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Original Article

Frequency of sucrose exposure on the cariogenicity of a biofilm-caries model

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ABSTRACT

Objective: Although sucrose is considered the most cariogenic carbohydrate in the human diet, the question of how many exposures are needed to induce damage on the hard dental tissues remains unclear. To approach this question, different frequencies of daily sucrose exposure were tested on a relevant biological caries model. **Materials and Methods:** Biofilms of the *Streptococcus mutans* were formed on enamel slabs and exposed to cariogenic challenges with 10% sucrose for 5 min at 0, 1, 3, 5, 8, or 10 times per day. After 5 days, biofilms were retrieved to analyze biomass, protein content, viable bacteria, and polysaccharide formation. Enamel demineralization was evaluated by percentage of microhardness loss (percentage surface hardness loss [%SHL]). **Results:** Biomass, protein content, polysaccharide production, acidogenicity of the biofilm, and %SHL proportionally increased with the number of daily exposures to sucrose ($P < 0.05$). One daily sucrose exposure was enough to induce 20% more demineralization than the negative unexposed control. Higher frequencies induced greater demineralization and more virulent biofilms, but eight and ten exposures were not different between them in most of the analyzed variables ($P > 0.05$). **Conclusions:** Higher sucrose exposure seems to increase cariogenicity, in a frequency-dependent manner, by the modification of bacterial virulent properties.

Key words: Caries, diet, nutrition, *Streptococcus mutans*, sucrose, sugar

INTRODUCTION

Dental caries is a multifactorial disease caused by an ecological imbalance of the dental biofilm.[1] For a carious lesion to be developed, it is mandatory the presence of a metabolically active bacterial biofilm and fermentable sugars which come mainly from the diet.[2,3] The disaccharide sucrose, formed by glucose and fructose, has been widely acknowledged as the most cariogenic sugar.[4-6] When in contact with the oral biofilm, sucrose is rapidly metabolized by the bacterial consortium and used as a substrate for the production of large amounts of organic acids, which in turn induces an important pH drop in the biofilm.[4] Under these environmental conditions, it is expected to observe hard tissue demineralization and a change in the composition and the virulence of the dental biofilm, including higher polysaccharide and protein production by the bacteria.[7,8] Frequent sucrose intake for caries onset, therefore, must be considered the main etiological factor.[9]

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Sucrose is a ubiquitous component of the modern diet. Besides cakes, confectionary, beverages, fruits, cereals, and dairy products containing variable amounts of sucrose,[10] pediatric drugs also contain this sugar.[11] It has been suggested that the total amount of sugar should not be higher than 5% of the total calorie intake which equals 25 mg for an adult with normal body mass index.[12,13] Caries experience derived from the consumption of dietary fermentable carbohydrates is dependent on the patterns of sugar intake, including physical presentation, type, quantity, and frequency.[6,14,15] The threshold where sucrose becomes a risk factor for caries, however, is elusive. In particular, how many intakes per day are capable to induce enamel lesions is unclear and of interest. The purpose of this study, therefore, was to test the effect of different frequencies of sucrose exposure to a *Streptococcus mutans* biofilm formed on enamel, in a relevant experimental caries model. Having an idea of the effect of different frequencies of sucrose exposure on lesion formation may help to support caries control programs in high-risk populations.

MATERIALS AND METHODS

Enamel slabs

Bovine incisors were obtained, disinfected with 5% NaOCl 5%, and stored in 0.9% NaCl until use, not longer than 30 days. Enamel slabs (4 mm × 7 mm × 1 mm) were prepared using a diamond saw machine (LECO VC50 Diamond Saw, St. Joseph, MI, USA) and polished with sequential polishing disks (Soflex, 3M-ESPE, St. Paul, MN, USA). Initial surface hardness $(SH)_i$ was assessed by three linear indentations of a Knoop microindenter with a microhardness tester (402 MVD, Wolpert Wilson Instruments, Norwood, USA), at 50 g for 5 s. To control tissue variability, only slabs with microhardness of 364.19 ± 36.4 kg/mm^2 (n = 18) were included. Samples were sterilized in an autoclave at 121°C for 15 min. Enamel slabs were covered for 30 min with ultra-filtered saliva obtained with 0.22 μm filters, from a healthy volunteer fasting for 12 h. To preserve the protein content and ensure the formation of the acquired pellicle on enamel to allow bacterial adhesion, saliva was mix with absorption buffer 0.1M containing phenylmethylsulfonyl fluoride 1:100 (v/v) and a protease inhibitor cocktail.[16] Slabs were suspended in the wells of a 24-well culture plate, with a metal holder made with orthodontic wire.

