UNIVERSIDADE ESTADUAL DE CAMPINAS
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Nilza Cristina Lopes Afonso de Valor Gonçalves

"EFEITO DA ASSOCIAÇÃO DE POLIÓIS E OUTROS AGENTES ANTICARIOGÊNICOS SOBRE ESTREPTOCOCOS DO GRUPO MUTANS E INIBIÇÃO DA DESMINERALIZAÇÃO DO ESMALTE DENTAL"

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas para a obtenção do título de Doutor em Odontologia – Área de concentração em Cariologia.

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Orientador: Prof. Dr. Jaime Aparecido Cury.

Banca Examinadora:
Prof. Dr. Francisco Carlos Groppo
Profa. Dra. Marcia Pinto Alves Mayer
Profa. Dra. Maria José de Carvalho Rocha
Prof. Dr. Pedro Luíz Rosalen

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Dedico este trabalho…

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“Deus nos fez perfeitos e não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende da nossa vontade e perseverança”

Albert Einstein

SUMÁRIO

RESUMO

Xilitol é um açúcar substituto não cariogênico e apresenta propriedades anticariogênicas e terapêuticas. Desta forma, xilitol têm sido incorporado a produtos utilizados para higiene bucal com o objetivo de prevenção da cárie dentária. O objetivo geral deste estudo foi avaliar a associação de xilitol, combinado ou não a sorbitol, a outras substâncias de conhecido efeito anticariogênico ou antibacteriano, sendo elas flúor e citrato de zinco. O trabalho foi conduzido em três etapas com objetivos específicos para cada estudo. Os estudos 1 e 2 avaliaram, respectivamente, *in vitro* o efeito da associação de fluoreto e misturas de xilitol/sorbitol no crescimento bacteriano e *in situ* o efeito na inibição da desmineralização do esmalte dental. No estudo 3 foi avaliado o efeito da associação de xilitol e citrato de zinco na produção de ácidos utilizando-se um modelo *in vitro* de biofilme bacteriano. Fluoreto e a mistura de xilitol/sorbitol mostraram-se eficazes na inibição do crescimento bacteriano *in vitro*, entretanto não apresentou efeito aditivo da combinação quando adicionados após o início do crescimento bacteriano. A adição de polióis à solução para bochecho fluoretada não promoveu efeito adicional na inibição da desmineralização do esmalte dental *in situ*, quando comparado a utilização de solução fluoretada isolada. Os resultados referentes a associação de citrato de zinco e xilitol mostraram um efeito adicional dos agentes na inibição da produção de ácidos a partir da fermentação da glicose e sacarose. Assim, os estudos apresentados neste trabalho sugerem que a associação de polióis a outras substâncias anticariogênicas ou antimicrobianas como o fluoreto e o citrato de zinco, respectivamente, pode ser vantajosa no controle e prevenção da doença cárie.

Palavras-chave: xilitol, sorbitol, fluoreto, citrato de zinco, biofilme, crescimento bacteriano, produção de ácido, desmineralização.

ABSTRACT

Xylitol is used as a noncariogenic sugar substitute and it has also been attributed anticariogenic or therapeutic properties. Thus, xylitol is currently used in oral care products for prevention of dental caries. The overall aim of this study was to evaluate the combination of xylitol, associated or not with sorbitol, and anticariogenic or antimicrobial agents as fluoride and zinc citrate respectively. The present study was conducted in three phases with specific objectives for each one. The studies 1 and 2 evaluated, respectively, the effect of fluoride and xylitol/sorbitol on the growth of planktonic cells of *S. mutans* and the effect of fluoride rinse containing sugar alcohol on the reduction of enamel demineralization *in situ*. In the study 3, the interactive effect of zinc citrate and xylitol on acid production by biofilm cells was evaluated. Fluoride and xylitol/sorbitol showed an additional effect on the inhibition of bacterial growth *in vitro*; however, no additive effect was found when the agent was added 3 h after inoculation. Xylitol/sorbitol does not enhance the effect of fluoride present in mouth rinses on the reduction of enamel demineralization *in situ*. Xylitol and zinc citrate acted in an additive way to inhibit biofilm acid production from glucose and sucrose. The combination of sugar alcohol and other anticariogenic or antimicrobial agents such as fluoride and zinc citrate may offer an interesting prospect in controlling and preventing dental caries.

Key-words: xylitol, sorbitol, fluoride, zinc citrate, biofilm, bacterial growth, acid production, enamel demineralization

1. INTRODUÇÃO

Cárie dental é uma doença bacteriana, infecciosa e açúcar dependente. O uso de substitutos dos açúcares tem como objetivo eliminar ou pelo menos reduzir o desenvolvimento da doença (Edgar and Dodds, 1985). Dentre os substitutos, os açúcares alcoólicos, como por exemplo xilitol e sorbitol, apresentam pouca ou nenhuma acidogenicidade (Trahan, 1995), sendo portanto considerados os mais promissores em relação à prevenção da cárie dentária.

Os estudos relacionados à utilização do xilitol iniciaram-se na década de 70, onde a substituição total ou parcial de sacarose por xilitol na dieta demonstrou significante redução da incidência de cárie (Scheinin et al., 1975; Scheinin, 1976). Baseado nestes estudos, gomas de mascar e outros produtos contendo xilitol foram avaliados em populações de vários países. Os dados clínicos reunidos sugeriram que o consumo de xilitol pode estar associado a uma grande redução do incremento de superfícies cariadas, perdidas e obturadas (CPOS) em indivíduos jovens e adultos (Mäkinen et al., 1996).

Além de ser utilizado como um açúcar substituto não cariogênico em gomas de mascar e pastilhas, propriedades anticariogênicas e terapêuticas também têm sido atribuídas ao xilitol (Trahan, 1995). Desta forma, este açúcar alcoólico tem sido incorporado em produtos utilizados para higiene bucal, como dentifrícios e enxaguatórios (Petersson et al., 1991; Cutress et al., 1992; Sintes et al.,1995; Lingström et al., 1997; Giertsen et al, 1999).

Dentre as hipóteses propostas para explicar o efeito do xilitol na redução da incidência da cárie dentária, efeitos específicos no metabolismo e crescimento bacteriano tem sido considerados. O mecanismo pelo qual xilitol inibe o crescimento e metabolismo bacteriano pode se parcialmente descrito pelo consumo de fosfoenolpiruvato (PEP), uma vez que o xilitol é transportado via frutose-sistema fosfotransferase (frutose-PTS) (Trahan et al., 1985), resultando em acúmulo intracelular de xilitol 5-fosfato. Este metabólito intermediário não é utilizado, sendo então desfosforilado e expelido do interior celular como xilitol

(Assev and Rölla, 1986a; Söderling and Pihlanto-Leppälä, 1989). Este ciclo fútil consome energia e resulta em inibição do crescimento e metabolismo bacteriano.

Sendo o xilitol um adoçante caro, combinações de xilitol e sorbitol ao invés de xilitol apenas, têm sido utilizadas (Topitsoglou et al, 1983; Söderling et al, 1989; Petersson et al., 1991). Embora sorbitol seja fermentado pelos microrganismos bucais, a adição de xilitol inibe a produção de ácidos a partir do sorbitol em suspensões de *S. mutans* e placa dental (Frostell, 1984; Sasaki et al., 1987). Além disso, a presença de sorbitol aumenta o potencial inibitório do xilitol (Assev and Rölla, 1986b). Entretanto, a proporção ideal de xilitol:sorbitol capaz de promover um efeito inibitório ótimo não é conhecida (Assev and Rölla, 1993).

Por outro lado, substâncias antibacterianas ou anticariogênicas que possam aumentar o efeito inibitório do xilitol no crescimento e metabolismo bacteriano ou mesmo atuar sobre outros mecanismos relacionados ao desenvolvimento da cárie dental, poderiam aperfeiçoar o controle e prevenção da doença cárie.

Dentre estas substâncias, o fluoreto é utilizado mundialmente no controle da cárie dentária. Seu principal efeito no desenvolvimento da doença é físico-químico, reduzindo a desmineralização e aumentando a remineralização do esmalte dentário (Dawes and ten Cate, 1990). Estudos *in vitro* têm sugerido que a associação de xilitol e fluoreto exerce efeito inibitório adicional no crescimento e produção de ácidos pelos microrganismos bucais (Scheie et al., 1988; Rogers and Bert, 1992). Além disso, considerando que o xilitol possui ação na acidogenicidade bacteriana, consumindo suas reservas energéticas durante seu transporte (Trahan et al., 1991), pode-se esperar um efeito adicional ao flúor na inibição da desmineralização do esmalte dentário.

Outra substância utilizada no controle da cárie dentária é o zinco (Zn^{++}). Ele é um antimicrobiano, geralmente adicionado em dentifrícios, sob a forma de citrato de zinco e apresenta efeito inibitório na produção de ácidos de bactérias da placa dental *in vitro* (Harrap et al., 1984) e *in vivo* (Skjörland et al., 1978; Oppermann and Rölla, 1980), além de inibir enzimas glicolíticas (Phan et al., 2004) como

sistema de transporte fosfoenolpiruvato: fosfotransferase (PTS), aldolase, gliceraldeido-3-fosfato desidrogenase e piruvato quinase. Associação de Zn^{++} e xilitol tem demonstrado aumento da concentração intracelular de xilitol-fosfato (Scheie et al., 1988), podendo desta forma potencializar o efeito inibitório na acidogenicidade bacteriana promovido individualmente por estes agentes. Entretanto, a efetividade do Zn^{++} e xilitol tem sido geralmente avaliada em culturas planctônicas (Scheie et al., 1988; Scheie et al., 1989), sendo estas geralmente menos resistentes a agentes antimicrobianos quando comparadas a bactérias crescidas em biofilme (Kinniment et al., 1996; Larsen et al., 1996).

Frente ao exposto, o presente estudo tem por objetivos: avaliar o efeito da associação de xilitol ou combinações de xilitol:sorbitol e flúor no crescimento bacteriano *in vitro*, avaliar o efeito da adição de xilitol:sorbitol em enxaguatório fluoretado na redução da desmineralização do esmalte dental *in situ* e avaliar o efeito da associação de xilitol e citrato de zinco na produção de ácidos em biofilme bacteriano *in vitro*.

2. CAPÍTULOS

Esta tese está baseada na Informação CCPG/001/98/Unicamp que regulamenta o formato alternativo para tese de Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato.

Desta forma, esta tese é composta de três artigos, os quais foram submetidos ou encontram-se em fase de submissão para publicação em revistas científicas, conforme descrito abaixo:

2.1. Artigo 1 – "Effect of fluoride and xylitol/sorbitol combinations on growth of *Streptococcus mutans*."

2.2. Artigo 2 – "Effect of rinse containing fluoride and sugar alcohol on enamel demineralization *in situ*." Artigo submetido à publicação na Caries Research (Anexo 1).

2.3. Artigo 3 – "Combined effect of xylitol and zinc citrate on acid production by *Streptococcus mutans* in suspensions and biofilms."

2.1. ARTIGO 1

EFFECT OF FLUORIDE AND XYLITOL/SORBITOL COMBINATIONS ON GROWTH OF *STREPTOCOCCUS MUTANS*.

N.C.L.A.V. Gonçalves, P.L. Rosalen, J.A. Cury

Faculty of Dentistry of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Running Title: Effect of fluoride and sugar alcohol on bacterial growth.

Key Words: fluoride; xylitol; sorbitol; bacterial growth inhibition.

Full address of the author to whom correspondence should be sent:
Prof. Jaime A. Cury
Av. Limeira 901
13414-903 Piracicaba, SP.
Brazil
Phone: #55-19-34125302
Fax: #55-19-34125218
E-mail: jcury@fop.unicamp.br

ABSTRACT

The aim of this study was to evaluate the effect of fluoride (F) and xylitol (X) combinations and establish the ratio of xylitol:sorbitol (X:S) on fluoride solution to exert inhibitory effect on growth of mutans streptococci. *Streptococcus mutans* IB1600 was grown in brain-heart infusion broth (BHI) containing various concentrations of xylitol (0.05 – 0.8 M) and containing or not 56 µgF/mL (as NaF). The effect of these substances on the bacterial growth was evaluated under two conditions: (1) *S. mutans* IB1600 was inoculated into BHI containing X, F, or XF; (2) *S. mutans* IB1600 was inoculated into BHI and X, F, or XF was added 3 h after inoculation (middle of log phase). The cultures were incubated at 37°C, 10% CO_2 and bacterial growth was assessed by optical density measurements at 650 nm, every two hours for 24 h. Other series of experiment were performed with 56 µgF/mL (NaF) and combinations of X:S in ratios from 3:1 to 1:3 (final concentration 0.4 M) following the same procedures for bacterial growth as cited above. Fluoride and xylitol showed an additive effect on the inhibition of bacterial growth; however, no effect was found when the agents were added 3 h after inoculation. Moreover, growth was essentially the same for xylitol:sorbitol ratios in the range from 3:1 to 1:3. The data suggest that fluoride and sugar alcohol combined may have additional effect on caries control that their use isolated.

INTRODUCTION

Caries is associated with an increase in the proportions of acidogenic and aciduric species, including mutans streptococci, in dental plaque. Fluoride reduces the incidence of dental caries, via effects on enamel such as by promoting remineralization and reducing demineralization [Dawes and ten Cate, 1990]. However, fluoride concentrations in plaque can reach the millimolar range [Gaugler and Bruton, 1982] and, consequently, could also exert inhibitory effects on the oral microflora.

The combined use of fluoride and other substances that also act on bacterial growth and metabolism may increase the anticariogenic effect of fluoride. Among these substances, polyols like xylitol has shown to be a useful sugar substitute for the prevention of dental caries and to exert specific effects on microbial growth and metabolism [Assev et al., 1983; Vadeboncoeur et al., 1983; Assev and Rölla, 1986a; Trahan, 1995]. This polyol has been incorporated in fluoride-containing oral health-care products, such as toothpastes and mouth rinses. *In vitro* studies have suggested that fluoride and xylitol exert an additive inhibitory effect on growth of and acid production by *Streptococcus sobrinus* [Scheie et al., 1988] and on acid production by *Streptococcus mutans* [Rogers and Bert, 1992]. The incorporation of xylitol in fluoride-containing oral health-care products may therefore be advantageous. However, the effective concentration of xylitol that could be added to a fluoride product is not clearly established.

