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Relação entre a Atividade da Isoenzima Anidrase Carbônica VI, o Fluxo Salivar e o pH do Biofilme Dental de Pré-escolares com Cárie na Infância

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, como requisito para obtenção do título de Mestre em Odontologia, Área de Odontopediatria.

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DEDICATÓRIA

“A DEUS, que sempre guia meus passos...”

“A toda minha família: pai e mãe, irmãs, cunhados, sobrinhas, sobrinhos, namorado, sogra, sogro, cunhada pela força e alegria que proporcionam a minha vida.”

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RESUMO

Este estudo teve como objetivos determinar a atividade da anidrase carbônica VI (ACVI) na saliva de pré-escolares com cárie e investigar a relação entre a experiência cárie (dmfs) e a atividade da ACVI, o fluxo salivar e pH do biofilme antes e após o bochecho com sacarose a 20% em pré-escolares. Trinta pré-escolares com idade entre 45,3 e 80,3 meses foram divididos em 2 grupos: grupo livres de cárie (LC) e grupo com cárie (C). Exames clínicos foram realizados por um examinador de acordo com os critérios da OMS + lesões iniciais de cárie. Em cada individuo, foi determinada a atividade da ACVI, o fluxo salivar e o pH do biofilme dental tanto antes quanto após o bochecho com sacarose. Os resultados foram submetidos aos testes de Wilcoxon, Mann-Whitney e correlação de Spearman (α=0,05). Os resultados mostraram que a atividade da ACVI antes do bochecho e a variação da atividade da ACVI foram maiores na saliva do grupo com cárie que do livres de cárie. Não houve diferença significativa entre os dois grupos após o bochecho. Porém, após o bochecho, a variação do pH do biofilme foi menor em ambos os grupos (p=0,0012 e p=0,0037 para C e LC, respectivamente). E, após o bochecho, houve um aumento significativo do fluxo salivar nos dois grupos (p=0,0003 e p=0,0037 para C e LC, respectivamente). Houve uma correlação negativa da variação entre a atividade da ACVI e a cárie (r=-0,501 e p=0,005). E uma correlação positiva entre a idade das crianças e a experiência de cárie (r=0,456 e p=0,011). Assim, os resultados sugerem que a variação da atividade da ACVI e a idade das crianças está associada com a cárie dentária em pré-escolares.

Palavras-chave: cárie dentária, anidrase carbônica VI, pré-escolares.

ABSTRACT

This study aimed to determine the activity of the carbonic anhydrase isoenzyme VI (CAVI) in saliva of preschool children with caries and investigate the relationship between caries and the salivary CAVI activity, salivary flow rate (SFR) and biofilm pH before and after a 20% sucrose mouthrinse in preschool children with caries. Thirty preschool children aging from 45.3 to 80.3 months were divided into two groups: caries-free group (CF) and caries group (C). Clinical examinations were conducted by one examiner (kappa=0.95) according to WHO criteria (dmfs) + early caries lesions. From each subject, CAVI activity, SFR and plaque pH were determined before and after a sucrose rinse. The results were submitted to Wilcoxon and Spearman correlation tests (α=0.05). The results showed that the pre-rinse CAVI activity and its variation were higher in saliva from caries children than from CF children. No difference was found between the two groups in the post- rinse salivary CAVI activity. After mouthrinse, the biofilm pH difference were lower in both groups (p=0.0012 and p=0.0037 for the C and CF group respectively). Also, after the sucrose rinse, SFR significantly increased in C and CF groups, (p=0.0003 and p=0.0037). The variation of salivary CA VI activity was negatively correlated with caries (r= ¯0.501 p=0.005). Child`s age showed a positive correlation with caries (r=0.456 and p=0.011). These results suggest that variation of salivary CA VI activity and child's age are associated with dental caries of preschool children.

Key-words: caries, carbonic anhydrase VI, primary dentition, preschool children.

SUMÁRIO

1. INTRODUÇÃO GERAL

No Brasil, a doença cárie, em crianças, apresenta-se como um problema de saúde pública. Já que crianças com idade de zero a cinco anos SB Brasil (Ministério da Saúde, 2003), evidenciou que o país não atingiu a meta estabelecida pela Organização Mundial de Saúde (OMS), a qual preconiza que 50% das crianças com idade de zero a cinco anos devem estar livres de cárie. Porém, no SB Brasil 2010 verificou-se que aumentou a proporção de crianças livres de cárie aos 12 anos que cresceu de 31% para 44%. Além disso, o mais importante é que a população infantil que tem experiência de cárie possui maior risco ao desenvolvimento de cárie futura (Sclavos *et al.,* 1988), sendo a experiência passada dessa doença considerada um dos preditores de risco mais significativos (Hausen, 1997; Zhan *et al.,* 2006).

