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Aluno: Antonio Pedro Ricomini Filho

Orientador: Prof. Dr. Jaime Ap. Cury

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Antonio Pedro Ricomini Filho

"Efeito da Sacarose na Seleção de Estreptococos Mutans e Lactobacilos em Biofilme Dental Formado *in situ*"

Monografia apresentada ao
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Orientador: Prof. Dr. Jaime Ap. Cury

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Aos meus pais

pelo, amor e incentivo.

A minha irmã pelo exemplo de vida,

a minha sobrinha Isabela e ao

meu cunhado pelo apoio

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Sumário

LISTA DE PALAVRAS E ABREVIATURAS EM LATIM

et al.= e outros (abreviatura de "et alii")

EPS= extracellular polysaccharide

MS= Mutans streptococci

LB= lactobacilli

PEC= polissacarídeos extracelulares

EM= Estreptococos mutans

LB= lactobacilos

IPS= intracellular polysaccharide

RESUMO

Estreptococos mutans (EM) e lactobacilos (LB) são bactérias acidúricas cujo crescimento no biofilme dental é favorecido pela freqüente exposição a açúcares fermentáveis. A sacarose além de ser fermentável, é substrato para a produção de polissacarídeos extracelulares (PEC) insolúveis, em especial por EM. Este estudo cruzado, duplo-cego, teve como objetivo avaliar o efeito da sacarose (fermentável + indutora de PEC) ou glicose + frutose (simplesmente fermentáveis) nas populações de EM e LB no biofilme dental formado *in situ*. Dezesseis voluntários utilizaram um dispositivo intrabucal palatino contendo 4 blocos de esmalte humano protegidos por uma tela. Os blocos foram expostos 8x/dia a água destilada e deionizada (ADD), solução de glicose a 10% + frutose a 10% ou solução de sacarose a 20%, por um período de 13 dias. O biofilme dental formado ao final de cada fase foi coletado para determinação de PEC insolúveis e para quantificação de EM (agar MSB, 10% CO_2, 48 h), LB (agar Rogosa SL, 10% CO_2, 24-48 h) e bactérias totais (agar sangue, anaerobiose, 72h). A média das contagens por mg de biofilme de bactérias totais ($7,4x10^6$), EM ($3,9\ x10^2$) e da %EM em relação às bactérias totais não foram diferentes entre os tratamentos ($p>0,05$). As contagens de LB (média±DP, $x10^6$/mg biofilme) e sua porcentagem em relação às bactérias totais foi significantemente maior no biofilme formado na presença de glicose + frutose (1,6±2,6, 24,8%) e sacarose (3,2±5,2, 39,7%) quando comparadas a ADD (0,0002±0,0005, 0,009%). O biofilme formado na presença de sacarose apresentou significantemente mais PEC insolúveis ($p<0,05$) do que aquele formado sob os outros dois tratamentos. Os resultados sugerem que a exposição a açúcares é mais relevante na predominância de LB no biofilme

dental que de EM e que os PEC insolúveis não interferem nas contagens desses microrganismos no biofilme.

PROPOSIÇÃO

O fator principal para a formação de um biofilme dental cariogênico é a exposição freqüente a açúcares fermentáveis. Sacarose é mais cariogênica que seus monossacarídeos componentes (glicose + frutose), mas a diferença não está na produção de ácidos e sim no fato que polissacarídeos extracelulares (PEC) são formados exclusivamente a partir da sacarose. Entretanto, a importância ecológica da mudança ambiental do pH associada à síntese de PEC é desconhecida. Assim esse trabalho teve como objetivo o estudo das alterações nas populações de estreptococos mutans e lactobacilos em biofilme formado in situ na presença de sacarose (fermentável + indutor de PEC) ou glicose + frutose (apenas fermentáveis).

CAPÍTULO

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Effect of Sucrose on the selection of Mutans streptococci and lactobacilli in dental biofilm formed *in situ*

L.M.A. Tenuta, A.P. Ricomini Filho, A.A. Del Bel Cury, J.A. Cury*

Faculty of Dentistry of Piracicaba, UNICAMP, Piracicaba, SP, Brazil
*Corresponding author, jcury@fop.unicamp.br

Address for correspondence:

Jaime A Cury
Av. Limeira 901
13414-903, Piracicaba, SP
Brazil
Phone: #55-19-21065303, Fax: #55-19-21065212
E-mail: jcury@fop.unicamp.br

Key Words: Acidogenicity, biofilm, sucrose, insoluble extracellular, polysaccharides, Lactobacilli, Mutans Streptococci

