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Composition of Dental Plaque Formed in the Presence of Sucrose and after its Interruption

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Since dental plaque reservoirs of fluoride (F), calcium (Ca) and inorganic phosphorus (P_i) are susceptible to decreases in pH, this *in situ* crossover study was conducted to test the hypothesis that the low concentration of these ions in plaque, formed in the presence of sucrose, could be attributed merely to the fermentation of this sugar. Eleven volunteers wore palatal appliances containing 6 human enamel blocks during two stages. In each stage, the treatments were either 20% sucrose solution or distilled deionized water, which were dripped onto the blocks 8 times a day. After 28 days, in each stage, the dental plaque formed on two blocks was collected, the treatment was inverted and after a further 24 and 48 h, the biofilm formed was collected from the other blocks. The concentration of acid-soluble F, Ca and P_i, and the concentration of insoluble polysaccharide (IP) were determined in the dental plaque. Statistically lower concentrations of F, Ca and P_i, and a higher concentration of IP were found in the 28-day biofilm formed in the presence of sucrose than in its absence; after the treatment inversion the change in F, Ca and P_i was not statistically significant, but the IP concentration changed significantly. The hypothesis was rejected because change in concentration of F, Ca and P_i is not due to fermentation of the sucrose.

Key Words: caries, dental plaque, fluoride, polysaccharide, sucrose.

INTRODUCTION

Dental caries is a dietary-bacterial disease and sucrose is considered to be the most cariogenic carbohydrate because, apart from fermentation, it is also transformed into extracellular polysaccharide in dental plaque. The presence of these polysaccharides in biofilm also increases the dental plaque matrix porosity, enhancing the cariogenicity of sucrose (1).

However, Cury et al. (2,3) recently showed that, in addition to the high concentration of insoluble polysaccharide, dental plaque formed in the presence of sucrose also shows a low concentration of fluoride (F), calcium (Ca), and inorganic phosphorus (P_i). Thus, considering the importance of the inorganic composition of dental plaque to caries development (4), the factors responsible for the lower concentration of F, Ca and P_i in dental plaque formed in the presence of sucrose must be determined and several hypotheses have been formulated (2,3): 1) repeated pH decreases, due to the fermentation of sucrose could have depleted plaque reservoirs of these ions, which then diffuse into the saliva; 2) the enamel could have incorporated the ions from the dental plaque during the cariogenic challenge; 3) lower bacteria density due to the large amount of insoluble polysaccharide, which in turn, results in few calcium-binding bacteria; 4) absence of anionic calcium-binding proteins or peptides. Data partially rejecting the first hypothesis were reported by Cury et al. (3); however, dental plaque was collected 12-h after the last exposure to sucrose and this period may not have been long enough for the released ions to replenish the plaque reservoirs.

Thus, the first hypothesis stated above was tested to evaluate whether the low concentrations of F, Ca and P_i found in dental plaque formed by repeated exposure to sucrose for 28 days would increase after this sugar exposure was interrupted for a longer time. As a control, we also evaluated whether the high concentrations of F, Ca and Pi in dental plaque formed during 28 days

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in the absence of sucrose would decrease when exposure to this sugar was introduced.

MATERIAL AND METHODS

Experimental Design

The study involved a crossover, blind design performed in two phases of 30 days each. Eleven healthy adult volunteers took part in this study approved by the Research and Ethics Committee of FOP/UNICAMP. They signed a written informed consent for participation.

Enamel blocks (3x3x3 mm) were prepared from impacted human third molars sterilized by immersion in 2% formaldehyde, pH 7.0, for at least one month (2,3,5). The surface of the enamel blocks was polished to remove a layer of 50 µm (6). The volunteers wore custom-made acrylic palatal appliances each containing six dental enamel blocks placed as closely as possible to the posterior teeth; 3 blocks on each side of the appliance (anterior, central and posterior position on the left and right sides). A 4.0-mm-deep space was created in the acrylic appliance, leaving a 1.0-mm space for plaque accumulation (3). For 28 days, dental plaque was allowed to form on the enamel blocks, which were protected from mechanical disturbance by a plastic mesh fixed to the acrylic surface. The volunteers were randomly assigned to one of two treatments: 8 times a day the appliances were removed and either 20% sucrose solution or distilled deionized water was dripped onto the enamel blocks. After 5 min, the appliances were replaced in the mouth. A washout period of at least 7 days was allowed between the phases to eliminate possible residual effects from the treatments. During a 10-day pre-experimental period and during the experimental period, the volunteers brushed their natural teeth with non-fluoride toothpaste, but drank fluoridated water (0.6-0.8 mg F/L). The volunteers received instructions to wear the appliances all the time, including at night, but to remove them during meals. The test subjects received oral and written information to refrain from using any antibacterial or fluoridated product during the pre-experimental and experimental periods. Considering that the study followed a crossover design, with the participation of the volunteers in both stages, the subjects did not receive any instructions regarding their daily diet.

