in 1000 μL of Eagle's minimum essential medium (MEM). Aliquots (100 μL) of this system (enamel block + test solution + MEM medium in each well of the 24-well plate) were aspirated, assigned to each experimental period, and incubated at 37 °C ± 1 °C, 90% ± 10% humidity, and 5.0% ± 1.0% CO_2/air for 15 min (the first 96-well plate), 30 min (the second 96-well plate) and 24 h (the third 96-well plate). Three aliquots, representing the test solutions, were obtained for each experimental period, for each treated block. The assays were performed in duplicate. Each culture well was considered to be an individual sample (n = 6).

2.4. Cytotoxicity assay

Control groups were prepared in order to perform the cell response evaluation. The first control group was cell detergent Tween-80 (polyoxyethylene-20-sorbitan, Sigma, St. Louis, Missouri, USA): blank – L-929 cells not exposed to the eluents; and negative control – bovine enamel blocks not treated. The controls were incubated in maintenance MEM, together with the testing solutions, for 15 min, 30 min and 24 h under the same experimental conditions. The cytotoxicity of the testing solutions was assessed using the neutral red dye uptake assay. Different experimental times were used: 15 min, 30 min and 24 h.

2.5. Neutral red dye uptake assay

This test is based on the ability of viable cells, with membrane integrity, to incorporate the neutral red dye through endocytosis, and accumulate it preferably on the lysosomes (Borenfreund & Puerner, 1985). Following incubation, 100 μL of 0.01% neutral red dye (Sigma, St. Louis, Missouri, USA) was added to each well of the microplates and incubated for 3 h at 37 °C. Sequentially, 100 μL of 4% formaldehyde solution in PBS (130 mmol NaCl, 2 mmol KCl, 6 mmol $Na_2HPO_4\ 2\,H_2O$, 1 mmol K_2HPO_4; pH 7.2) was included in each well to promote cell attachment to the plate. After 5 min, 100 μL of 1% acetic acid and 50% methanol were added in order to remove the dye not taken up by the cells. After 20 min, a spectrophotometer (BioTek, Winooski, Vermont, USA), set at a wavelength of 492 nm, was used to determine the absorbance (Dos Santos et al., 2009). Cytotoxicity was rated as a percentage, based on cell viability relative to the blank. The solutions were classified as cytotoxic if they demonstrated a less than 70% cell survival rate, as suggested by international standards for the evaluation of medical materials (ISO 10993-5, 2009).

2.6. Bacterial strain and inoculum preparation to determine minimum bactericidal concentration (MBC)

Streptococcus mutans (ATCC 25175) was used to formulate the inoculum. This bacterial sample was assessed in order to validate its degree of purity. Subsequently, isolated bacterial colonies were selected and transferred to a brain heart infusion (BHI, Oxoid Ltd., Hampshire, England) medium broth, until an optical density of 0.15 at 520 nm (Libra S2 Colorimeter, Biochrom, Cambridge, England), corresponding to approximately 10^8 CFU/mL, was obtained (Cardoso et al., 2016). The antibacterial potential of the nanocomplexed solutions was examined by MBC, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). The MBC was performed in the 96-well microplates, in which the samples were inoculated with 5×10^5 CFU/mL, in 100 μL of BHI medium. The concentrations of the solutions ranged

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