Since xylitol is an expensive sweetener, attempts have been made to use mixtures of xylitol and sorbitol instead of xylitol alone. Although sorbitol is fermented by oral microorganisms, addition of xylitol inhibited acid production from sorbitol in suspension of

S. mutans and dental plaque [Frostell, 1984; Sasaki et al., 1987]. Moreover, the presence of sorbitol enhances the inhibitory potential of xylitol [Assev and Rölla, 1986b]. However, the proportion between xylitol:sorbitol to inhibit bacterial growth is not known (Assev and Rölla, 1993).

Thus, the present study was performed in order to evaluate the effects of fluoride and xylitol on the growth of mutans streptococci and to establish the optimal ratio of xylitol:sorbitol (X:S) in a fluoride solution able to exert an inhibitory effect on the bacterial growth.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Streptococcus mutans Ingbritt-1600 (kindly donated by the Eastman Department of Dentistry, Rochester, USA) were stored at –80^{o}C in brain-heart infusion (BHI) containing 20% glycerol, and subcultured on BHI agar plates prior to transfer to BHI medium.

Test solutions

Fluoride (as NaF) (Merck), xylitol (Sigma) and sorbitol (Difco) solutions were sterilized separately by filtration though a filter (Millipore Co., pore size 0.22µm) and then added aseptically to the medium in order to obtain the concentrations specified below.

Growth studies

The microorganisms were grown in BHI broth at 37^{o}C, 10% CO_2 for overnight. Aliquots (0.5 mL) of this culture were inoculated into 9.5 mL BHI medium tubes with additional xylitol concentrations (0.05, 0.1, 0.2, 0.4 and 0.8 M – final medium

concentration) and containing or not 56 µg F/mL (as NaF) (final medium concentration). A culture without extra carbohydrate and fluoride was included as a positive control. The test tubes were incubated at 37°C, 10% CO_2 and bacterial growth was assessed by optical density measurements at 650 nm, every second hour for 12 hours and at 18 and 24 hours. Also, 0.5 mL aliquots of a overnight culture were inoculated into 9.5 mL BHI medium tubes. The test tubes were incubated at 37°C, 10% CO_2 and fluoride (56 µg F/mL – final medium concentration), xylitol (0.05, 0.1, 0.2, 0.4 and 0.8 M – final medium concentration) or both were added 3 h after inoculation (middle of log phase – as determined previously). A culture without extra carbohydrate and fluoride was included as positive control. Bacterial growth was allowed to proceed for another 21 h and was assessed as described above.

In order to evaluate the effect of xylitol:sorbitol combinations and fluoride on bacterial growth, 0.5 mL of an overnight culture of *S. mutans* IB1600, incubated at 37°C, 10% CO_2 was added to 9.5 mL of BHI supplemented with fluoride (56 µgF/mL – final medium concentration) and xylitol:sorbitol ratios from 3:1 to 1:3 (0.4 M – final sugar alcohol medium concentration). Indeed, cultures were incubated at 37°C, 10% CO_2 for 3 h, whereafter fluoride and xylitol:sorbitol ratios were added. The test tubes were incubated at 37°C, 10% CO_2 and bacterial growth was assessed by optical density measurement as described above.

The area under the curve (optical density X time) was calculated by Gauss method and results expressed as mean ± SD. The growth studies were performed three times in duplicate.

Fermentation test

Fermentation test was carried out as previously described [Nuuja et al., 1993]. Briefly, bacteria were grown in BHI broth for 18 h at 37^{o}C, cells were harvested by centrifugation (3,000 *g*, 15 min, 4^{o}C), washed three times with 0.1 M PBS and suspended in 0.9% NaCl to an optical density of 0.135 at 650 nm (1-2 x 10^{8} colony-forming units (CFUs)/mL). Equal volumes of standardized bacterial suspension and test solutions (fluoride, xylitol or both) plus 10% sucrose were used. The test vials were incubated at 37°C, 10% CO_2, and the pH suspension was measured every 15 min for 1 hour. The difference from the initial pH to the end pH was calculated. The experiment was carried out three times in duplicate.

Statistical Analysis

The data were analysed by analysis of variance and subsequent Tukey-test. All assumptions were tested and, if necessary, corrected through data transformation. The BOX-COX procedure was used to detect the optimal power transformation. For all the analyses a significant level of 5% was undertaken and the data were analysed using the SAS System, SAS Institute Inc. release 8.0, 1999.

RESULTS

Fluoride and xylitol combinations

The effects of fluoride and xylitol on the growth of *S. mutans* IB 1600 are presented in Figure 1 and 2, Table 1, 2, and 3. Inhibition of bacterial growth by xylitol increased with increasing xylitol concentrations. Addition of fluoride further increased the growth-

inhibiting effect. Moreover, fluoride and xylitol showed an additional inhibitory effect (Analysis of variance - $p < 0.05$) when added at inoculation time (Table 1 and Figure 1). The inhibitory effect of 0.05 M xylitol plus fluoride or 0.2 M xilitol plus fluoride was not different from that 0.2 M xilitol or 0.4 M xilitol, respectively ($p>0.05$). However, when the agents were added to the medium growth 3 h after inoculation (Figure 2), no apparent additive effect was found (Analysis of variance – $p > 0.05$). Thus, the effect of fluoride and xylitol were analyzed individually. The data showed that fluoride (Table 2) and xylitol (Table 3) were effective to inhibit bacterial growth (Tukey test – $p < 0.05$).

For sucrose fermentation test, fluoride and xylitol did not show additive effect (Analysis of variance – $p > 0.05$). Thus, the effect of fluoride and xylitol were analyzed individually and the data are presented at Table 4 and Table 5, respectively. The data showed that Fluoride and high concentration of xylitol (0.4 or 0.8M) were able to avoid the pH drop reduction (Tukey test – $p < 0.05$).

Fluoride and xylitol:sorbitol ratios

The effect of fluoride and xylitol:sorbitol ratios on bacterial growth when the agents were added at inoculation time are shown on Table 6 and Figure 3. Table 7 and Figure 4 show the effect of the agents on bacterial growth when they were added to the medium 3 h after inoculation. All xylitol:sorbitol combinations inhibited the growth of bacteria similar to xylitol alone in fluoride-containing medium.

DISCUSSION

The present study shows that the combination of fluoride and xylitol was effective to inhibit bacterial growth. Our results on growth inhibition are in accordance with Scheie et al. [1988], who reported an additive effect of xylitol and fluoride. The present data suggest that if a fluoride mouth rinse containing xylitol could be prepared, the concentration of xylitol should be 2-4-times lower than a mouth rinse containing xylitol alone to reduce bacterial growth.

However, the reported additive effect of fluoride and xylitol on acid production [Scheie et al., 1988; Roger and Bert, 1992] was not confirmed by our data. No additive effect of XF was found regarding pH drop reduction and only high concentrations of xylitol (0.4 and 0.8 M) were able to reduce pH drop in comparison to control treatment (without xylitol) ($p < 0.05$).

The apparent additive effect of xylitol and fluoride may be explained by the increased intracellular concentration of xylitol metabolites [Scheie et al., 1988]. Xylitol-5-phosphate cannot be utilised by xylitol-sensitive cells and may be even toxic to bacteria [Trahan et al., 1985]. It must therefore be dephosphorylated and expelled from the cell as xylitol [Assev and Rölla, 1986a; Söderling and Pihlanto-Leppälä, 1989]. Fluoride probably inhibits this dephosphorylation [Reizer et al., 1985].

In addition, the present data show that *in vitro* growth-inhibitory effect of xylitol:sorbitol combinations is similar whether xylitol represented 100% or 25% of a total sugar alcohol concentration of 0.4 M added to a fluoride solution. This finding is in agreement with Assev and Rölla [1993], who also found no optimal inhibitory

concentrations of xylitol:sorbitol combinations. Thus, it seems that the addition of sorbitol into a xylitol-fluoride solution did not affect its inhibitory growth effect. Moreover, plaque studies involving chewing gums with xylitol:sorbitol ratios varing from 1:4 to 7:1 have, as a rule, reduced the amount of plaque [Birkhed et al., 1983; Topitsoglou et al., 1983; Kandelman ang Gagnon, 1987; Söderling et al., 1989].

In conclusion, the data indicated that fluoride and xylitol act in an additive way to inhibit bacterial growth. Combinations of fluoride and sugar alcohols (0.4 M final concentration) were also efficient to inhibit bacterial growth in various xylitol:sorbitol ratios. However, considering that bacteria living in biofilms as dental plaque are generally more resistant to antimicrobial agents than bacteria in broth cultures, it should be of interest to evaluate the effect of these agents against biofilm cells.

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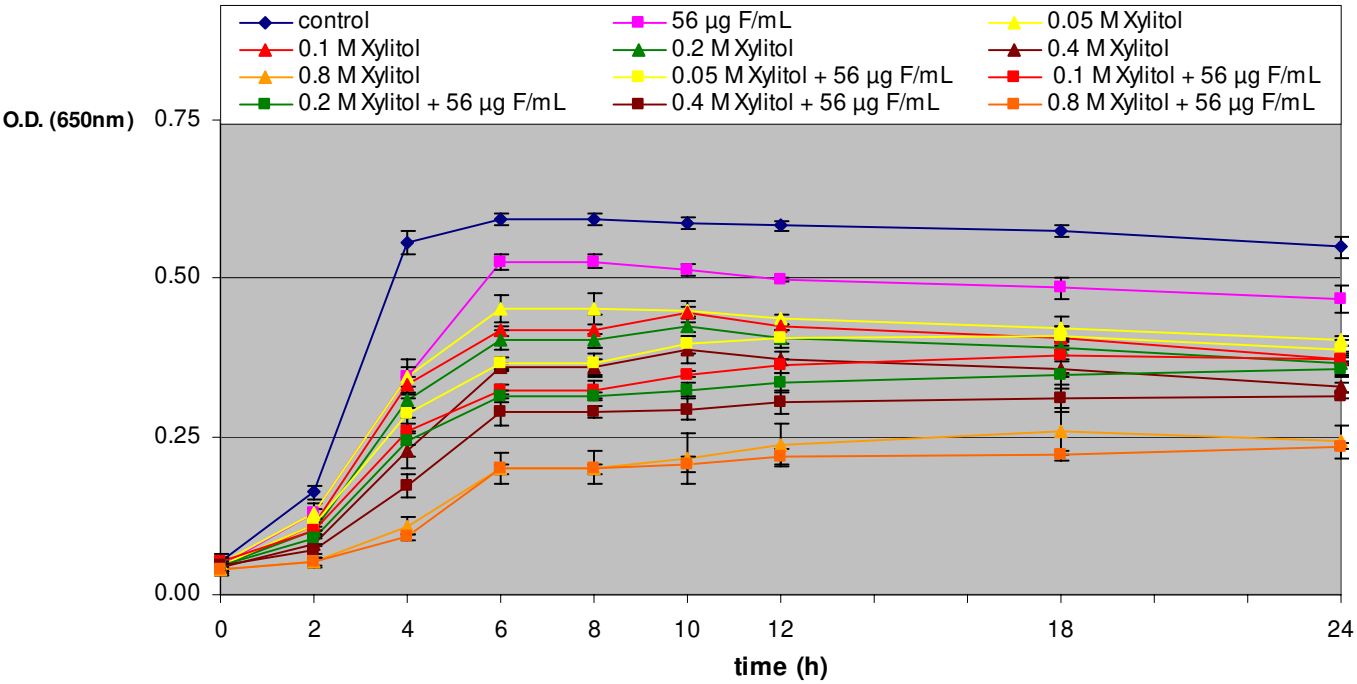

Figure 1: *Streptococcus mutans* IB 1600 grown in BHI supplemented with xylitol and / or fluoride.

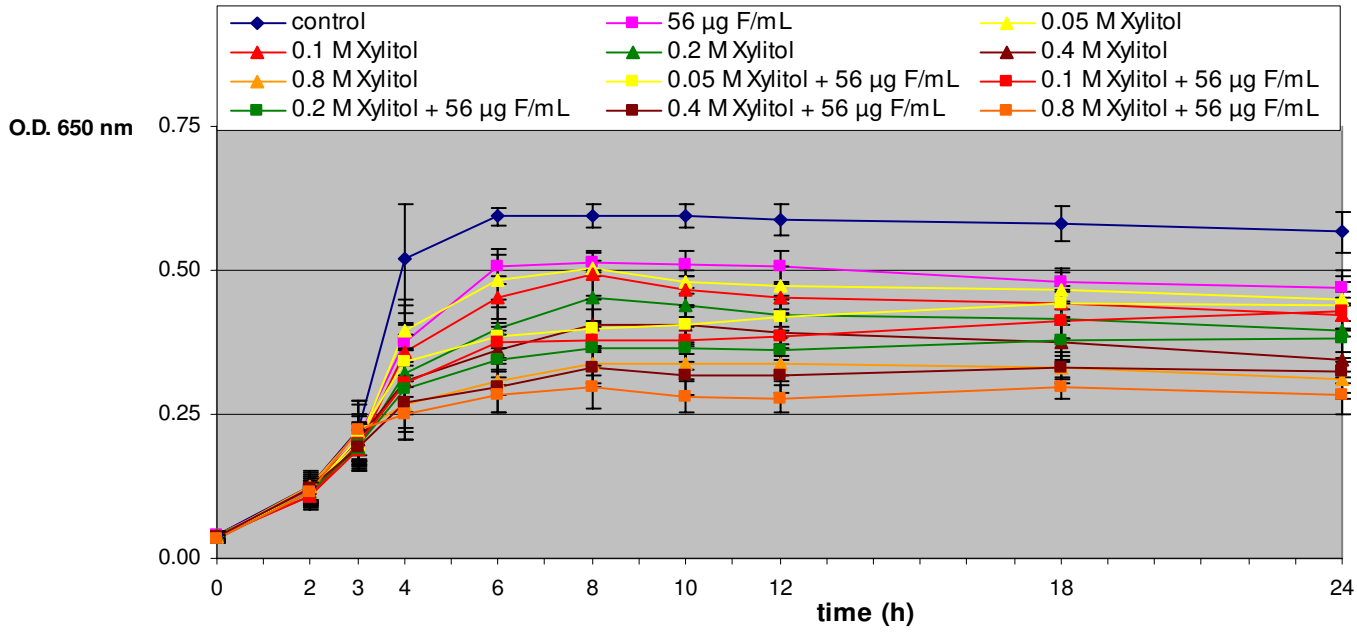

Figure 2: *Streptococcus mutans* IB 1600 grown in BHI, xylitol and / or fluoride were added 3 hours after inoculation (middle of log phase).

Table 1: Area of growth curves (Means ± SD) of *Streptococcus mutans* IB 1600 grown in BHI with various concentrations of xylitol and containing or not 56 µgF/mL (n = 6). Test solutions added at inoculation time.