Streptococcus mutans biofilms

Frozen stocks of *S. mutans* UA159 (kindly donated by Prof. J.A. Cury, UNICAMP, SP, Brazil) were reactivated in Brain Heart Infusion medium (BHI, Merck, Darmstadt, Germany) supplemented with 1% glucose and incubated 18 h at 37°C and 10% CO_2 (Panasonic, MCO-19M, Osaka, Japan). Slabs were inoculated with 100 μL of the *S. mutans* culture at an optical density of 0.8–1.0 at 600 nm and cultured for 8 h in BHI supplemented with 1% sucrose at 37°C and 10% CO_2 to facilitate the formation of an adherent biofilm.[16] To allow biofilm maturation before the treatments, cultures were kept in BHI supplemented with 0.1 mM glucose for additional 16 h, which resembles basal glucose concentration in saliva.[17]

Sucrose exposure

Once mature and visible after 24 h, slabs were randomly allocated and exposed to 10% sucrose for 5 min 1, 3, 5, 8, or 10 times per day at defined times. A slab/biofilm exposed 10 times per day to 0.9% NaCl served as negative control. After each sucrose exposure, biofilms were washed 3 times with 0.9% NaCl and relocated in a well containing BHI supplemented with 0.1 mM glucose until the following exposure. To maintain samples under similar conditions, except for the number of sucrose exposures, all the slabs were manipulated in a similar fashion. Thus, when samples receiving 10% sucrose were placed in their corresponding treatment well, those without treatment were immersed in 0.9% NaCl for the same 5 min. The assay lasted 5 days and all the conditions were done in triplicate in two independent experiments (n = 6).

Biofilm-derived acidogenicity

A pH-cycling model was used. Cariogenic challenges with sucrose occurred during the day, while at night, the biofilms were maintained only under a basal concentration of glucose.[18] To verify acid production by the biofilm, pH of the spent medium was measured twice per day, in the morning after overnight culture and the evening after cariogenic pulses with sucrose during the day, with the aid of a microelectrode (HI 1083B, Hanna Instruments, Rumania).

Enamel slab demineralization

Demineralization induced by sucrose on the enamel slabs was estimated by Knoop's SH.[17] After the 5-day experimental phase, slabs were mounted on a glass plate and performed three indentations separated 100 μm from each other, adjacent to the initial indentations, considered final SH $(SH)_f$ (kg/mm^{-2}). Mean from SH_i and SH_f were used to calculate percentage of SH loss (%SHL), interpreted as demineralization: (mean SH_i − mean de SH_f) × 100/SH_i.

Biofilm assessment

Once completed the experimental phase, slabs were washed 3 times with 0.9% NaCl and agitated with vortex (Maxi Mix II 37600 Mixer, Thermolyne, Iowa, USA) in 1 mL of 0.9% NaCl for 30 s to detach the biofilms from the slabs. The resulting biofilm suspension was further used to determine biomass,[16] viable prokaryotic cells,[17] intra- and extra-cellular polysaccharides,[18] and soluble protein content.[19] Protocols have been previously reported, so the reader is referred to the original sources.

Statistical analysis

Data were analyzed by the SPSS software version 15.0 (IBM, USA) for Windows. Dependent variables of acidogenicity, %SHL, biomass, polysaccharides, and proteins were compared across the treatment groups by one-way ANOVA followed by the Tukey *post hoc* test. Differences were considered statistically significant when *P* value was lower than 0.05.

RESULTS

Biofilm acidogenicity is depicted in Figure 1. At 32 h from the beginning of the experiments, medium pH containing the slabs unexposed and those exposed once per day to 10% sucrose did not show significant differences. Slabs and the biofilms exposed 3 and 5 times per day showed a pH drop ($P < 0.05$). The pH of the samples exposed 8 and 10 times per day was lower than the rest of the slabs ($P < 0.05$), without differences between them ($P > 0.05$). From 56 h to 104 h, medium pH of the samples exposed once per day was lower ($P < 0.05$) than that of the unexposed controls. Similar to 32 h, medium pH of the biofilms exposed 3, 5, 8, and 10 times decreased proportionally to the number of sucrose exposures.