ABSTRACT

Microorganisms are selected in dental biofilm by the acidic environment created by sugar fermentation, but the effect of extracellular polysaccharide (EPS) on the counts of cariogenic bacteria is not clear. Dental biofilm was formed in situ for 13 days under exposure 8 times a day to distilled-deionized water, glucose + fructose or sucrose solutions. Mutans streptococci (MS) counts were not different among the groups, but lactobacilli (LB) were significantly higher in glucose + fructose and sucrose groups, without significant difference between them, irrespective of the higher insoluble EPS concentration in the sucrose biofilm matrix. The data suggest that exposure to sugar is more relevant for the predominance of LB in dental biofilm than for MS and that insoluble EPS does not change the counts of these microorganisms in the biofilm.

INTRODUCTION

Frequent exposure to fermentable carbohydrates is related to a microbiological shift in dental biofilm, selecting acidogenic and aciduric bacteria [Marsh, 1994], such as mutans streptococci (MS) and lactobacilli (LB). Sucrose is considered the most cariogenic carbohydrate because besides being fermented to acids, it is also a substrate for extracellular polysaccharide (EPS) production in dental biofilm [Rølla, 1989]. These EPS improve the adhesion of new bacteria to the growing biofilm [Rølla, 1989] and increase the porosity of the matrix [Dibdin and Shellis, 1988; Van Houte et al., 1989], allowing the diffusion of fermentable substrates to the inner part of the biofilm, where they are converted to acids near the enamel surface. However, the effect of EPS on the population of MS and LB in the biofilm has not been experimentally studied.

Thus, this study aimed at evaluating the changes in the population of MS and LB caused by sucrose (acidogenic + EPS inductor) compared with its component monosaccharides, glucose and fructose (only acidogenic), in dental biofilm formed *in situ*.

MATERIALS AND METHODS

Experimental Design

The study involved an in situ double-blind crossover design approved by the Research and Ethics Committee of FOP/UNICAMP (Protocol 015/2002). During three phases of 14 days, 16 healthy adult volunteers, aged 19–33 years, with past caries experience ranging from 1 to 41 (mean DMFS = 12.4) but no active lesions, wore acrylic palatal appliances containing 4 human enamel

blocks of 4 x 4 mm, placed 1 mm below the acrylic level and covered by a plastic mesh to allow accumulation of dental biofilm [Cury et al., 2000]. Before the beginning of the study, volunteers' MS and LB salivary levels were determined; MS ranged from 2.5 x 10^2 to 8.3 x 10^5 CFU/ml and for LB, 4 volunteers had less than 50 CFU/ml, and the others presented from 5.0 x 10 to 3.5 x 10^5 CFU/ml. In each experimental phase, 8 times a day, volunteers removed the appliance from the oral cavity and dripped the treatment solutions on the dental blocks: distilled and deionized water (negative control), 10% glucose + 10% fructose solution (active control), or 20% sucrose solution (experimental). Five minutes after the application, the appliance was replaced in the mouth. On day 13 of each phase, the pH of the biofilm (baseline) was determined in situ after overnight fasting and 5 min after dripping a 20% glucose solution. Glucose was used to avoid complications due to synthesis of EPS by some organisms as well as acid production. The solution was dripped onto two blocks extraorally, and after 1 min the appliance was replaced in the mouth, the in situ pH being measured after additional 4 min (pH 5 min) using a contact microelectrode (WPI, MEPH-3L, Sarasota, Fla., USA) and a reference electrode (Orion, 9002, Boston, Mass., USA) connected to a pH meter (Orion, 720-A) [Ribeiro et al., 2005]. After the measurement, the biofilm formed on these blocks was collected for MS and LB counts. Volunteers continued using the appliance until the next day, when the biofilm formed on the other two blocks was collected for determination of insoluble EPS and intracellular polysaccharide (IPS) concentration.

Throughout the experiment, volunteers used a silica-based dentifrice containing 1,100 μg F/g (NaF) to brush their own teeth and the appliance, except for the

plastic mesh area, consumed fluoridated water (0.76 ± 0.03 mg F/l) and received instructions as previously described [Cury et al., 2000]. A washout interval of 7 days was established between each experimental phase. No restriction was made with regard to the volunteers' diet, but they were instructed to remove the appliances during meals [Cury et al., 2000].