Test solutions	Area of growth curve
Control	12.47 ± 0.16 ^{A}
56 µgF/mL	10.45 ± 0.21 ^{B}
0.05 M Xylitol	9.08 ± 0.20 ^{C}
0.1 M Xylitol	8.67 ± 0.14 ^{D}
0.2 M Xylitol	8.32 ± 0.11 $^{D E}$
0.05 M Xylitol plus 56 µgF/mL	8.26 ± 0.14 ^{E}
0.1 M Xylitol plus 56 µgF/mL	7.58 ± 0.22 ^{F}
0.4 M Xylitol	7.40 ± 0.12 $^{F G}$
0.2 M Xylitol plus 56 µgF/mL	7.10 ± 0.23 ^{G}
0.4 M Xylitol plus 56 µgF/mL	6.25 ± 0.27 ^{H}
0.8 M Xylitol	4.72 ± 0.61 ^{I}
0.8 M Xylitol plus 56 µgF/mL	4.35 ± 0.12 ^{I}

* Analysis of variance - interactive effect of xylitol and fluoride was statisticaly significant ($p<0.05$). Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

Table 2: Area of growth curves (Means ± SD) of *Streptococcus mutans* IB 1600 grown in BHI with or without 56 µgF/mL (n = 36). Test solutions added 3 h after inoculation.

Test solutions	Area of growth curve
no Fluoride	9.76 ± 1.81 ^{A}
56 µgF/mL	8.58 ± 1.52 ^{B}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

[WX1]

Table 3: Area of growth curves (Means ± SD) of *Streptococcus mutans* IB 1600 grown in BHI with with various concentrations of xylitol (n = 12). Test solutions added 3 h after inoculation.

Test solutions	Area of growth curve
no Xylitol	11.82 ± 1.17 ^{A}
0.05 M xylitol	9.95 ± 0.85 ^{B}
0.1 M xylitol	9.42 ± 0.82 $^{B\,C}$
0.2 M xylitol	8.78 ± 1.02 $^{C\,D}$
0.4 M xylitol	7.96 ± 0.93 $^{D\,E}$
0.8 M xylitol	7.10 ± 0.75 ^{E}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

Table 4: Fermentation of sucrose by *Streptococcus mutans* IB 1600 incubated in the presence or not of fluoride (n = 36). Results (Means ± SD) shown as the difference from final to initial pH after 60 mim ($\Delta pH_{60\text{-}0\ min}$).

Test solutions	$\Delta pH_{60\text{-}0\ min}$
no Fluoride	- 0.78 ± 0.26 ^{A}
56 µgF/mL	- 0.49 ± 0.22 ^{B}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

Table 5: Fermentation of sucrose by *Streptococcus mutans* IB 1600 incubated in the presence of various concentrations of xylitol (n=12). Results (Means ± SD) shown as the difference from final to initial pH after 60 mim ($\Delta pH_{60\text{-}0\ min}$).

Test solutions	$\Delta pH_{60\text{-}0\ min}$
no Xylitol	- 0.88 ± 0.38 ^{A}
0.05 M xylitol	- 0.66 ± 0.30 $^{A\,B}$
0.1 M xylitol	- 0.66 ± 0.28 $^{A\,B}$
0.2 M xylitol	- 0.61 ± 0.17 $^{A\,B}$
0.4 M xylitol	- 0.54 ± 0.14 ^{B}
0.8 M xylitol	- 0.47 ± 0.22 ^{B}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

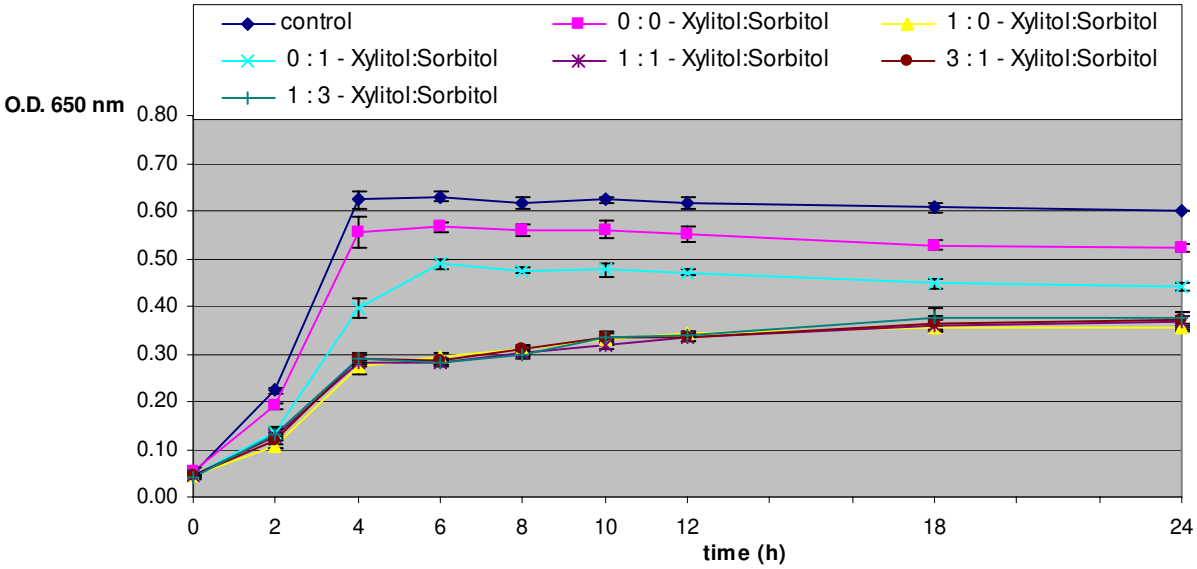

Figure 3: *Streptococcus mutans* IB 1600 grown in BHI supplemented with fluoride and xylitol:sorbitol ratios.

Table 6: Area of growth curves (Means ± SD) of *Streptococcus mutans* IB 1600 grown in BHI containing 56 µgF/mL and xylitol:sorbitol ratios (0.4 M final concentration) (n = 6). Test solutions added at inoculation time.

Test solutions **Xylitol:Sorbitol ratios**	**Area of growth curve**
0 : 0	11.84 ± 0.20 ^{A}
0 : 1	9.89 ± 0.10 ^{B}
1 : 3	7.44 ± 0.16 ^{C}
3 : 1	7.36 ± 0.14 ^{C}
1 : 1	7.26 ± 0.11 ^{C}
1 : 0	7.24 ± 0.07 ^{C}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

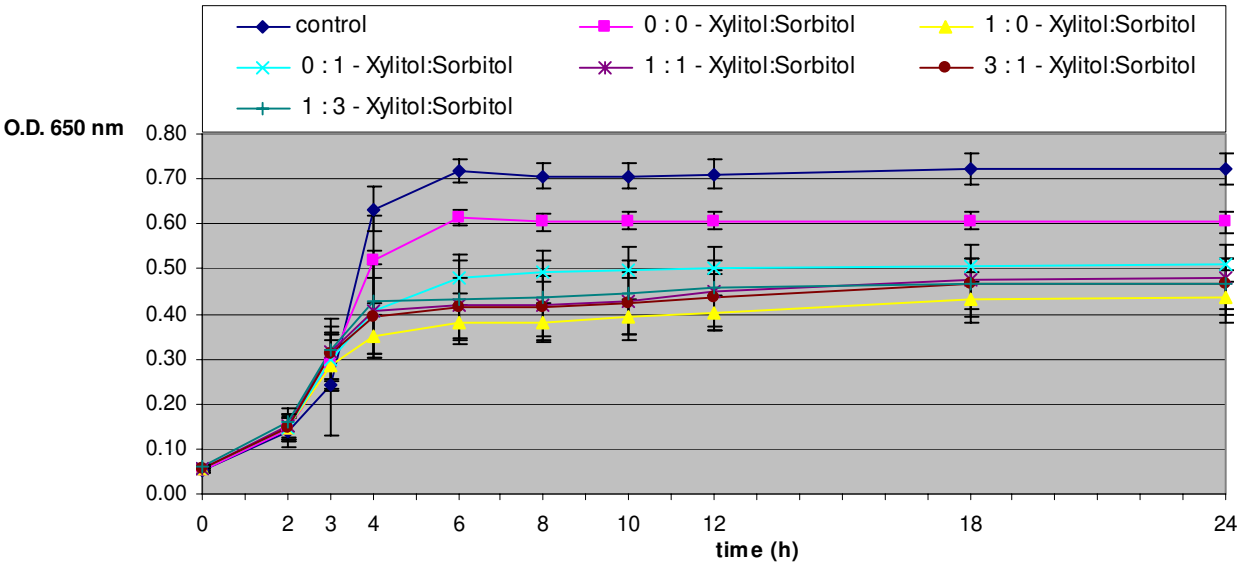

Figure 4: *Streptococcus mutans* IB 1600 grown in BHI, fluoride and xylitol:sorbitol ratios were added 3 hours after inoculation (middle of log phase).

Table 7: Area of growth curves (Means ± SD) of *Streptococcus mutans* IB 1600 grown in BHI containing 56 µgF/mL and xylitol:sorbitol ratios (0.4 M final concentration) (n = 6). Test solutions added 3 h after inoculation.

Test solutions **Xylitol:Sorbitol ratios**	**Area of growth curve**
0 : 0	13.48 ± 0.44 ^{A}
0 : 1	11.39 ± 0.58 ^{B}
1 : 3	10.67 ± 2.03 ^{B}
1 : 1	10.49 ± 1.34 ^{B}
3 : 1	10.29 ± 1.32 ^{B}
1 : 0	9.59 ± 0.88 ^{B}

* Treatment whose means are followed by distinct superscripts letters differ statistically ($p<0.05$) – Tukey test.

2.1. ARTIGO 2

EFFECT OF RINSE CONTAINING FLUORIDEAND SUGAR ALCOHOLS ON ENAMEL DEMINERALIZATION *IN SITU*

N.C.L.A.V. Gonçalves, A.A.Del Bel Cury, G.S. Simões, A.T. Hara, J.A. Cury

Faculty of Dentistry of Piracicaba, State University of Campinas, Piracicaba, SP, Brazil

Running Title: Effect of fluoride and sugar alcohols mouth rinses on enamel demineralization

Key Words: fluoride; xylitol; sorbitol; mouth rinse; enamel demineralization

Full address of the author to whom correspondence should be sent:
Prof. Jaime A. Cury
Av. Limeira 901
13414-903 Piracicaba, SP.
Brazil
Phone: #55-19-34125302
Fax: #55-19-34125218
E-mail: jcury@fop.unicamp.br

ABSTRACT

Since the additive anticariogenic effect of combining sugar alcohols with fluoride (F) in a rinse is unknown, this crossover study was conducted in situ using the known IEDT short-term model. Ten volunteers were submitted to the following treatments: (I) Distilled and deionized water, as a negative control; (II) F (226 µg F/mL as NaF); (III) X:S (xylitol/sorbitol 1:3 – final concentration 1.6 M); (IV) X:S+F (same final concentration that groups II and III); (V) CHX (0.12% chlorhexidine digluconate, as a positive control). The volunteers wore palatal appliances containing 4 bovine enamel blocks of known surface microhardness (SMH), which were covered with a 'test plaque' of mutans streptococci. The appliances were immersed during 1 min in one of the allocated treatment solutions, simultaneously that the volunteers rinsed their mouths with the same solution. Immediately after the rinsing, the appliances were put in the mouth and after 20 minutes, 20% sucrose solution was rinsed. After further 45 minutes the 'test plaque' was collected for F analysis. The SMH of enamel was again determined and the percentage of change in relation to baseline was calculated; F uptake in enamel was also determined. With respect to all the analyses made, the groups treated with X:S and CHX did not differ from the negative control, but the groups treated with F and F+X:S did ($P < 0.05$). The results suggest that xylitol/sorbitol does not enhance the effect of fluoride present in mouth rinses on the reduction of enamel demineralization.

INTRODUCTION

Fluoride is a recognized substance used worldwide to control dental caries. There is a consensus that the main effect of fluoride (F) is to interfere physicochemically with caries development by reducing demineralization and enhancing remineralization of dental enamel [Dawes and ten Cate, 1990]. The use of substances that act on bacterial metabolism inhibiting acid production may increase the anticariogenic effect of fluoride. Among these substances, xylitol, a five-carbon sugar alcohol which has shown specific effects on microbial growth and metabolism [Assev et al., 1983;Vadeboncoeur et al., 1983; Assev and Rölla, 1986a; Trahan, 1995] could account to enhance the effect of fluoride products.

The bacteriostatic effect of xylitol may partly be attributed to the consumption of the bacteria energy since it is taken up, by the oral bacteria, via an energy-dependent transport system [Trahan et al., 1985]. The resulting intracellular xylitol-5-phosphate is not metabolizable being dephosphorilated and transported out of the cell. This energetic 'futile cycle' provoked by xylitol results in inhibition of growth and acid production by mutans streptococci [Assev and Rölla, 1986a; Söderling and Pihlanto-Leppälä, 1989].

Xylitol has been incorporated into sweets, candies, and chewing gums and also in many other products such those for oral hygiene. Since it is an expensive sweetener, attempts have been made to use mixtures of xylitol and sorbitol instead of only xylitol [Topitsoglou et al, 1983; Söderling et al, 1989; Petersson et al., 1991]. Although sorbitol is fermented by oral microorganism, addition of xylitol inhibited acid production from sorbitol in suspension of *S. mutans* and dental plaque [Frostell, 1984; Sasaki et al., 1987].

Moreover, the presence of sorbitol enhances the inhibitory potential of xylitol [Assev and Rölla, 1986b].

The incorporation of these polyois, xylitol and sorbitol, in fluoride-containing oral health-care products may therefore be advantageous [Sintes et al., 2002]. *In vitro* studies have suggested that fluoride and xylitol exert an additive inhibitory effect on growth of and acid production by oral microorganisms [Scheie et al., 1988; Rogers and Bert, 1992]. However, the effect of xylitol/sorbitol on enamel demineralization when combined with fluoride has not been well studied.

Thus, the present study was undertaken to evaluate the effect of xylitol/sorbitol present in a 0.05% sodium fluoride mouthrinse on the reduction of enamel demineralization using a short-term in situ model.