The dental plaque formed on two opposite enamel blocks was collected with plastic curettes at three different periods of time 12 h after the last exposure to the solutions. The first collection, from anterior left and central right blocks, was made 28 days after the treatments. Immediately after this first plaque collection, the volunteers wore the appliances for 48 h longer, but the treatment was exchanged. Thus, the volunteers who had been dripping sucrose onto the blocks 8 times a day for 28 days started using water at the same frequency and vice versa. The second plaque collection, from central left and posterior right dental blocks, occurred 24 h after exposure to the solutions had been exchanged; the third collection, from posterior left and anterior right blocks, was made 48 h after the solution inversion. In the second stage of the study, the treatment was crossed for 28 days and exchanged for a further 48 h.

Analysis of Dental Plaque

Dental plaque was placed in coded pre-weighed microcentrifuge tubes and the wet weight of each sample was determined to ± 10 µg. Hydrochloric acid (0.5 M) was added to the tubes in the proportion of 50 µL/mg plaque wet weight. After extraction for 3 h at room temperature under constant agitation, the same volume of TISAB II pH 5.0 (containing 20 g NaOH/L) was added as a buffer (2,3). The samples were then centrifuged (11,000 *g*) for 1 min and the supernatant retained for determination of acid-soluble F, Ca and P_i. To the precipitate, 1.0 N NaOH (100 µL/mg plaque wet weight) was added. The samples were vortexed for 1 min, agitated for 3 h at room temperature and the concentration of insoluble polysaccharide (IP) in the resulting supernatant was determined. In the plaque acid extract, F was analyzed using an ion-selective electrode (Orion 96-09; Boston, MA) and an ion analyzer (Orion EA-940), Ca was analyzed by atomic absorption spectrophotometry using lanthanum to suppress interference and P_i (7) and IP (8) were colorimetrically determined.

Statistical Analysis

Statistical analysis using the Shapiro-Wilks normality test detected heterogeneous variances in most of the variables. Thus, a nonparametric analysis was used for all variables, considering that variables with normal

distribution show the same power when analyzed either by nonparametric or parametric tests. Wilcoxon test was used to compare 28-day dental plaque formed in the presence or absence of sucrose. The Friedman test was used to compare the effect of treatment inversion during the subsequent 48 h of biofilm formation. The non-parametric test of multiple comparisons was applied to determine significant differences among the experimental conditions. The relationship between F, Ca and P_i concentrations in dental plaque was evaluated by Pearson's correlation (9). For all analyses, the significance level was set at 5%.

RESULTS

Data on inorganic composition and concentration of insoluble polysaccharide in 28-day dental plaque formed in the presence or absence of sucrose are reported in Tables 1 and 2. Dental plaque formed in the presence of sucrose had a significantly lower concentrations of F, Ca and P_i and higher IP than that formed in its absence ($p<0.05$).

Tables 1 and 2 also show the results of F, Ca, P_i and IP in dental plaque 24 and 48 h after the treatments were exchanged. The inorganic concentration neither increased (Table 1) nor decreased (Table 2) significantly when exposure to sucrose for 48 h was interrupted or started, respectively. The concentrations of F and Ca did not change significantly ($p>0.05$) in either situation evaluated (Tables 1 and 2). With regard to P_i concentration, the only statistically significant difference found was between 48 h after the interruption of sucrose exposure in comparison with 24 h (Table 1). The IP concentration fell in the dental plaque previously formed in the presence of sucrose when exposure to this sugar was interrupted for 48 h (Table 1). In contrast, IP concentration increased when dental plaque formed in the absence of sucrose for 28 days was exposed to this sugar for 48 h (Table 2).

Statistically significant correlations ($p<0.05$) were found between concentrations (mmol/kg) of F x Ca, F x P_i and Ca x P_i both when the biofilm was formed in the presence of sucrose and when the exposure was interrupted (Table 3). This table also shows that a statistically significant correlation was found for Ca x P_i both when the plaque was formed in the absence of sucrose for 28 days and after the exposure to this sugar for an additional 48 h.