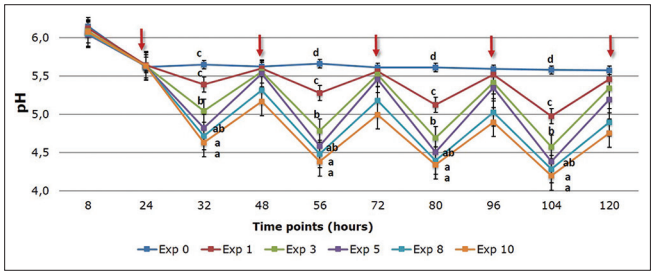

Figure 1: *Streptococcus mutans* biofilm acidogenicity after exposure to different frequencies of sucrose. Biofilms were treated with different daily frequencies of 10% sucrose for 5 min, from 0 to 10 times, for 5 days (120 h), as indicated. Plot depicts medium pH at 24 h from biofilm formation and then twice per day. The arrows indicate replenishment of the medium. Surface hardness was measured in each enamel slab before and after the experiment and the percentage of surface hardness loss was calculated. Bars denote mean values of two independent experiments, each in triplicate ($n = 6$). Error bars show the standard deviation. Different letters represent statistically significant differences ($P < 0.05$)

Regarding demineralization (%SHL) [Figure 2], slabs exposed 1, 3, 5, 8, and 10 times resulted in higher values than the unexposed controls and increased proportionally to the number of daily sucrose exposures ($P < 0.05$), albeit 8 and 10 times failed to show differences between them ($P > 0.05$). Slabs/biofilms exposed 1, 3, 5, 8, and 10 times per day to sucrose induced 21%, 36%, 60%, 73%, and 77% more demineralization than the unexposed controls, respectively.

Polysaccharide production by *S. mutans* is shown in Table 1. No differences were detected in soluble extracellular polysaccharides (SEPS) [Table 1] in any of the experimental groups, except the group exposed 10 times ($P < 0.05$). Conversely, biofilms exposed 5, 8, and 10 times to 10% sucrose showed significantly more insoluble extracellular polysaccharides (IEPS) formation that the rest of the groups [Table 1]. Despite a trend for higher IEPS formation, biofilms exposed 1 and 3 times to sucrose did not differ from the unexposed controls ($P > 0.05$). The IPS fraction [Table 1] did not show differences among any of the experimental groups ($P > 0.05$).

S. mutans biofilms exposed three or more times per day to sucrose resulted in higher biomass ($P < 0.05$) than those unexposed or exposed only once daily [Table 1]. Although no differences were detectable among samples exposed 3, 5, and 8 times per day ($P < 0.05$), those exposed 10 times per day induced more biomass than the rest ($P < 0.05$). Consistent with the biomass results, protein content in the biofilms showed a similar trend [Table 1].

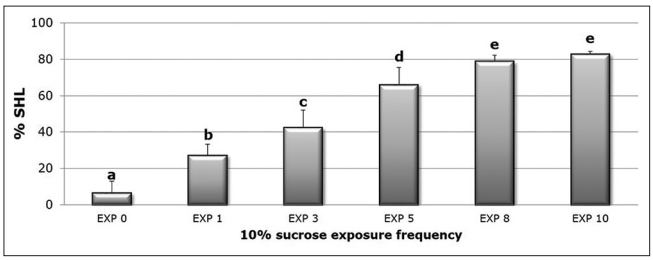

Figure 2: *Streptococcus mutans* biofilm enamel slab demineralization after exposure to different frequencies of sucrose. Surface hardness was measured in each enamel slab before and after the experiment and the percentage of surface hardness loss was calculated. Bars denote mean values of two independent experiments, each in triplicate ($n = 6$). Error bars show the standard deviation. Different letters represent statistically significant differences ($P < 0.05$)

Table 1: Biofilm properties after to exposure to different frequencies of sucrose