MATERIALS AND METHODS

Experimental Design

A crossover *in situ* study was carried out in 5 phases, using an intra-oral enamel demineralization test model (IEDT) described by Zero et al. [1992]. Ten healthy, adult volunteers wore palatal appliances containing 4 bovine enamel blocks, with pre-determined surface microhardness (SMH). The blocks were covered with a layer of bacteria ('test plaque') obtained from a culture of *Streptococcus mutans* and fixed on palatal appliances using acrylic holders [Zero et al., 1992; Cury et al., 2003]. This study was approved by the Ethics in Research Committee of Faculty of Dentistry of Piracicaba, and the volunteers were submitted to the following groups of treatments: (I) DDW (distilled and deionized water, as a negative control); (II) Fluoride (226 µg F/mL, as NaF); (III) Xylitol/Sorbitol

(1:3 – final concentration 1.6 M); (IV) Xylitol/Sorbitol plus Fluoride (same final concentration of groups II and III); (V) Chlorhexidine (0.12% chlorhexidine digluconate, as a positive control). The effect of the treatments was evaluated by the reduction of enamel surface demineralization induced by sucrose rinse. The volunteers were randomly assigned to the treatments at the beginning of the study and after 5 steps all of them were submitted to all treatments. At the end of each experimental period, the bacteria that settled on the enamel blocks ('test plaque') were collected for analysis of fluoride concentration, and the enamel was analyzed for surface microhardness and fluoride uptake.

The sugar alcohol concentrations and xylitol:sorbitol ratio used were based in previous *in vitro* studies [Gonçalves et al., 2002a; Gonçalves et al., 2002b].

Preparation of enamel blocks and baseline SMH determination

Enamel blocks measuring 5 x 5 x 2 mm were cut from bovine incisors, which were sterilized by storage in 2% formaldehyde solution, pH 7.0, for at least one month [Cury et al., 2003]. The enamel surface of each block was polished flat as described by Zero et al. [1990]. Two marks were made on the upper corner of one side of the enamel blocks to ensure proper positioning of the blocks in the holders with regard to the side were the baseline indentations were made. SMH were made at center of this side as described previously [Cury et al., 2003], but in the present study additional measurements were made at distances of 1500, 2000, and 2500 μm from the edge of the block. Thus, the measurement sites correspond to plaque thickness from 50 to 2500 μm [Zero, 1995]. Enamel microhardness was measured using a Knoop indenter with a 50-g load for 5 s and a Future-Tech FM microhardness tester coupled to software FM-ARS.

Palatal appliance mounting

'Test plaque' was prepared from *S. mutans* Ingbritt-1600 (kindly donated by the Eastman Department of Dentistry, Rochester, USA), as described by Zero et al [1992] and Cury et al. [2003]. Palatal appliances capable of carrying two plastic holders were constructed for each volunteer. Two enamel blocks were mounted in each holder, with enamel surface in contact with the 'test plaque'. The plastic holders were secured to the palatal appliance, with the side where the baseline measurements were made facing the center of the palatal appliance. Further details can be seen in Cury et al. [2003].

Intra-oral Test

The volunteers rinsed their mouths for 1 min with one of the allocated treatment solution, while the palatal appliances set with the blocks covered with the 'test plaque' were kept in the same solution. After rinsing, the appliances were put in the mouth. Twenty minutes later, the devices were removed and immersed in 20% sucrose solution, simultaneously that the volunteers rinsed their mouth for 1 min with 15 ml of 20% sucrose solution. The appliances were put again into the mouth for further 45 min. After this period, the devices were removed and the bacteria that settled on the enamel blocks ('test plaque') were collected for analysis of fluoride concentration, and the enamel was analyzed for surface microhardness and fluoride uptake. During the time that the apparatus was kept into the mouth, the volunteers were instructed to refrain from talking, drinking or eating.

Analysis of fluoride in 'test plaque'

The 'test plaque' on all the enamel blocks were recovered, pooled, weighed (± 0.01 mg), suspended in distilled and deionized water (62.5 mg/ml), and homogenized by Vortex

agitation. To determine fluoride ion (F^-) concentration, 0.40 ml of the suspension was added to a test tube containing 0.040 ml of TISAB III. Fluoride was analyzed with an ion specific electrode, Orion 96-09, and an ion-analyzer, previously calibrated with various standard fluoride solutions (0.05 to 0.50 μg F/ml) prepared like the samples. The results were expressed in μg F/g of 'test plaque'.

Surface Microhardness Analysis

The enamel blocks were removed from the holders, washed with distilled and deionized water and surface microhardness (SMH) was measured again as already described. The indentations were made at 100 μm right and left from the baselines and at 50, 75, 100, 200, 300, 400, 500, 1000, 1500, 2000, and 2500 μm from the block edge [Cury et al., 2003]. From this block edge, mouth rinse, sucrose and saliva had access to the enamel surface covered by the 'test plaque', simulating dental plaque thickness of up to 2.5 mm. The percentage of surface microhardness change (%SMC) was calculated [%SMC = (SMH after treatments - baseline) x 100/ baseline]. The results found in the four blocks at each distance for each volunteer were averaged and submitted to statistical analysis.

Analysis of Fluoride in Enamel

After SMH measurements, each enamel block was sectioned removing two slices of approximately 1.25 mm from the lateral parts of the blocks and preserving the central part where the indentations had been made. This area of enamel was determined (± 0.01 mm) and all the other sides of the block were covered with an acid-resistant nail varnish. A layer of enamel was removed by immersion of this slice in 0.25 ml of 0.5 M HCl for 15 s under

agitation and fluoride acid-extracted was determined [Cury et al., 2000]. Fluoride concentration in enamel was expressed as µg F/cm^2 of enamel. The results found in the 4 blocks for each volunteer were averaged and submitted to statistical analysis.

Statistical Analysis

The assumptions of homogeneity of variances and normal distribution of errors were tested for the response variables tested. Since they were not satisfied data were transformed, according to the Box-Cox method. For the response %SMC, a *split-plot* analysis of variance (ANOVA) followed by the Tukey test and regression analyses was performed. Responses of fluoride in 'test plaque' and fluoride in enamel were analyzed by ANOVA followed by Tukey tests. All the analyses were carried out with SAS System 6.11 software (SAS Institute, Cary, NC, USA) and the significance level was set at 5%.

RESULTS

Data of %SMC were submitted to the root square transformation. Treatments containing fluoride (Fluoride and Xylitol/Sorbitol + Fluoride treatments) did not differ from each other and had a significant lower %SMC than the other treatments in reducing enamel demineralization ($p < 0.05$) irrespective of the 'test plaque' thickness (Figure 1). The Xylitol/Sorbitol, Chlorhexidine and negative control groups did not differ from each other ($p > 0.05$).

The %SMC was affected by the increase of the plaque thickness in the negative control, Xylitol/Sorbitol and Chrorhexidine treatment groups; an increase followed by a

decrease in enamel demineralization was observed the deeper into the 'test plaque', fitting a 3rd degree polynomial regression ($p < 0.05$). For the two fluoride-containing treatments, no effect of plaque thickness was observed.

Data of fluoride in the 'test plaque' and in enamel were submitted to the log transformation. Table 1 shows that both mouth rinses containing fluoride were able to significantly increase the concentration of fluoride ion (F) in 'test plaque' in comparison with negative control, Xylitol/Sorbitol and Chlorhexidine treatments ($p<0.05$). With regard to fluoride uptake in enamel, the highest concentrations were found for the fluoride-containing mouth rinse treatments (Table 1) and the difference compared with that of the negative control group was statistically significant ($p<0.05$).

DISCUSSION

The present study investigated the immediate effect of Xylitol/Sorbitol and its combination with fluoride on enamel demineralization, simulating a single rinse used in a clinical situation. The findings (Figure 1) showed that fluoride rinse treatment was able to reduce enamel demineralization ($p<0.05$), while the mixture of polyois (Xylitol/Sorbitol) was ineffective ($p>0.05$). As a consequence, the statistically significant effect of the combination Xylitol/Sorbitol + Fluoride treatment may be attributed to the isolated effect of fluoride.

The data of reduction of enamel demineralization are supported by the analyses of F in the 'test plaque' and enamel (Table 1). The increased F concentration found in 'test plaque' when fluoride-containing treatments (with or without Xylitol/Sorbitol) were used may be responsible for the reduction of enamel demineralization found (Figure 1). Fluoride

can interfere with de-remineralization process of enamel either by reducing the ability of *S. mutans* to ferment sucrose in acids or by a physicochemical mechanism. In the present study, an inhibition of acidogenicity did not seem reasonable since the F concentrations found in 'test plaque' are lower than the minimum concentration known to interfere with oral bacteria acidogenesis [Bradshaw et al., 2002]. On the other hand, the physicochemical effect is supported by the highest F concentration in enamel treated with fluoride-containing rinses (Table 1). The uptake of F by enamel can be explained by the fact that, during the dynamic of caries process, there is loss of more soluble minerals, but in presence of F part of these minerals are rebuilt as fluorapatite [Larsen, 1990; ten Cate, 1990]. As a consequence, there is a reduction of demineralization and an increase of F in enamel. The increased F concentration in enamel is coherent with the F concentration in 'test plaque' when fluoride-containing treatments were used (Table 1).

However, the lack of significant inhibitory effect on enamel demineralization observed when Xylitol/Sorbitol treatment was used previously to the cariogenic challenge suggests that the 'futile xylitol cycle' [Trahan et al., 1991] may not occurred under the experimental conditions tested. It is possible that the limited exposure time rinsing (1-min rinse) or the low xylitol dose used in the present study could account for the observed xylitol/sorbitol ineffectiveness. Indeed, the 'futile xylitol cycle' has only been proven to operate under highly non-physiological conditions both in pure culture and plaque samples incubated *in vitro* with xylitol [Waaler et al., 1985; Assev and Rölla, 1986a; Waaler, 1992]. Moreover, mouth rinses with higher xylitol concentrations (20 or 40%) than that used in the present study (xylitol 6% - 0.4 M and sorbitol 22% - 1.2 M) have shown no evidence for effects on the acidogenic potential of plaque [Giertsen et al., 1999]. Thus, the data suggest

that the xylitol-induced effects on plaque acidogenic potential, *in vivo*, may be marginal, and probably discernible only under extreme conditions. However, the experimental design used in this study did not allow the evaluation of other effects of xylitol such as decrease of dental plaque adhesiveness [Verran and Drucker, 1982; Mäkinen et al., 1985; Söderling et al., 1987] or reduction of plaque formation [Larmas et al, 1975; Söderling et al., 1989].

In the present study, chlorhexidine was used as a positive control because it is considered the most effective anti-plaque and antimicrobial agent currently in use [Jones, 1997]. However it did not inhibit enamel demineralization. The finding is coherent with the limited effect of chlorhexidine in biofilm compared to batch culture [Kinniment et al., 1996]. Thus, the data suggest that the IEDT model used presents the same response to chlorhexidine than a more complex biofilm [Pratten and Wilson, 1999; Zaura-Arite et al., 2001].

In conclusion, this study shows that a single rinse of xylitol/sorbitol did not inhibit enamel demineralization induced by sucrose fermentation while fluoride did. Moreover, xylitol/sorbitol does not enhance the effect of fluoride present in mouth rinses on the reduction of enamel demineralization. However, the present study does not exclude a possible effect of sugar alcohols on caries if used for a long time.

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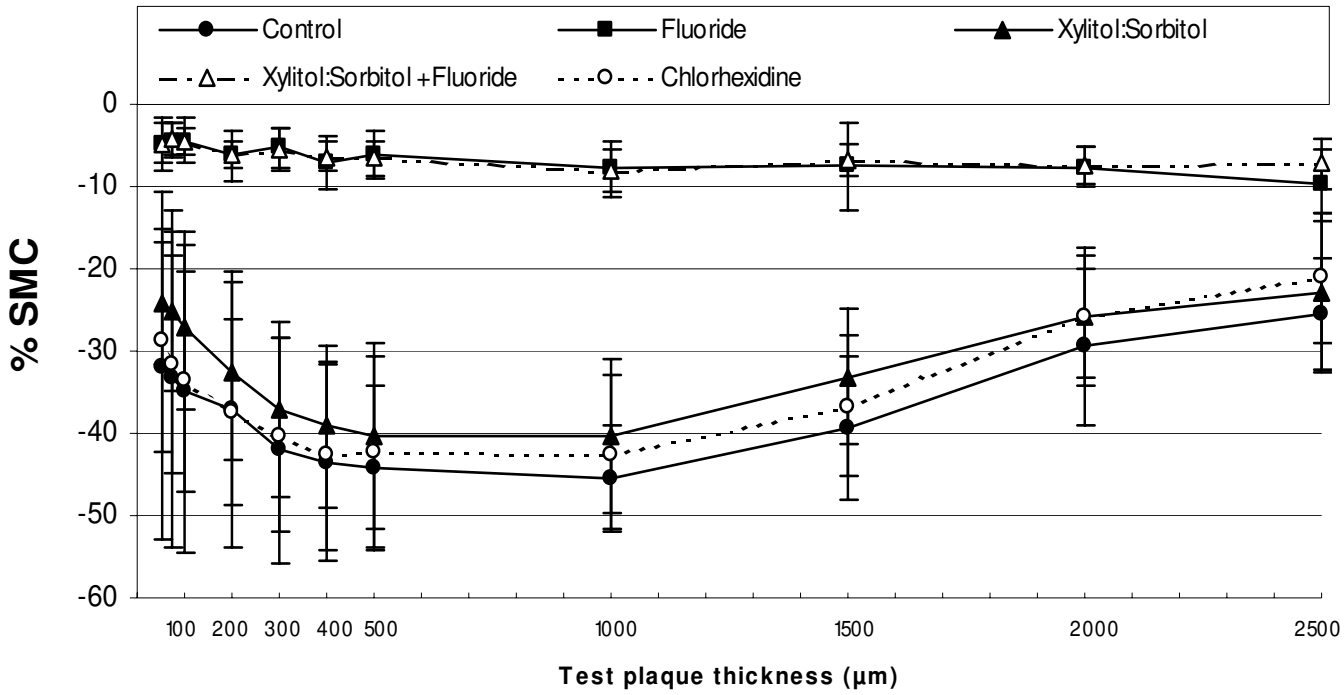

Figure 1: Means (n=10) of percentage of enamel surface microhardness change (% SMC) (original data), according to the treatments and "test plaque" thickness (µm). Fluoride and Xylitol/Sorbitol + Fluoride groups differed statistically from Control, Xylitol/Sorbitol and Chlorhexidine groups. Bars denote standard deviation.

Table 1: Fluoride concentration (mean ± SD) in 'test plaque' (µg F/g) and in enamel (µg F/cm^2) (original data) according to the treatment groups (n = 10).