Table 1. Composition of dental plaque formed in the presence of sucrose for 28 days and changes after exposure interruption.

Conditions	F, µg/g (n=8)	Ca, mg/g (n=9)	P_i, mg/g (n=9)	IP, mg/g (n=11)
Sucrose exposure (28 days)	1.1 ± 0.3[a]	1.2 ± 0.7[a]	0.2 ± 0.04[a,b]	51.1 ± 13.6[a]
Sucrose interruption				
24 h	1.6 ± 0.4[a]	1.6 ± 0.6[a]	0.2 ± 0.06[a]	49.7 ± 11.4[a]
48 h	2.7 ± 0.6[a]	3.0 ± 1.5[a]	0.4 ± 0.07[b]	40.6 ± 9.6[b]

Data are reported as average ± SE.
Different letters indicate statistically significant differences ($p<0.05$).

Table 2. Composition of dental plaque formed in the absence of sucrose for 28 days and changes after sucrose exposure.

Conditions	F, µg/g (n=8)	Ca, mg/g (n=9)	P_i, mg/g (n=9)	IP, mg/g (n=11)
Sucrose absence (28 days)	63.3 ± 23.6[a]	12.2 ± 1.9[a]	4.3 ± 1.5[a]	4.6 ± 0.5[a]
Sucrose exposure				
24 h	86.1 ± 38.6[a]	18.8 ± 4.5[a]	4.9 ± 1.3[a]	10.0 ± 2.7[a,b]
48 h	67.9 ± 37.5[a]	14.8 ± 4.0[a]	3.9 ± 1.1[a]	11.4 ± 2.3[b]

Data are reported as average ± SE.
Different letters indicate statistically significant differences ($p<0.05$).

DISCUSSION

The inorganic composition of dental plaque matrix is potentially important in the development of dental caries (4). Thus, it is relevant to understand the mechanisms that lead to a lower concentration of F, Ca and P_i in biofilm formed in the presence of the cariogenic carbohydrate sucrose.

The present findings confirmed our previous studies (2,3) showing that dental plaque formed during exposure to sucrose for 28 days resulted in biofilm with a low

inorganic concentration of F, Ca, P_i and a high IP content (Tables 1 and 2). Our findings are also in agreement with Pearce et al. (10) with regard to Ca and IP changes. However, the aim of this study was to evaluate the effect of repeated decreases in pH on the depletion of inorganic reservoirs present in dental plaque. Thus, if this low concentration of F, Ca and P_i were a simple consequence of decreased pH by sugar fermentation, the plaque reservoirs would be replenished again when the supply of sucrose was interrupted. By contrast, the high concentration of these ions in dental plaque formed in the absence of sucrose would be depleted when repeated exposure to this sugar was started.

Data in Tables 1 and 2 show that these ions neither increase nor decrease significantly in dental plaque when these conditions were tested. There are two known reservoirs in dental plaque that are sensitive to pH change: minerals (11) or Ca-F bridge bound to bacteria (12). The first reservoir would explain the change of F, Ca and P_i, and the second would explain F and Ca changes by pH. Thus, the present data suggest that the low inorganic concentration in biofilm formed in the presence of sucrose is a result of changes in the structure of the biofilm formed, rather than depletion of the inorganic pools by organic acids. The biofilm structure or unknown reservoirs seem to be the key answer to the binding of the ions. If the binding sites, such as anionic proteins or bacteria, are not available, then the simple interruption of a pH decrease will not allow favorable conditions for ion binding again. The high relationship found (Table 3) between F, Ca and P_i, mainly when biofilm was formed in the presence of sucrose as opposed to its absence, suggests that the structure of this biofilm is more complex than has been previously considered.

On the other hand, the findings could be explained by the fact that 48 h was too short a period to evaluate the change in the inorganic composition of biofilm. It may have been insufficient for the depleted plaque reservoirs of F, Ca and P_i to be replenished by these ions when the sucrose supply was interrupted, or for the release of the high amount of F, Ca and P_i stored in plaque formed in the absence of sucrose. However, this possibility is small because kinetics data have shown that F concentration in dental plaque reaches or approaches baseline values at 12 h (13) or less (14,15) after the use of dentifrice or mouth rinse. Furthermore, Ca and P_i are natural ions in saliva and they may maintain a fine balance with the reservoirs of the mouth, including dental plaque.