Frequency of sucrose exposure	Biomass (mg)	Proteins (mg/mg biomass)	IEPS (percentage/mg biomass)	SEPS (percentage/mg biomass)	IPS (percentage/mg biomass)
Exp 0	0.75 (0.27)^{a}	0.06 (0.01)^{a}	0.20 (0.16)^{a}	0.3 (0.31)^{a}	0.36 (0.09)^{a}
Exp 1	1.34 (0.41)^{a}	0.12 (0.03)^{ab}	0.25 (0.05)^{a}	0.4 (0.21)^{a}	0.37 (0.09)^{a}
Exp 3	2.08 (0.20)^{b}	0.15 (0.02)^{bc}	0.49 (0.09)^{ab}	0.5 (0.03)^{ab}	0.35 (0.07)^{a}
Exp 5	2.33 (0.26)^{bc}	0.19 (0.04)^{cd}	0.68 (0.10)^{b}	0.7 (0.07)^{ab}	0.38 (0.09)^{a}
Exp 8	2.70 (0.45)^{bc}	0.21 (0.04)^{cd}	0.80 (0.14)^{b}	0.8 (0.09)^{ab}	0.45 (0.13)^{a}
Exp 10	2.83 (0.52)^{c}	0.22 (0.05)^{d}	0.89 (0.28)^{b}	1.0 (0.12)^{b}	0.52 (0.08)^{a}

Different letters represent statistically significant differences (P<0.05). Mean (SD), n=6. Exp 0-Exp 10: Slabs exposed to 10% sucrose for 5 min 1, 3, 5, 8, or 10 times per day. IEPS: Insoluble extracellular polysaccharides, SEPS: Soluble extracellular polysaccharides, IPS: Intracellular polysaccharides, SD: Standard deviation

DISCUSSION

Although the role of sucrose as a fermentable sugar in the development of carious lesions is vastly acknowledged, the frequency of exposure to this disaccharide that leads to an increased caries risk is rather unclear. It has been widely stated that the frequency of sugar consumption is the most crucial factor associated with caries.[15] Indeed, a systematic review of the literature suggests that frequency, but not quantity, of sugar consumption is positively associated with caries.[6] Although frequency is important, other variables in consumption such as type, physical presentation, and quantity may also play a role on the relation between sugar and caries.[5,14,20] In this context, this study aimed to quantitatively elucidate the frequency of sugar consumption capable to induce an imbalance in the biofilm, predisposing to caries. It would be more clinically relevant to scale these results to a clinical trial for more accurate conclusions. Ethical obvious reasons, nonetheless, preclude those types of approaches. Given this limitation, therefore, this experimental caries model may serve the purpose to mimic the clinical situation. We have attempted to model sucrose consumption of a whole range of clinical situations. Thus, 0 times per day could be representative of a person unexposed to sucrose as the source of dietary simple sugars. One time per day exposure should represent low consumption, three moderate to high consumption, and five and over high sucrose consumption. Eight and 10 times per day exposures were included to model the situation of people with extremely high sucrose consumption. The results suggest that an increase in the daily exposure to sucrose enhances biofilm cariogenicity, in a dose-dependent manner. Importantly, the effect was not only on the acidogenicity [Figure 1] but also on the composition and characteristics of the *S. mutans* biofilm [Table 1].

It has been shown that steady daily sucrose consumption of at least 3 times per day is associated with a 179% increase in caries experience.[21] Moreover, a prospective 4-year study reported a dose–response relationship between frequency of sucrose intake and increasing caries rates in adults, with 3 or more exposures representing a 33% higher decayed-missing-filled tooth as compared to those that reported no consumption.[22] Here, we have used three exposures to sucrose as a moderate consumption pattern, based on the fact that just one sucrose-containing soft drink (like a Cola drink) contributes 295 kilocalories per day, which represent about 35% of the sugar needed per day.[23] It has been stated that a reduction in sucrose consumption below 15–20 kg/person/year or 40–55 g/day or 10% of total energy intake, reduces caries risk.[12] Although data from NHANES III showed that sucrose from soft drinks may have an cumulative effect on caries after years of consumption,[24] it is important to make the point that high frequency is not the same as a long period exposure to dietary sucrose. The model used here is designed to test dietary substances for a period that is enough to induce caries-like lesions. The variable "exposure time," therefore, is controlled to allow frequency to be the independent variable under study. Hence, we have tried to model different sucrose consumption patterns over a long period. Given the ecological nature of the disease, it is perfectly possible that someone with a highly frequent consumption of sucrose does not develop lesions over time, if this high consumption is reverted before the onset of the clinical lesions. Collectively interpreting the results, it is possible to state that one daily exposure to sucrose induces mild, three moderate, and five or more severe modifications on the cariogenic and virulent traits of the biofilm. Fermentable sugars, mainly mono- or di-saccharides, induce a rapid pH drop from neutral to pH 5.0 or below.[25] Although a one per day exposure to sucrose does not show differences with the unexposed control in medium pH at 32 h, after 56 h of biofilm, growth differences are readily detectable. It is plausible that a 32 h biofilm is still immature to evidence changes that