Treatments groups	'test plaque', µg F/g	Enamel, µg F/cm^2
Control	0.8 ± 0.8^{a}	0.7 ± 0.1^{a}
Fluoride	3.2 ± 1.7^{b}	1.2 ± 0.4^{c}
Xylitol:Sorbitol	0.6 ± 0.2^{a}	0.6 ± 0.2^{a}
Xylitol:Sorbitol + Fluoride	3.0 ± 1.5^{b}	$1.0 \pm 0.2^{b,c}$
Chlorhexidine	0.5 ± 0.2^{a}	$0.8 \pm 0.1^{a,b}$

* Treatment groups whose means are followed by distinct superscripts letters differ statistically ($p < 0.05$).[WX1]

2.1. ARTIGO 3

COMBINED EFFECT OF XYLITOL AND ZINC CITRATE ON ACID PRODUCTION BY *STREPTOCOCCUS MUTANS* IN SUSPENSIONS AND BIOFILMS.

N.C.L.A.V. Gonçalves [1]; R.E. Marquis [2]; J.A. Cury [1].

[1]Faculty of Dentistry of Piracicaba, UNICAMP – Brazil; [2]Department of Microbiology and Immunology, University of Rochester Medical Center, USA.

Running Title: Effect of Xylitol and zinc citrate on acid production by *Streptococcus mutans*

Key Words: xylitol; zinc citrate; biofilms; *Streptococcus mutans*; glycolysis.

Full address of the author to whom correspondence should be sent:
Dr. Robert E. Marquis
Box 672, University of Rochester Medical Center,
Rochester, NY, 14642-8672, USA
Phone: #01- 585- 275-1674
Fax: #01- 585- 473-8973
E-mail: mutansst@aol.com

ABSTRACT

Xylitol is a useful sugar substitute for prevention of dental caries, but its anticaries effects when combined with zinc have not been well studied. ***Objective:*** The purpose of the present study was to investigate combined actions of xylitol and zinc citrate on acid production from glucose or sucrose by *S.mutans* in suspensions or in biofilms. ***Methods:*** Cells of *S.mutans* GS-5 from suspension cultures or 5-day biofilms grown on glass slides were used. Acid production from glucose or sucrose was assessed with standard pH-drop assays or by determining production of titratable acid at a constant pH value of 5.0. All experiments were performed at least three times. ***Results:*** Glycolysis by cells in suspensions with excess sugar was inhibited by xylitol and zinc citrate in an additive way as indicated by slower pH drop and higher final pH values or reduced rates of acid production at pH 5. The two agents acted in an additive way also to inhibit biofilm acid production, which was slower than that of cells in suspensions, probably mainly because of diffusion limitation in the biofilms. The combination of xylitol and zinc citrate was inhibitory for pH drop and acid production from glucose and sucrose. 50% inhibitory concentrations for biofilms were some 0.02 and 0.05 mM for zinc citrate with glucose or sucrose as substrate, while levels of xylitol equal to those of the cariogenic sugars used (11.1 or 5.9 mM for glucose or sucrose) were required. ***Conclusion:*** The data indicate that xylitol and zinc citrate act concert to inhibit acid production from glucose and sucrose by mutans streptococci.

INTRODUCTION

Zinc is added as an antimicrobial agent to many oral care products, notably toothpaste. It is commonly added along with citrate, which has anti-calculus action. Zinc has been shown to be an inhibitor of growth and acid production by human dental plaque *in vitro* [Harrap et al., 1984] and *in vivo* [Skjörland et al., 1978; Oppermann and Rölla, 1980]. Although chelators such as citrate or EDTA can lower the antimicrobial potency of zinc [Watson et al., 1991], zinc citrate has been found [Phan et al., 2004] to be more potent than the sulfate or chloride salts for inhibiting acid production by cariogenic bacteria. Moreover, zinc is known to inhibit certain glycolytic enzymes, such as the phosphoenolpyruvate: sugar phosphotransferase system (PTS), aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, with resultant reduction of glycolytic production of acid [Phan et al., 2004].

The use of metabolic inhibitors to enhance the actions of zinc against oral bacteria has potential for better control of acid production by cariogenic plaque. Xylitol, a five-carbon sugar alcohol is currently used in oral care products, chewing gum and sweets as a sugar substitute for prevention of dental caries. The inhibitory effects of xylitol on glycolysis by oral bacteria are generally ascribed to competition for phosphoenolpyruvate (PEP) since xylitol is transported into cells by a PEP-dependent PTS [Trahan et al., 1985]. The product of xylitol uptake, xylitol-5-phosphate, cannot be catabolized by xylitol-sensitive bacteria and is toxic to them. It can be dephosphorylated, and the xylitol produced is then expelled from the cell [Assev and Rölla, 1986; Söderling and Pihlanto-Leppälä,

1989]. Thus, a futile xylitol cycle is set up, which consumes even more PEP and enhances inhibition of growth and acid production.

Combinations of zinc, xylitol and fluoride have been found [Scheie et al., 1988] to have mutual inhibitory effects on bacterial growth and metabolism, possibly related to the agents acting on different targets in the glycolytic system. Moreover, the intracellular concentrations of phosphorylated xylitol were higher in cells exposed to zinc than in those exposed to xylitol alone [Scheie et al., 1988]. However, the effectiveness of zinc and xylitol combinations against cells in biofilms has not been determined. Considering that the biofilm state is the common lifestyle for most microorganisms in nature, it should be of interest to evaluate the effect of these agents against biofilm cells.

Thus, the purpose of the present study was to investigate combined actions of zinc citrate and xylitol on acid production from glucose or sucrose by mutans streptococci in biofilms compared with suspensions.

MATERIALS AND METHODS

Bacteria

Streptococcus mutans GS-5 are maintained routinely in the laboratory with weekly subculture on tryptic-soy agar plates (Difco, Detroit, MI) to avoid selecting rapidly growing variants. Long term storage is at –70^{o}C in 50% glycerol solution.

For work with cell suspensions, bacteria were grown in static cultures in a standard 37^{o}C incubator in tryptone-yeast-extract (TY) medium containing 1% (w/v) glucose until the early stationary phase of growth, when they had undergone acid adaptation [Belli and Marquis, 1991].

Mono-organism biofilm were grown on glass slides following procedures described previously [Phan et al., 2000; Burne and Marquis, 2001]. The slides were placed initially in TYSucrose medium in glass staining dishes and transferred to a new medium daily until dense biofilms formed, usually some 3 X 10^8 cell/mm^2 on slides with a total area of 18.75 cm^2. Then, on the day before the biofilms were to be used for experiments (4 days after inoculation), they were transferred overnight to the final medium, such as TYGlucose. Biofilms were harvested by removing them from the final growth medium, washing then with salt solution (50 mM KCl plus 1 mM $MgCl_2$) and then using them directly for experiments.

Glycolysis assays

Initial evaluations of the effects of xylitol and/or zinc citrate on glycolysis involved standard pH-drop experiments with thick cell suspensions or biofilms following procedures described previously [Belli and Marquis, 1994]. Briefly, cells from suspension cultures were harvested, washed once with salt solution (50 mM KCl plus 1 mM $MgCl_2$), and resuspended in salt solution to give suspensions with approximately 0.4 mg cell dry weight per ml. The suspension pH was adjusted to 7.2 and sugars were added - 0.2% (11.1 mM) glucose or 0.2% (5.8 mM) sucrose. The subsequent fall in pH was recorded with a glass electrode.

In addition to recording pH drop, glycolysis was also assayed by determining production of titratable acid. Acid production was assayed under pH-stat conditions at a maintained pH value of 5 by recording the amount of alkali required to maintain constant pH. Acid production was expressed in terms of μequivalents/mg cell dry weight and the percentage of inhibition regarding to the control (without xylitol and zinc citrate) was

calculated. For assays with cells in suspensions, starved cells from suspension cultures were harvested, washed once with salt solution (50 mM KCl plus 1 mM $MgCl_2$), and resuspended in 20 mM phophate buffer (pH 7.0) to give suspensions with approximately 2.0 mg cell dry weight per ml. When biofilms were used, 5-day biofilms were used and an entire, intact biofilm on it slide was immersed in salt solution in a tube. Dry weights of biofilms were determined by scraping them from the glass sides into water, centrifuging the cells and washing again with water. Samples of the final water suspensions were dried to constant dryness in an oven.

All the assays were carried out in duplicate on at least three different occasions.

RESULTS

The effect of zinc citrate and xylitol on pH drop in suspensions of *S. mutans* GS-5 with glucose and sucrose are presented in Figure 1A and 1B, respectively. Rates of pH drop reflect acidogenic capacities of the cells, while final pH values of the suspensions also reflect acid tolerance. For acid-tolerant mutans streptococci, the final pH values are typically slightly below 4, as shown here for the GS-5 strain. When 2 times equimolar xylitol was added, there was only a slight effect on the rate of acidification from glucose. However, it did not seem to affect the pH drop from sucrose on planktonic cells. The addition of 0.010 mM zinc citrate showed only a small effect on the rate and extent of acidification either to glucose or to sucrose. However, combination of xylitol and zinc citrate was able to inhibit glycolysis by cells in suspension with glucose or sucrose in an additive way as indicated by slower pH drop and higher final pH values (Figure 1A and 1B).

The combination of xylitol and zinc citrate was inhibitory for acid production from glucose and sucrose both in suspension (Figure 2) and biofilm cells (Figure 3) by *S. mutans* GS-5 under pH-stat conditions, here at a pH value of 5. Control values (0% inhibition) in terms of µmol acid produced per mg dry weight per hour are Fig. 2A – 1.59, Fig. 2B - 0.37, Fig. 3A – 0.19 and Fig. 3B – 0.21. The two agents acted in an additive way also to inhibit biofilm acid production, which was slower than that of cells in suspensions, probably mainly because of diffusion limitations in the biofilms. 50% inhibitory concentrations for biofilms were some 0.02 and 0.05 mM for zinc citrate with glucose or sucrose as a substrate, while equal or double levels of xylitol to those of glucose or sucrose used (11.1 or 5.9 mM for glucose or sucrose) were required (Figure 3).

DISCUSSION

The present study indicates that glycolysis and acid production by suspension or biofilm cells were inhibited either by zinc and xylitol combination or by the individual agents. Zinc citrate and xylitol seem to act in an additive way as indicated by slower pH drop and higher final pH values or reduced rates of acid production at pH 5 (Figure 1, 2 and 3). The inhibitory effect on the PTS activity and glycolytic enzymes by zinc [Phan et al., 2004] in addition to the phosphoenolpyruvate (PEP) and energy depletion by xylitol [Assev and Rölla, 1986; Trahan et al., 1991] probably may account for the reduced acid production from glucose and sucrose observed in the present study. Furthermore, combination of zinc and xylitol increases intracellular concentrations of xylitol metabolites [Scheie et al., 1988], however it is still unknown whether this phenomena is due to increased uptake or to decreased or retarded expulsion of xylitol metabolite.

In the present study, acid production was slower in biofilms than in suspensions, although the difference was less when sucrose was the added catabolite compared with glucose. The slower rates for biofilms are probably due at least in part to diffusional limitations on glycolysis because of the thickness of the biofilms. Moreover, increased resistance of bacteria in biofilm to antibacterial agent compared with planktonic bacteria have already been reported by other authors [Larsen and Fiehn, 1996; Shani et al., 2000; Zaura-Arite et al., 2001].

In terms of % inhibition values, the agents appeared to be actually more effective against biofilms than against cells in suspensions when sucrose was the catabolite, but this finding may be related to the overall slower pace of acid production in the biofilms.

In conclusion, under the above-mentioned experimental conditions, the data indicate that xylitol and zinc citrate act concert to inhibit acid production from glucose and sucrose by mutans streptococci. However, further studies should be done to investigate the exact mechanism by which xylitol and zinc citrate act to inhibit bacterial metabolism.

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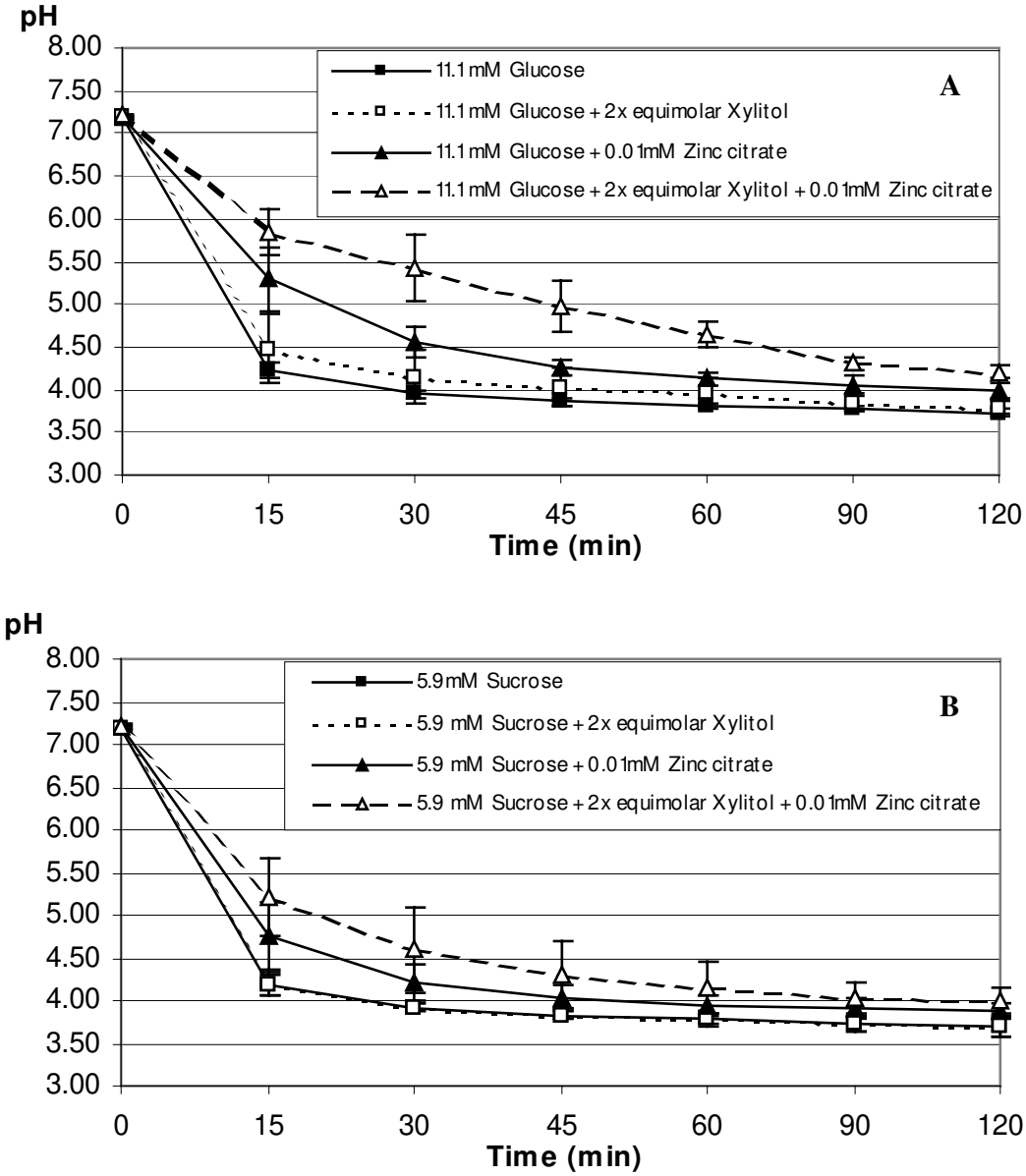

Figure 1: Effect of zinc citrate and xylitol on glycolytic pH drop by cells in suspensions given glucose (A) or sucrose (B) just after initial pH adjustment. Error bars indicate standard deviation with n = at least 3.