Another explanation for the data is that F, Ca and P_i concentration in dental plaque formed in the presence of sucrose for 28 days did not significantly increase when this sugar supply was cut off for 48 h, due to the limited diffusion of these ions throughout this biofilm. Although the biofilm formed in the presence of sucrose is deeper than that formed in its absence, it is more porous and the diffusion from plaque is not a limitation (16). Furthermore, the concentration of F, Ca and P_i may not have decreased when sucrose was supplied for 48 h to plaque formed for 28 days in the absence of sugar, due to the decrease in pH being limited to the outer part of the plaque.

Statistically significant differences were, however, observed with regard to the IP concentration in dental plaque (Tables 1 and 2). Thus, simultaneously to its decrease in concentration in 28-day dental plaque which had been formed in the presence of sucrose, when the supply of this sugar was interrupted (Table 1), it increased when the exposure to sucrose started for 48 h (Table 2). The decrease in IP concentration could not be explained by the breakdown of these polysaccharides for energy production because they have a structural function and are not easily metabolized. This reduction could, however, be a consequence of the

Table 3. Pearson's correlation between F, Ca and P_i according to dental plaque formation.

Conditions	Correlations [values of *r* and (*p*)]		
	F x Ca	F x P_i	Ca x P_i
28 days of sucrose exposure	0.9817 (0.0001)	0.9803 (0.0001)	0.9920 (0.0001)
28 days of sucrose absence	-0.3192 (0.4032)	-0.4296 (0.2481)	0.7834 (0.0125)
28 days of sucrose exposure and 48 h of sucrose absence	0.9959 (0.0001)	0.9451 (0.0001)	0.9602 (0.0001)
28 days of sucrose absence and 48 h of sucrose exposure	0.5713 (0.1080)	0.4909 (0.1795)	0.9764 (0.0001)

Concentrations in mmol/kg

formation of a new layer of dental plaque which, since formed in the absence of sucrose, would not be able to synthesize IP. Thus, the IP formed for 28 days, by exposure to sucrose, would have been diluted by the *de novo* plaque formed for 48 h in the absence of this sugar. However, we did not find any statistically significant difference between the wet weights of these plaques (p=0.0943; data not shown). It is also possible that the alkali extracted some degradable extracellular polysaccharide that had been trapped in the plaque matrix, but was resistant to the first extraction with acid.

The increase in IP when the supply of sucrose was started can, however, be explained because this type of polysaccharide is exclusively synthesized in the presence of this carbohydrate. Thus, IP is formed at the same time that sucrose is utilized as a source of energy for the growth of a new biomass of plaque. We found that the 28-day plaque wet weight formed in the absence of sucrose increased significantly from 6.46 to 9.79 mg (51%) after 48 h of sucrose supply (p=0.0256; data not shown) and IP had a statistically significant increase of 60% (Table 2).

In conclusion, the findings of the present study suggest that the low concentration of F, Ca and P_i observed in dental plaque formed in the presence of sucrose cannot be explained by the repeated pH decrease due to the acid production from fermentation of this carbohydrate. It may be explained by structural changes in dental plaque formed and research in this direction is in progress.

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RESUMO

Desde que os reservatórios de flúor (F), cálcio (Ca) e fósforo inorgânico (P_i) na placa dental são suscetíveis a quedas de pH, este estudo *in situ* cruzado foi conduzido para testar a hipótese de que baixas concentrações destes íons na placa, formada na presença de sacarose, poderiam ser atribuídas simplesmente à fermentação deste açúcar. Onze voluntários utilizaram dispositivos palatinos contendo seis blocos de esmalte dental humano durante duas fases. Em cada fase os tratamentos foram solução de sacarose a 20% ou água destilada deionizada, que foram gotejadas sobre os blocos 8 vezes ao dia. Após 28 dias, em cada fase, a placa dental formada sobre dois blocos foi coletada, o tratamento foi invertido e após um tempo adicional de 24 e 48 horas, o biofilme formado foi coletado dos outros blocos. A concentração de F, Ca e P_i solúvel em ácido e a concentração de polissacarídeo insolúvel (PI) foram determinadas na placa dental. Concentrações estatisticamente menores de F, Ca e P_i, e uma concentração maior de PI foram encontradas no biofilme de 28 dias formado na presença de sacarose do que na sua ausência; após a inversão do tratamento a mudança no F, Ca e P_i não foi estatisticamente significante, mas a concentração de PI mudou significativamente. A hipótese foi rejeitada porque a mudança na concentração de F, Ca e P_i não é devida à fermentação da sacarose.

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