Microbiological Analyses

Stimulated saliva, collected for 5 min, was serially diluted in 0.9% NaCl, and drops of 20 μl of each dilution were plated, in duplicate, on mitis salivarius-bacitracin (MSB) agar [Gold et al., 1973] for enumeration of MS, and on Rogosa SL agar for enumeration of LB. Plates were incubated at 37 °C and 10% CO_2 for 24– 48 h.

Biofilm formed on enamel blocks was collected with a plastic spatula, weighed to ±10 μg (Analytical Plus AP 250D, Ohaus Corp., Florham Park, N.J., USA) in sterile microcentrifuge tubes, suspended in 0.9% NaCl (1 ml/mg biofilm wet weight) and sonicated using Sonifier Vibra Cell (Sonics and Materials, Danbury, Conn., USA) at 40 W, 5% amplitude, 6 pulses of 9.9 s each [Bowen et al., 1988]. The suspensions were serially diluted in 0.9% NaCl and drops of 20 μl were inoculated in duplicate in MSB agar, Rogosa SL agar, and blood agar, this for enumeration of total bacteria. The plates were incubated at 37 °C, in atmosphere of 10% CO_2 (MSB and Rogosa) or in anaerobiosis (blood agar), for 24–72 h. The colony-forming units (CFU) were counted using a stereomicroscope, and the results expressed in colony-forming units per milligram of dental biofilm wet weight. Different colony morphologies were

identified by Gram staining and morphology. Additionally, sugar fermentation (mannitol, sorbitol, melibiose, raffinose) was used to identify MS colonies.

Polysaccharide Analyses

Dental biofilm was collected and weighed (± 10 μg). After extraction of acid-soluble ions using 0.5 M HCl (data not shown), 1 M NaOH was added to the precipitate (0.5 ml/10 mg biofilm wet weight) for extraction of insoluble EPS [Cury et al., 2000]. After 3 h at room temperature under constant agitation, supernatant was collected for analysis. To the precipitate, 1 M NaOH (0.5 ml/ 10 mg biofilm wet weight) was added for extraction of IPS, which was done at 100 °C for 1 h. Supernatants containing the polysaccharides were precipitated with 75% ethanol and analyzed for total carbohydrate according to Dubois et al. [1956].

Statistical Analyses

The statistical analyses were done using SAS software (SAS Institute Inc., version 8.01, Cary, N.C., USA) employing a significance level fixed at 5%. For all the analyses, the experimental unit considered was the volunteer. The null hypothesis assumed no differences among treatments. Data that violated the assumptions of equality of variances and normal distribution of errors were transformed [Box et al., 1978] before they were analyzed by ANOVA, followed by Tukey test. When no transformation was adequate to normalize data (variables MS and % MS in relation to total bacteria), they were analyzed by the Friedman nonparametric test.

RESULTS AND DISCUSSION

Table 1 shows the acidogenicities, biochemical and microbiological analyses of the biofilms according to the treatments. The lowest baseline pH was found in biofilmes formed under exposure to sucrose ($p < 0.05$), and both carbohydrate treatments presented lower baseline pH than the negative control group ($p < 0.05$). This could be explained by the significantly higher concentration of IPS in the glucose + fructose and sucrose groups, suggesting their use by bacteria as energy source during the fasting period [Tanzer et al., 1976]. After the sugar challenge, pH decreased for all groups, but the significantly greater pH fall for the glucose + fructose and sucrose groups, when compared to the negative control, indicates a higher acidogenic potential which could be due to the bacterial composition of these biofilms.

Acid production and survival in acidic environment are known as virulence factors of MS and LB in the cariogenic dental biofilm [Marsh, 1994]. Considering that sucrose would additionally promote the adhesion of bacteria expressing surface glucosyltransferases, an increase in MS [Rølla, 1989] and also in LB [Kralj et al., 2004] could be suspected in the biofilm formed in its presence.

With respect to MS, however, their counts in dental biofilm did not differ significantly among the experimental groups. This result could be explained by the volunteers' low salivary MS counts, which could have impaired the initial colonization of enamel blocks with these bacteria [Van Houte and Green, 1974]. However, while some studies [De Stoppelaar et al., 1970; Staat et al., 1975; Bowen et al., 1980] observed a positive relation between sugar in the diet and

MS counts in the dental biofilm, others only found a trend for increase in individuals with high salivary MS counts [Aranibar Quiroz et al., 2003] or did not find an increase in MS in the biofilm in diets with high frequency of sugar exposure [Scheie et al., 1984]. In in situ studies, where the exposure to carbohydrates and biofilm accumulation are experimentally controlled, no such direct relation between exposure to sucrose and increase in MS in the biofilm has been demonstrated [Macpherson et al., 1990; Cury et al., 2001]. In situ, LB were observed to increase 4–10 times more than MS in biofilms formed under exposure to sucrose, when compared to a control group [Minah et al., 1981]. The lack of increase in MS following sucrose exposure suggests that the structural change provoked by these microorganisms in dental biofilm may be more relevant than their abundance [Cury et al., 2001].