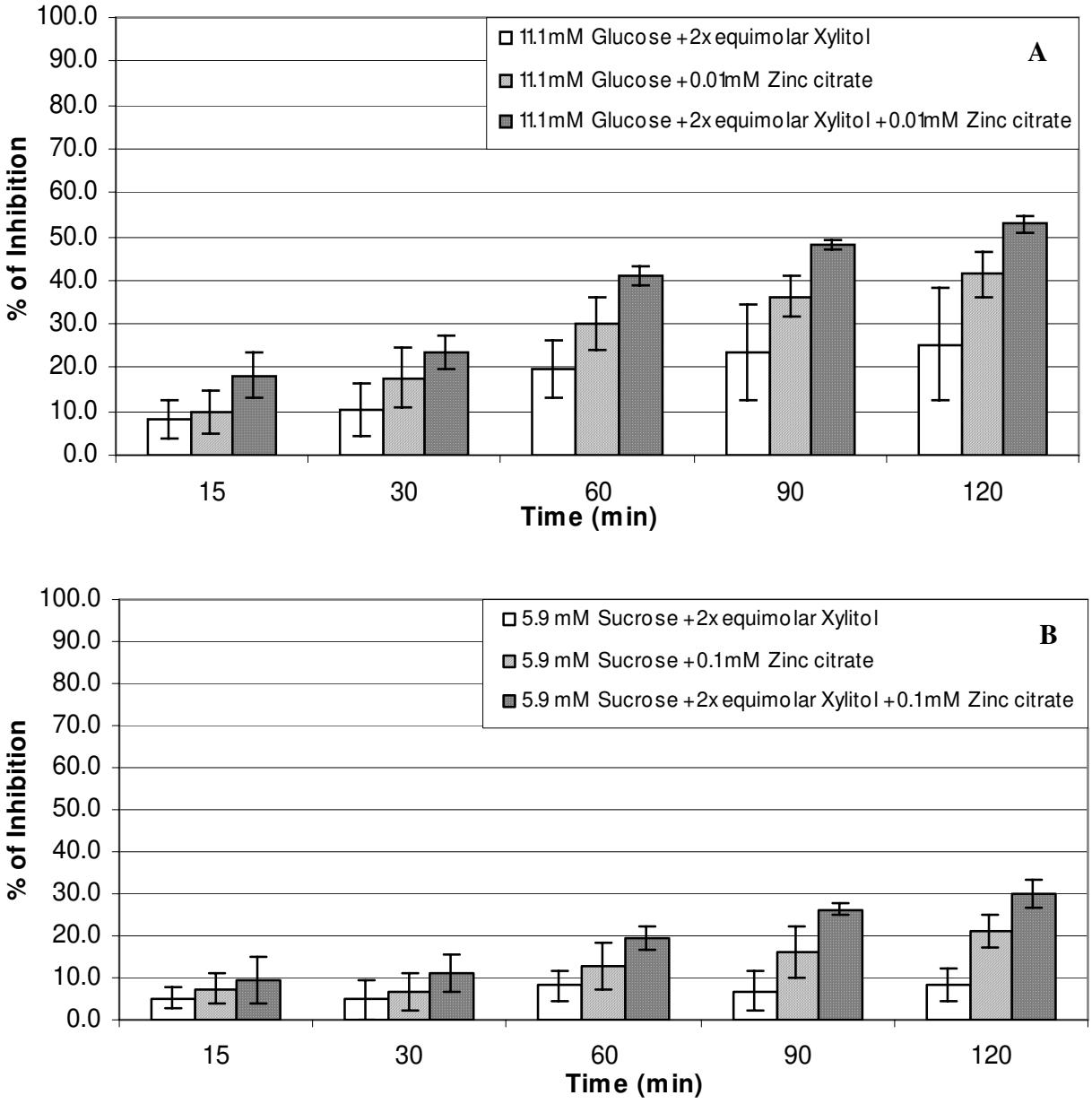

Figure 2: Effect of zinc citrate and xylitol on acid production by suspension cells of *S. mutans* GS-5 in the presence of glucose (A) and sucrose (B) at a constant pH of 5. Error bars indicate standard deviation with n = at least 3.

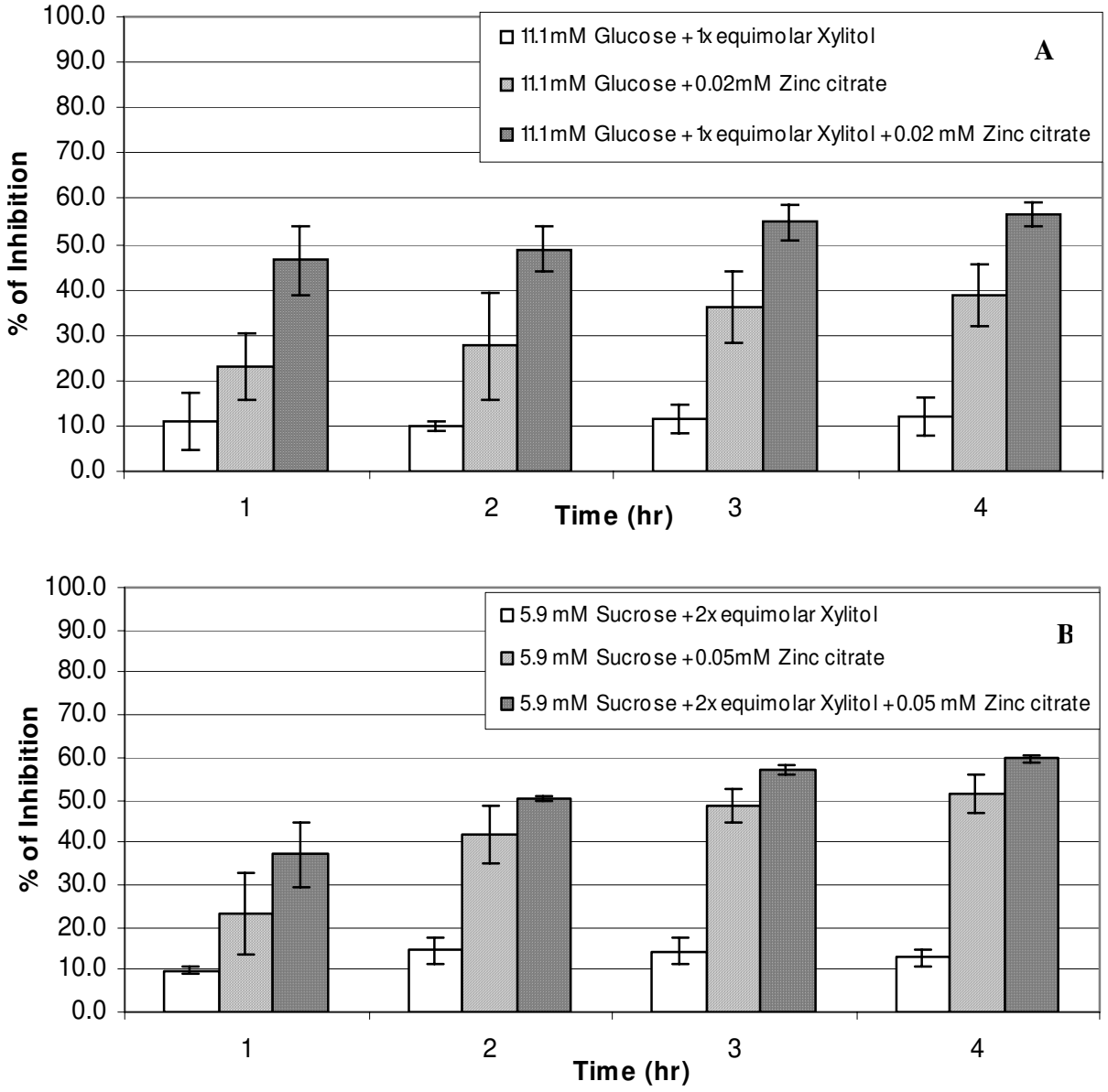

Figure 3: Effect of zinc citrate and xylitol on acid production by biofilm cells of *S. mutans* GS-5 in the presence of glucose (A) and sucrose (B) at a constant pH of 5. Error bars indicate standard deviation with n = at least 3.

3. CONCLUSÃO

Os estudos apresentados neste trabalho sugerem que a associação de polióis a outras substâncias anticariogênicas ou antimicrobianas como o flúor e o citrato de zinco, respectivamente, pode ser vantajosa no controle e prevenção da doença cárie.

Conclusões específicas:

- A associação de flúor e xilitol mostrou-se eficaz na inibição do crescimento bacteriano *in vitro* quando comparado ao efeito individual dos agentes. Além disso, a adição de sorbitol não interferiu no efeito inibitório do xilitol e flúor (estudo 1);

- A adição de polióis a solução para bochecho fluoretada não promoveu efeito adicional na inibição da desmineralização do esmalte dental *in situ*, quando comparado a utilização de solução fluoretada apenas (estudo 2);

- A associação de citrato de zinco e xilitol indicaram um efeito adicional dos agentes na inibição da produção de ácidos a partir de glicose e sacarose em biofilme bacteriano *in vitro* (estudo 3).

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Trahan L. Xylitol: a review of its action on mutans streptococci and dental plaque – its clinical significance. **Int Dent J**.1995; 45: 77-92.

Anexo 1

----- Original Message -----
From: "Peter Shellis" <r.p.shellis@bristol.ac.uk>
To: <jcury@fop.unicamp.br>
Sent: Friday, February 27, 2004 6:56 AM
Subject: Re: Request for review

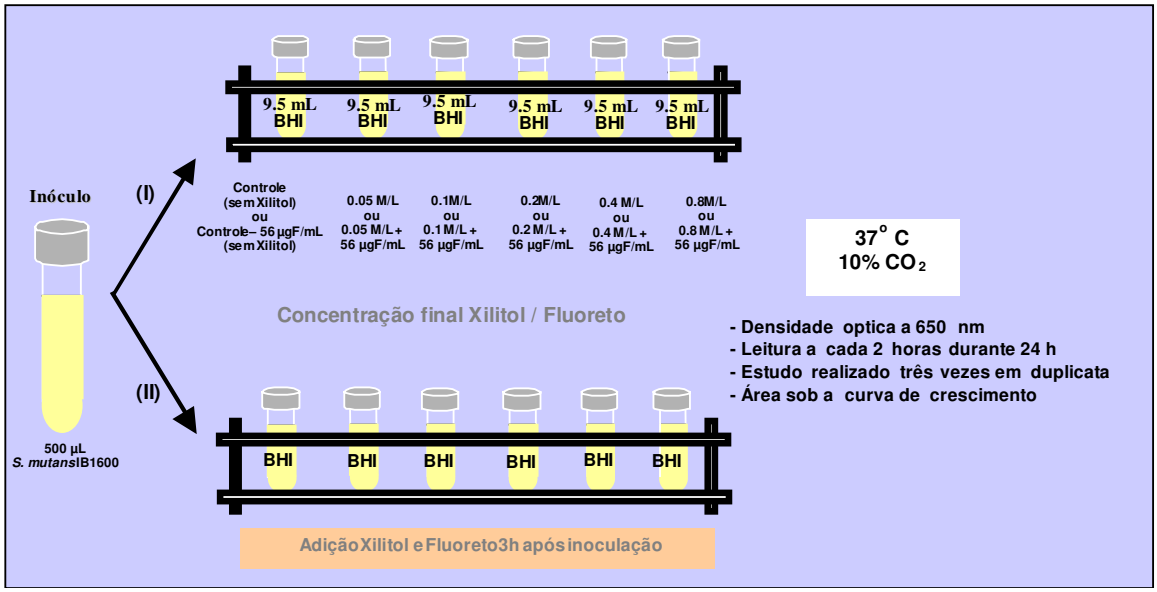
> Dear Jaime
> I remember now. I received your email but as it said that you would also be
> sending the paper by ordinary mail as well, I was waiting for that to
> arrive before entering it in the database and sending an acknowledgement.
> As the mail version has not arrived, that accounts for the delay. Shall I
> just proceed with the emailed version? In any case, I have entered it in
> the database, with the number 32/04.