begin to show after 56 h [Figure 1]. Biofilm traits are also modified by the different daily frequencies of sucrose exposure. This sugar is the raw material for extracellular polysaccharide production by the bacteria.[26] Promoting bacterial adhesion to the hard dental tissues through to the acquired pellicle and cohesion and aggregation between prokaryotic cells, polysaccharides are key for the development of a mature oral biofilm.[27] Our results indicate that polysaccharide production, both SEPS and IEPS increase proportionally to the daily frequency of sucrose exposure to the biofilm [Table 1]. There was an interesting difference in the way *S. mutans* cells react to sucrose frequency in terms of polysaccharide production. While 5 daily exposures to sucrose were enough to show differences in IEPS production with just one, only 10 exposures resulted higher than the rest for SEPS [Table 1]. It is possible to speculate that sucrose availability is differentially used by the biofilm, regulating substances that may result more crucial for biofilm growth and survival. Unlike polysaccharides, demineralization described a dose-dependency pattern, showing differences between each sucrose dose [Figure 2]. Further studies should explore the nature of these discrepancies. Polysaccharide production by bacteria depends on the activity of the enzyme glycosyltransferase (Gtf).[28] It has been demonstrated that when growing in biofilms, *S. mutans* is capable to produce more Gtf B and C than when growing as planktonic cells.[29] Likewise, type and quantity of the consumed carbohydrates induce changes on *gtfB* y *gtfC* expression with consequential variations on the synthesis of extracellular polysaccharides.[30] Consistent with our results, *in situ* approaches have shown that high concentration and frequency of sucrose exposure increase the concentration of extracellular polysaccharides within the biofilm matrix.[31,32] Clinical studies have also suggested that the synthesis of extracellular polysaccharides is related with caries activity in children.[33,34] Besides acid production, an excess on sugar availability leads to an increased synthesis of IPS. These carbohydrate reservoir is used as a source of nutrients when there is scarcity in the environment.[35] Herein, no differences were detected among the experimental conditions [Table 1]. Despite the pH-cycling nature of the caries model used in our experiments, there is always a basal glucose concentration in the medium and the famine periods may not be long enough as to induce the synthesis of IPS. Besides the regulation of polysaccharide production, there was an increase in the biofilm protein content upon higher sucrose frequencies [Table 1]. Increased metabolic activity of the bacterial consortium imposed by sucrose exposure leads also to enhanced protein and enzyme synthesis necessary to form a mature biofilm.[36] When polysaccharide and protein content was augmented by frequent exposure to fermentable carbohydrates, the biomass content was also higher [Table 1]. The latter is consistent with previous studies.[37,38] It has been reported that when *S. mutans* biofilms grow in the presence of sucrose, adhesion protein (Gbp), Gtf B and Gtf C, and competence stimulating peptide (ComDE) significantly rise as compared with growth under only glucose.[29,39]

In spite of the possibility that this study provides support for recommendations in terms of the frequency in which sucrose intake may represent higher caries risk, the nature of the study requires caution. This is an *in vitro* approach using a relevant biological experimental model. Thus, caries is sugar-biofilm dependent disease, but many other factors modulate the interaction of the necessary factors involved in initiation of the process.[1] In particular, saliva has been widely acknowledged to have a protective function in caries.[40] This fluid contains a high number of substances that have been reported as anticariogenic. Further studies should include saliva and other key elements in modeling caries. Thus, exposure to protective or risk factors must also be taken into consideration to assess the effect that the frequency of sucrose exposure will have at the individual level. Clinical studies, therefore, are strongly recommended to better judge the importance of the frequency of sugar consumption.

CONCLUSIONS

Results from this study suggest that:

- Increased daily frequency of sucrose consumption enhances cariogenicity of a *S. mutans* biofilm
- Cariogenicity is dose-dependent
- As little as one daily exposure to sucrose may initiate carious lesion formation.

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Conflicts of interest
There are no conflicts of interest.

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