A cárie é uma consequência da dissolução dos tecidos duros dos dentes quando na presença de condições cariogênicas. Os fatores primários relacionados à etiologia da cárie dental são a presença de bactérias cariogênicas, a ingestão de carboidratos fermentáveis e um hospedeiro/superfície dentária susceptível, que interagem em determinado período de tempo (Harris *et al.,* 2004; Selwitz *et al.,* 2007). Dentre esses fatores, a frequência de exposição à sacarose tem sido apontada como responsável por alterações orgânicas/inorgânicas (Cury *et al.,* 1997; Nobre dos Santos *et al.,* 2002; Tenuta *et al.,* 2006; Ccahuana-Vásquez *et al.,* 2007) e microbiológicas (Loesche, 1986; Nobre dos Santos *et al.,* 2002) no biofilme dental.

Em relação à saliva, sabe-se que ela desempenha um papel fundamental na homeostasia oral e que as alterações quantitativas na secreção salivar podem levar a efeitos adversos locais, tais como infecções orais e cárie (Ship et al., 2003). Diante de tantos fatores relacionados à dinâmica do processo carioso, outro fator importante a ser citado é a capacidade dos fluídos orais tamponarem os ácidos presentes no meio bucal, sendo o sistema de tamponamento do bicarbonato/fosfato o mais relevante na cavidade bucal. Este

sistema é diretamente influenciado por uma proteína salivar, a isoenzima anidrase carbônica VI (ACVI), que penetra no biofilme dental e facilita a neutralização ácida pelo bicarbonato salivar. A ACVI catalisa a reação reversível do dióxido de carbono na reação $H^+ + HCO^-_3 \leftrightarrow CO_2 + H_2O$. Acredita-se que pela catalisação dessa reação, essa enzima seja capaz de prover uma maior neutralização dos ácidos do biofilme dental (Kimoto, 2006). Assim, um biofilme com maior capacidade tampão poderia ter um efeito protetor contra a cárie dental. No que diz respeito à saliva, foi demonstrado que em crianças livres de cárie, a expressão da ACVI foi maior do que naquelas com lesões de cárie ativa (Szabó,1974). Posteriormente, foi observada uma relação negativa entre a concentração salivar de anidrase carbônica VI e o CPOD (índice de dentes cariados, perdidos e obturados) em adultos com higiene bucal deficiente (Kivelä *et al.,* 1999). No entanto, a evidência de que existe uma alta concentração da ACVI na saliva pode não necessariamente significar que toda a isoenzima esteja ativa e dessa forma, seja capaz de neutralizar os ácidos após um desafio cariogênico. Assim, faz-se necessário determinar a atividade da ACVI na saliva de pré-escolares com cárie e verificar se existe correlação entre a atividade dessa isoenzima e o pH do biofilme dental bem como o fluxo salivar antes e após o bochecho com sacarose a 20% nos pré-escolares.

2. CAPÍTULOS

Essa dissertação está baseada na Resolução CCPG/002/06/UNICAMP que regulamenta o formato alternativo para teses de Mestrado e Doutorado e permite a inserção de artigos científicos de autoria ou co-autoria do candidato (Anexo 1). Por se tratar de pesquisas envolvendo seres humanos, o projeto de pesquisa destes trabalhos foi submetido à apreciação do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba, tendo sido aprovado (Anexo 2). Assim sendo, essa tese é composta por um capítulo como descrito a seguir:

Capítulo 1: "Relationship among Salivary Carbonic Anhydrase VI Activity and Flow Rate, Biofilm pH and Caries in Primary Dentition". Frasseto F, Parisotto TM, Peres RCR, Marques MR, Line SRP, Nobre-dos-Santos M.

Relationship among Salivary Carbonic Anhydrase VI Activity and Flow Rate, Biofilm pH and Caries in Primary Dentition

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Short title – Carbonic anhydrase VI activity and dental caries

Key words – Caries, carbonic anhydrase VI; primary dentition, preschool children

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Declaration of interests

The authors declare that there is no potential conflict of interest as none of the authors have any personal or financial relationship that might introduce bias or affect their judgment.