----- Original Message -----
From: Jaime Cury
To: Peter Shellis
Sent: Friday, February 13, 2004 4:12 PM
Subject: MS submission
Dear Dr Shellis
I have sent you by airmail the attached manuscript (**EFFECT OF RINSE CONTAINING FLUORIDEAND SUGAR ALCOHOLS ON ENAMEL DEMINERALIZATION IN SITU**) to be submitted to publication in the Caries Res. The letter signed by all the authors will be also sent.
Please, could send me by e-mail an advance acknowledge receipt about it.
Have you a nice weekend
Jaime A Cury
Prof. Titular de Bioquímica, FOP-UNICAMP
Av. Limeira 901
13414-903 Piracicaba SP
Tel: 0xx-19-3412-5302/5303
Fax: 0xx-19-3412-5218

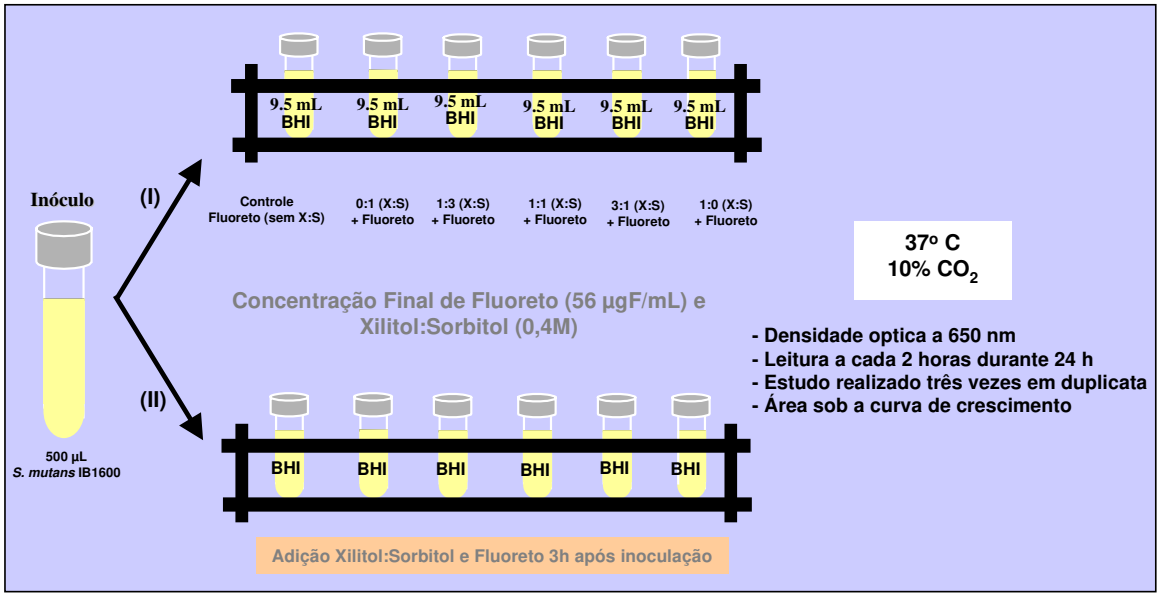
Anexo 2

Fluxograma do Teste de Crescimento Bacteriano – Artigo 1

- Efeito da adição de xilitol e fluoreto no crescimento bacteriano



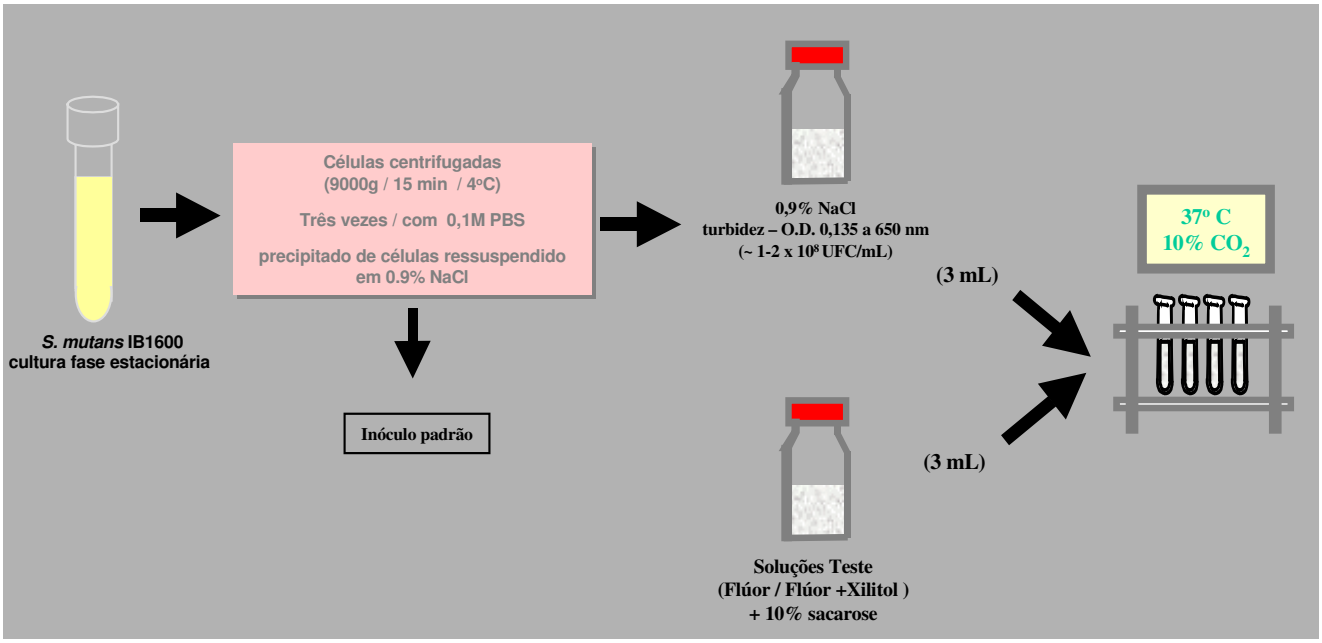
- Efeito de proporções de xilitol:sorbitol e flúor no crescimento bacteriano



Anexo 3

Fluxograma do Teste de Fermentação – Artigo 1

- Efeito da adição de xilitol e fluoreto na fermentação da sacarose



- Leitura pH: inicial e a cada 15 min durante 1 h
- • $pH_{0\text{-}60min}$ (final - inicial).

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
CEP-FOP-UNICAMP

CERTIFICADO

Certificamos que o Projeto de pesquisa intitulado "Efeito da associação de fluor e xilitol/sorbitol na inibição da desmineralização do esmalte dental – estudo ***in situ***", sob o protocolo nº **36/2000**, do(a) Pesquisador(a) **Nilza Cristina Lopes Afonso de Valor Gonçalves**, sob a responsabilidade do(a) Prof(a). Dr(a). **Jaime Aparecido Cury**, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa – FOP.

Piracicaba, 03 de maio de 2000

We certify that the research project with title "Effect of association of fluoride and xylitol/sorbitol on reduction of demineralization of enamel – ***in situ*** evaluation", protocol nº**36/2000**, by Researcher**Nilza Cristina Lopes Afonso de Valor Gonçalves**, responsibility by Prof. Dr.**Jaime Aparecido Cury**, is in agreement with the Resolution 196/96 from National Committee of Health/Health Department (BR) and was approved by the Ethical Committee in Research at the Piracicaba Dentistry School/UNICAMP (State University of Campinas).

Piracicaba, SP, Brazil, May 03 2000

Prof. Dr. Pedro Luiz Rosalen
Secretário - CEP/FOP/UNICAMP

Prof. Dr. Antonio Bento Alves de Moraes
Coordenador - CEP/FOP/UNICAMP

Anexo 5

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Nome do voluntário:_____

As informações contidas neste prontuário foram fornecidas por Nilza Cristina Lopes Afonso de Valor Gonçalves (pós-graduanda - doutoramento), Prof. Dr. Jaime A. Cury (Orientador), Profa Dra. Altair A. Del Bel Cury (Co-orientadora da parte clínica) objetivando firmar acordo escrito mediante o qual o voluntário da pesquisa autoriza sua participação, com pleno conhecimento da natureza dos procedimentos e riscos a que se submeterá, com a capacidade de livre arbítrio e sem qualquer coação.

1. Título do Trabalho Experimental

Efeito da associação de flúor e xilitol/sorbitol na inibição da desmineralização do esmalte dental – estudo in situ.

2. Objetivo

Comparar o efeito in situ de soluções para bochecho contendo flúor e/ou xilitol/sorbitol em relação à inibição da desmineralização do esmalte dental.

3. Justificativa

Existe um consenso mundial de que o efeito do flúor no controle da cárie dental é essencialmente físico-químico, reduzindo a desmineralização e ativando a remineralização do esmalte-dentina (ten CATE & REMPT, 1986). O efeito do xilitol na redução da acidogenicidade bacteriana também tem sido comprovado (SÖDERLING et al., 1989). Por outro lado, o efeito da redução da quantidade de ácidos produzidos na desmineralização do esmalte não tem sido demonstrado. Deve ser enfatizado que a maioria dos estudos relata o efeito do xilitol na remineralização do esmalte (SMITS & ARENDS, 1985; MANNING et al., 1992; SCHEININ et al., 1993). Entretanto, considerando que o xilitol possui ação na acidogenicidade bacteriana, consumindo suas reservas energéticas durante seu transporte (LOESCHE, 1993), pode-se esperar um efeito adicional ao flúor na inibição da desmineralização do esmalte.

4. Procedimento da fase experimental

Avaliação da inibição da desmineralização: o estudo in situ será do tipo cruzado, sendo que dez voluntários serão divididos em cinco grupos de tratamentos e utilizarão dispositivos intra-orais contendo oito blocos de esmalte bovino, recobertos por uma massa bacteriana (‘placa teste’) de *S. mutans* IB-1600 (ZERO et al., 1986). Os voluntários utilizarão dentifrício placebo por uma semana antes de cada tratamento. O estudo será realizado duas horas após os voluntários terem comido e escovados seus dentes com dentifrício não fluoretado. Os tratamentos estão descritos abaixo:

Grupo 1 – solução de fluoreto de sódio a 0,05%: os voluntários realizarão bochecho com a solução de fluoreto de sódio a 0,05% durante um minuto. Ao mesmo tempo, os dispositivos intra-orais, contendo os blocos de esmalte dental bovino, serão imersos por um minuto na mesma solução. Depois da realização do bochecho, os dispositivos serão

imediatamente introduzidos na boca. Após 20 minutos, o voluntário removerá o dispositivo intra-oral, bochechará 15 ml de solução de sacarose 20% , por 1 minuto. Ao mesmo tempo, o dispositivo intra-oral do voluntário será imerso na mesma solução de sacarose 20%. Após o bochecho, os voluntários utilizarão os dispositivos por mais 45 minutos. Após este período, o dispositivo será removido e os blocos de esmalte dental e amostras de placa teste serão analisados.

Grupo 2 - solução de xilitol/sorbitol: os voluntários realizarão os mesmos procedimento do grupo anterior, modificando apenas a solução para bochecho a ser utilizada, no caso, solução de xilitol/sorbitol (1,6M - 1:3).

Grupo 3 - solução de fluoreto de sódio a 0,05% adicionada de xilitol/sorbitol: os voluntários realizarão os mesmos procedimento dos grupos anteriores, modificando apenas a solução para bochecho a ser utilizada, no caso, solução de fluoreto de sódio a 0,05% adicionada de xilitol/sorbitol (1,6M - 1:3).

Grupo 4 - solução de digluconato de clorexidina 0,12%: os voluntários realizarão os mesmos procedimentos dos grupos anteriores, modificando apenas a solução para bochecho a ser utilizada, no caso, solução de digluconato de clorexidina 0,12%. Este grupo será o controle positivo do estudo.

Grupo 5 - solução placebo: os voluntários realizarão os mesmos procedimento dos grupos anteriores, modificando apenas a solução para bochecho a ser utilizada, no caso, placebo de flúor e xilitol/sorbitol (água destilada). Este grupo será o controle negativo do estudo.

Entre os tratamentos será obedecido um período de “wash-out” de sete a dez dias para eliminar possível efeito residual das substâncias anteriormente utilizadas.

5. Desconforto ou riscos esperados e benefícios

O uso da sacarose será apenas durante o experimento, não implicando em qualquer aumento de cárie dental nos voluntários. O dispositivo intra-oral pode causar um leve desconforto, que é, entretanto, semelhante ao desconforto causado por um aparelho ortodôntico móvel e será utilizado apenas por algumas horas. A placa artificial poderá trazer um leve gosto de fermento de pão. A utilização de pequenas doses diárias de xilitol não apresenta efeitos colaterais adversos, ainda que a solução para bochecho não será ingerida, e sim dispensada após o tempo de 60 segundos de bochecho. Bochechos comerciais de clorexidina a 0,12% existem no mercado nacional (Periogard) e nos EUA (Peridex), sendo que os efeitos colaterais do uso 2 vezes /dia, durante o período de três meses é a presença de manchamento dental extrínseco, o qual é removido por profilaxia dental. No presente estudo, manchamento dental não é esperado, uma vez que esta solução será utilizada uma única vez. O benefício será um auxílio indireto, contribuindo para a realização deste projeto e para a ciência como um todo.

6. Forma de acompanhamento e assistência

Haverá esclarecimento do experimento. Os pesquisadores envolvidos na pesquisa darão toda a assistência aos voluntários durante a pesquisa.

7. Garantia de esclarecimentos

O voluntário tem garantia de que receberá respostas a qualquer pergunta ou esclarecimento de qualquer dúvida quanto aos procedimentos, riscos, benefícios e outros assuntos relacionados com a pesquisa. Também os pesquisadores supracitados assumem o compromisso de proporcionar informação atualizada obtida durante o estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando. Qualquer dúvida ou problema, por favor comunicar-nos com a maior brevidade possível.

Tel: 3412-5303 (Laboratório de Bioquímica) / 3433-4736 (Residência Profa. Altair/Prof. Jaime) / 3411-6936 (Residência Nilza).

8. Retirada do consentimento

O voluntário tem a liberdade de retirar seu consentimento a qualquer momento e deixar de participar do estudo sem prejuízo de ordem pessoal-profissional com os responsáveis pela pesquisa.

9. Garantia de sigilo

Os pesquisadores asseguram a privacidade dos sujeitos quanto aos dados confidenciais envolvidos na pesquisa.

10. Formas de ressarcimento

Os voluntários serão ressarcidos de eventuais despesas com o transporte para a participação no experimento.

11. Formas de indenização

Não há danos previsíveis decorrentes desta pesquisa.

Eu _____, certifico que tendo lido as informações acima e suficientemente esclarecido(a) de todos os itens pela Pós-graduanda Nilza Cristina Lopes Afonso de Valor Gonçalves, Prof. Dr. Jaime A. Cury (Orientador), Prof a Dra. Altair A. Del Bel Cury (Co-orientadora da parte clínica), estou plenamente de acordo com a realização do experimento. Assim, eu autorizo a execução do trabalho de pesquisa, exposto acima, em mim.

Piracicaba, ___ de _____ de _____.

Nome (por extenso):_____

Assinatura:_____

1ª via: Instituição

2ª via: Voluntário

Anexo 6

ANEXO AO TERMO DE CONSENTIMENTO

INSTRUÇÕES AOS VOLUNTÁRIOS:

1- Inicialmente, os voluntários deverão utiliar dentifrício placebo por 7 dias antes do início do estudo e não deverão utilizar nenhum bochecho ou substância enxaguatória.

2- Manter sua dieta normal.

3- Os dias de realização dos estudo estão marcados em anexo.

4- No dia do experimento, não comer, não beber e não conversar 2 horas antes do experimento. Beber água é permitido.