However, LB counts were significantly higher in the biofilm formed under exposure to glucose + fructose and sucrose than in the control group (table 1). This is in agreement with Bradshaw et al. [1989] and Bradshaw and Marsh [1998], who observed a predominance of LB in in vitro multispecies communities according to the decreased pH of the media. The selection of LB has also been found in situ when the biofilm was formed in the presence of sucrose [Pecharki et al, 2005] or starch [Ribeiro et al., 2005] in comparison with the control group. Even though the mean LB salivary level of volunteers was moderate (6.2 x 10^4 CFU/ml saliva), in 7 of them it was below 10^3 CFU/ml saliva, suggesting that the somewhat higher acidogenicity and aciduricity of LB in relation to MS [Bradshaw et al., 1989] may have played a role in promoting their selection in the biofilm during the frequent pH drops caused by the carbohydrates for 13 days. Although Bradshaw et al. [1989] have demonstrated

a similar selection of MS and LB in bacterial communities caused by pH drops, their in vitro study comprised 10 pulses of exposure to carbohydrates and consequent pH fall. In the present in situ study, the selection of LB should have been greater because of the higher frequency and period of exposure to the carbohydrates. Furthermore, even in vitro the selection of LB was 10 times higher than that of MS [Bradshaw et al., 1989]. The similar counts for LB in the glucose + fructose and sucrose groups, irrespective of the significantly higher concentration of insoluble EPS in the biofilm formed in the presence of sucrose, suggest that insoluble EPS is not an additional factor for an increase of the species in the biofilm. It is possible that the experimental model used, where the biofilm is undisturbed for 13 days, may resemble proximal plaque or the ecological niche of a carious lesion, where LB are known to be abundant [Loesche and Syed, 1973]. Considering that LB are not involved with caries initiation, the data have implications for caries progression either in enamel or dentine. Also, the findings have implications for the cariogenicity of dietary carbohydrates other than sucrose, such as starch [Ribeiro et al., 2005].

This in situ study, suggesting that insoluble EPS are not relevant to select MS and LB in dental biofilm, agrees with recent in vitro research done with mixed biofilm containing *Streptococcus mutans gtf* mutants [Thurnheer et al., 2006]. However, our data were observed using 13 days of biofilm formation and shorter times should be further evaluated.

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Table 1: Mean ± SD (n) of the variables analyzed, according to the treatments.

Variables	Treatments		
	Negative Control	Glucose+Fructose	Sucrose
Baseline biofilm pH	7.51 ± 0.30 [a] (14)	6.99 ± 0.48 [b] (16)	6.47 ± 0.80 [c] (15)
Biofilm pH 5 min after sugar exposure	6.27 ± 0.46 [a] (16)	5.54 ± 0.44 [b] (16)	5.30 ± 0.54 [b] (16)
Insoluble EPS (mg/g)	3.00 ± 1.39 [a] (16)	4.02 ± 1.51 [a] (16)	38.9 ± 20.5 [b] (16)
IPS (mg/g)	1.93 ± 0.73 [a] (16)	6.56 ± 4.43 [b] (16)	6.71 ± 2.47 [b] (16)
Total bacteria (CFU/mg biofilm x 10^6)	8.0 ± 7.4 [a] (16)	7.7 ± 6.9 [a] (16)	6.5 ± 5.5 [a] (16)
Mutans streptococci (CFU/mg biofilm x 10^2)	1.1 ± 2.0 [a] (16)	6.4 ± 22.3 [a] (16)	4.3 ± 8.1 [a] (16)
Mutans streptococci (% of total viable count)	0.004 ± 0.009 [a] (16)	0.01 ± 0.04 [a] (16)	0.009 ± 0.016 [a] (16)
Lactobacilli (CFU/mg biofilm x 10^6)	0.0002 ± 0.0005 [a] (16)	1.6 ± 2.6 [b] (16)	3.2 ± 5.2 [b] (16)
Lactobacilli (% of total viable count)	0.009 ± 0.02 [a] (16)	24.8 ± 34.7 [b] (16)	39.7 ± 40.5 [b] (16)

Treatments for which the means are followed by distinct letters differ statistically ($p < 0.05$). (n) = Number of volunteers.