5- No início do experimento, os voluntários devem bochechar 15 ml da **solução para bochecho** oferecida pelo pesquisador, durante 1 minuto e cuspir.

6- Ao mesmo tempo, o dispositivo intra-oral do voluntário será imerso na mesma solução, por 1 minuto.

7- Introduzir o dispositivo intra-oral palatino na boca imediatamente após a realização do bochecho e permanecer por 20 minutos com o dispositivo na boca. **Importante:** o voluntário não pode comer, falar, beber ou deglutir saliva com muita força durante estes 20 minutos.

9- Após 20 minutos, o voluntário removerá o dispositivo intra-oral, bochechará 15 ml de solução de sacarose 20% , por 1 minuto e cuspir. Ao mesmo tempo, o dispositivo intra-oral do voluntário será imerso na mesma solução de sacarose 20%.

10- Após bochecho com sacarose, permanecer com o dispositivo na boca por mais 45 minutos. Não falar, comer ou beber durante o uso do dispositivo intra-oral.

Anexo 7

Ilustração da metodologia do modelo *in situ* de curta duração – Artigo 2

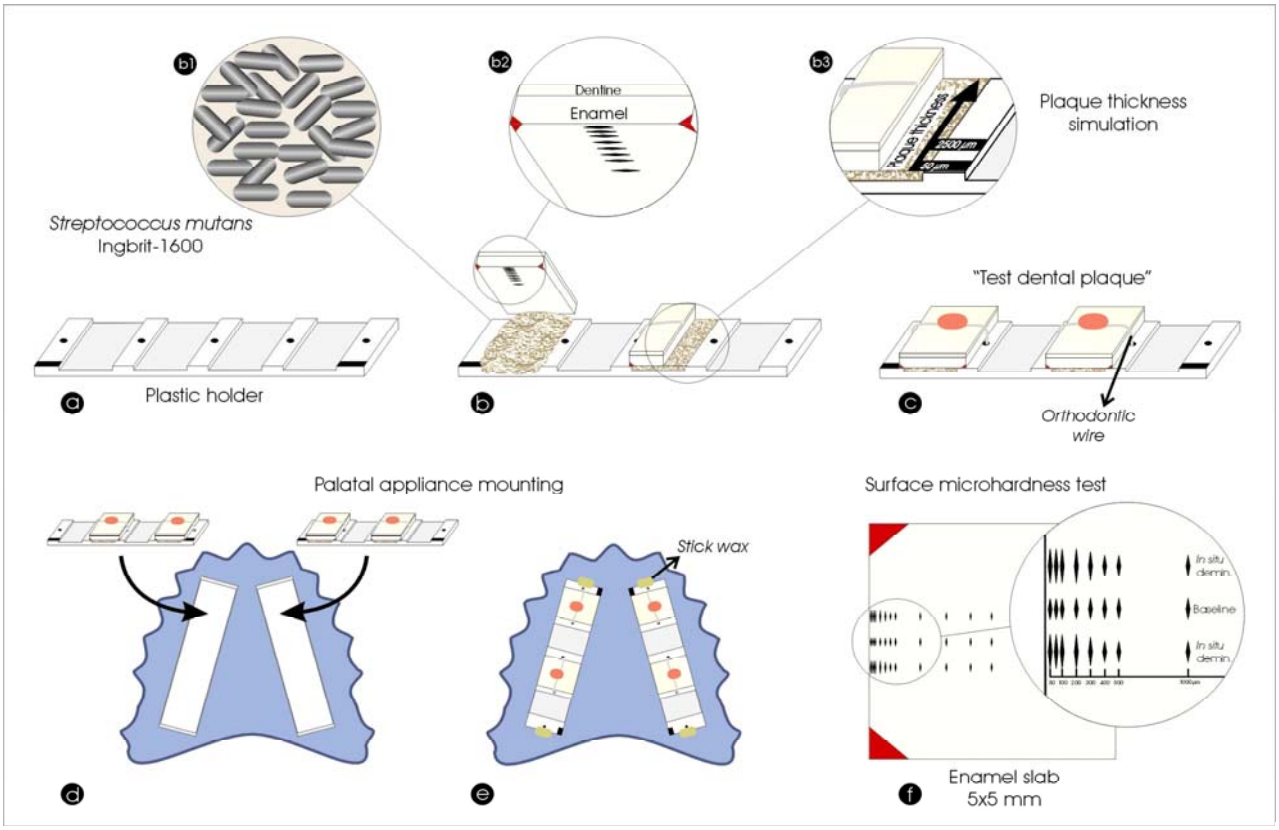
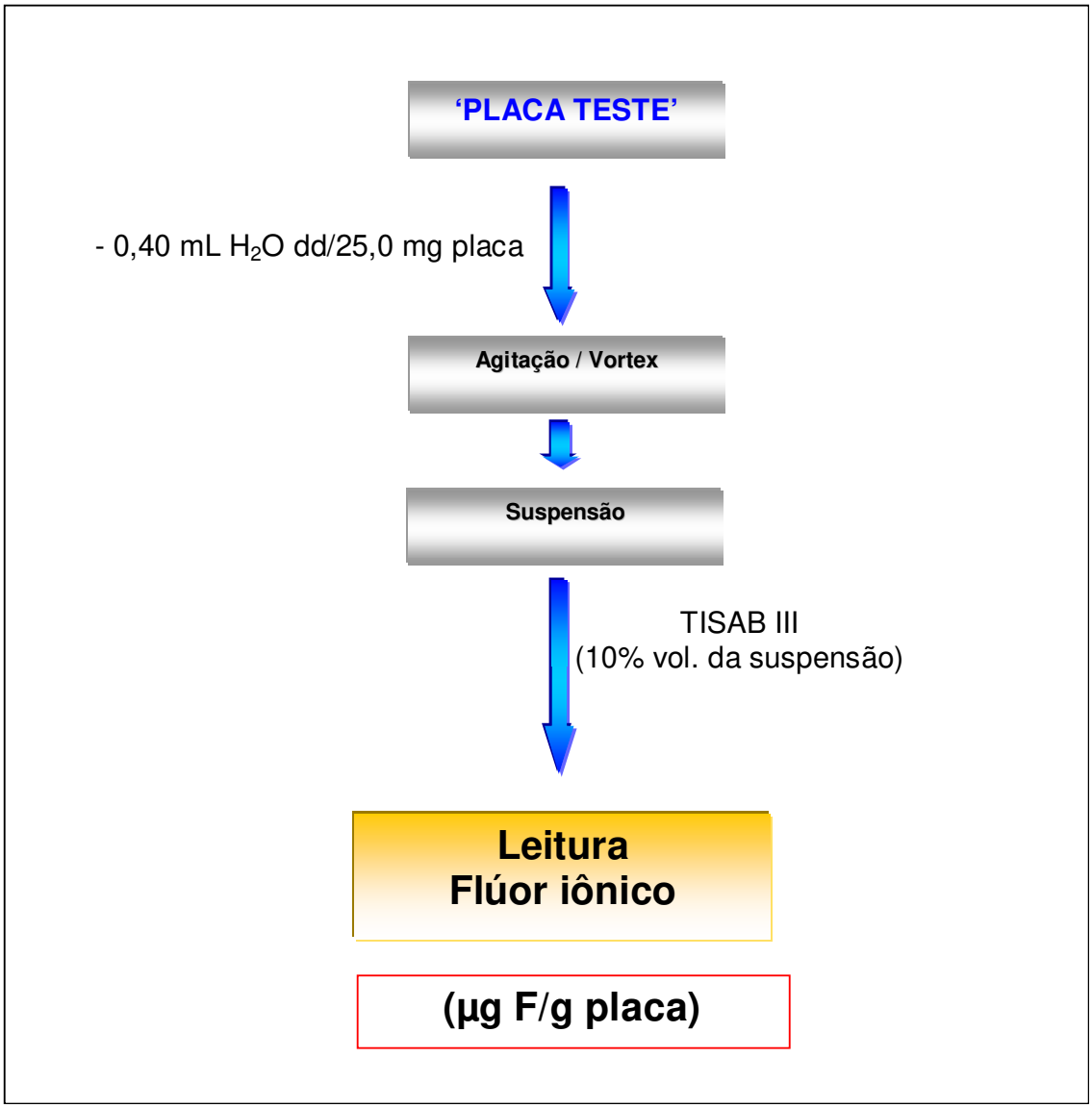


Figura: Ilustração da metodologia do modelo *in situ* de curta duração utilizado no Capítulo - Artigo 2. (a) " holder" acrílico; (b) seqüência da montagem do "holder" contendo a 'placa teste' e os blocos de esmalte dental; (b1) 'Placa teste' – *S. mutans* IB 1600; (b2) blocos de esmalte dental mostrando as marcações e indentações pré-tratamento realizadas; (b3) simulação da espessura de placa e direção da saliva, substrato e difusão do tratamento; (c) montagem completa dos blocos de esmalte no "holder"; (d, e) montagem dos dispositivos intra-orais palatinos; (f) ilustração esquemática das indentações realizadas pós-tratamento.

Anexo 8

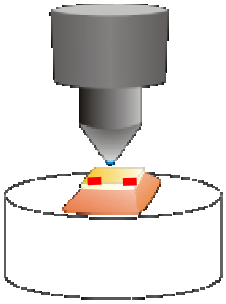
Análise da concentração de flúor na 'placa teste' – Artigo 2



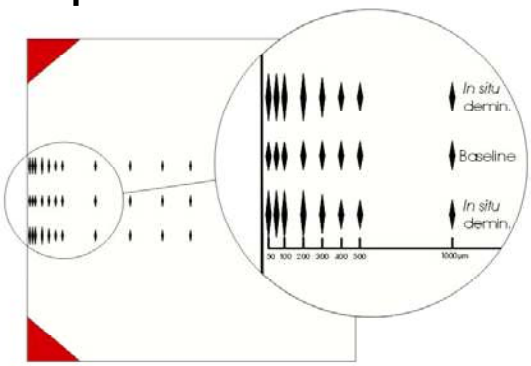
Anexo 9

Análises no esmalte dental – Artigo 2

Microdureza de superfície



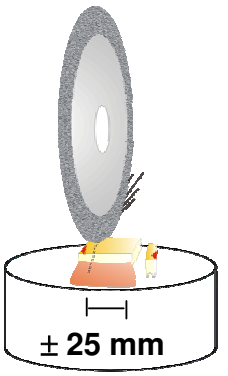
Análise do bloco dental no microdurômetro



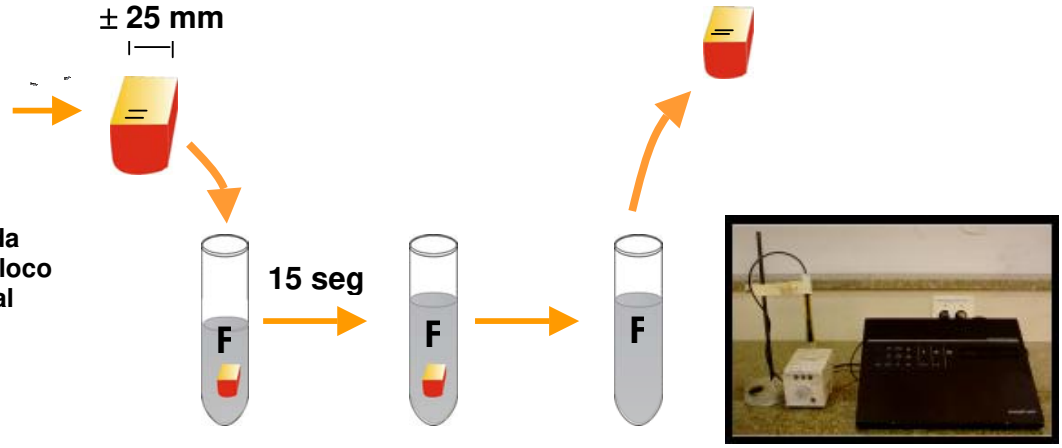
Bloco dental (5 X5) após a análise

% PDS = Dureza Pós-Tratamento – Dureza Inicial X 100
Dureza Inicial

Concentração de flúor no esmalte dental



Seccionamento da porção central do bloco de esmalte dental



0,25 mL HCl 0,5M

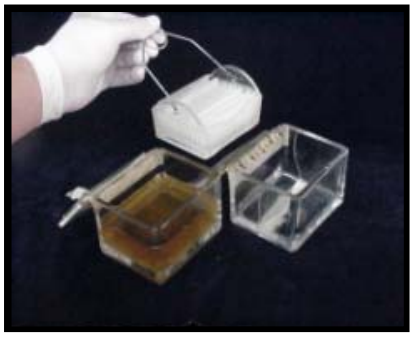
0,25 mL TISAB + 20g NaOH/L

µg F/cm²

Anexo 10

Ilustração da metodologia de Biofilme Bacteriano *in vitro* – Artigo 3

A - Caixa, cestas e lâminas.



B - Formação do biofilme bacteriano
- troca dos meios de cultura
(3º – 4º dia TY Sacarose)
(5º dia TY Glicose).

C - Biofilme formado – 6º dia.

D - Transferência das lâminas com Biofilme formado, para tubos cônicos

E - Teste de queda de pH e Inibição da Produção de ácido – Titulação.

Phan et al., 2000; Burne & Marquis, 2001.

Anexo 11

Outros trabalhos publicados ou em publicação durante o período de doutorado:

Gonçalves NCLAV.; Valsecki Jr., A.; Salvador, S.L.S.; Bergamo, G.C. Efeito de soluções fluoretadas contendo xilitol/sorbitol no número de estreptococos do grupo mutans na saliva de seres humanos. ***Rev.Panam. Salud Publica/Pan. Am.J.Public Health***. 2001; 9 (1): 30-4.

Hayacibara MF, Paes Leme AF, Lima YBO, **Gonçalves NCLAV**, Queiroz CS, Gomes MJ, Kozlowski FC. Alkali-soluble fluoride deposition on enamel after professional application of topical fluoride *in vitro.* ***J Applied Oral Sci***, 2004. (aceito)

Tenuta LMA; Ribeiro CCC; **Gonçalves NCV**; Del Bel Cury AA; AiresCP; Tengan C; Tagliaferro EPS; Pecharki GD; Napimoga MH; Tabchoury CPM; Cury JA. Anticariogenic potential of ionomeric materials evaluated *in situ* by a short-term model. ***Caries Res.*** (submitted)

Cury JA; Simões GS; Del Bel Cury AA; **Gonçalves NC**; Tabchoury CPM. Effect of a calcium carbonate-based dentifrice on in situ enamel remineralization. ***Caries Res***. (submitted)