Abstract

This study aimed to determine the activity of the carbonic anhydrase isoenzyme VI (CAVI) in saliva of preschool children with caries and investigate the relationship between caries and the salivary CAVI activity, salivary flow rate (SFR) and biofilm pH before and after a 20% sucrose mouthrinse in preschool children with caries. Thirty preschool children aging 45.3 to 80.3 months were divided into two groups: caries-free group (CF) and caries group (C). Clinical examinations were conducted by one examiner (kappa=0.95) according to WHO criteria (dmfs) + early caries lesions. From each subject, CAVI activity, SFR and plaque pH were determined before and after a sucrose rinse. The results were submitted to Wilcoxon and Spearman correlation tests (α=0.05). The results showed that the pre-rinse CAVI activity and its variation were higher in saliva from caries children than from CF children. No difference was found between the two groups in the post- rinse salivary CAVI activity. After mouthrinse, the biofilm pH difference were lower in both groups (p=0.0012 and p=0.0037 for the C and CF group respectively). Also, after the sucrose rinse, SFR significantly increased in C and CF groups, (p=0.0003 and p=0.0037). The variation of salivary CA VI activity was negatively correlated with caries (r= ¯0.501 p=0.005). Child`s age showed a positive correlation with caries (r=0.456 and p=0.011). These results suggest that variation of salivary CA VI activity and child's age are associated with dental caries of preschool children.

Introduction

Dental caries is a consequence of dental hard tissues dissolution under cariogenic conditions of dental biofilm. The etiology of caries is multifactorial and it is thought to include dietary carbohydrate consumption, the microbial composition and the pH-lowering ability of dental biofilm, and the action of saliva [Bowen et al., 2005; Selwitz, 2007; Llena and Forner 2008].

Concerning dental biofilm, dietary habits can result in biofilm acidogenicity, which might influence the caries process. There is some controversy about biofilm acidogenicity and dental caries [Fejerskov et al., 1992; Dong et al., 1999, Shimizu et al., 2008]. While some studies show that the biofilm pH samples may not be the primarily responsible for caries [Pearce, 1991], others demonstrate that biofilm pH fall is higher in caries-active than in caries-inactive subjects [Dong et al., 1999; van Ruyven et al., 2000].

In respect to saliva, it is known that saliva plays a critical role in oral homeostasis and quantitative changes in salivary secretion may lead to local adverse effects such as oral infections and caries [Ship et al., 2003]. Whole saliva is a mixture of the secretions from the parotid, submandibular, sublingual and minor salivary glands and gingival crevicular fluid. Saliva contains inorganic compounds and multiple proteins that affect conditions in the oral cavity and locally on the tooth surfaces. The salivary buffering capacity, based primarily on bicarbonate ions, is a factor of primary importance in protecting the enamel surface from caries [Wolinsky, 1994]. Among the defense systems of saliva, salivary carbonic anhydrase isoenzyme VI (CAVI) is the only known secreted isoenzyme of the carbonic anhydrase family, which has been detected in the saliva secreted by the serous acinar cells of mammalian parotid and submandibular glands. It catalyzes the reversible reaction of carbon dioxide in a reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ [Kivela et al., 1999]. By catalyzing this reaction, CAVI is believed to

provide a greater buffering capacity to the saliva by penetrating dental biofilm and facilitating acid neutralization by salivary bicarbonate [Kimoto et al., 2006].

Regarding the association of CAVI with dental caries, different results have been provided by literature. The study from Oztürk et al. [2008] found no significant difference in CAVI concentration when caries and caries-free young adults were compared. In the other side, the investigation performed by Kivela et al. [1999] has shown that a low CAVI concentration is associated with a higher caries index, and a negative correlation between CAVI concentration and DMFT index in individuals with poor oral hygiene. In the same way, Szabó [1974] found a higher concentration of CAVI in 7-14 year–old, caries-free children than in caries children. However, a high concentration of the CAVI in saliva may not necessarily means that all isoenzyme present in the media is active. Thus, determining the activity of salivary CAVI instead of just its concentration would provide further evidence of the effect of this isoenzyme in protecting teeth from dental caries. Considering the above, the aims of this study were to determine the CAVI activity in preschool children with caries and investigate the relationship between dental caries and the salivary CAVI activity, salivary flow rate and plaque pH before and after a mouthrinse with 20% sucrose in preschool children with caries.

Materials and Methods

Ethical considerations

This study was approved by the Ethical Committee in Research of Piracicaba Dental School/State University of Campinas (UNICAMP), in agreement with the Declaration of Helsinki under protocol #090/2008. The daycare and preschools granted permission for the study and an informed written consent was given by the children's guardians.

Subjects

After examined 62 children of a nursery schools in Piracicaba town, 30 preschools of both gender was selected, all of low socioeconomic level, aged 45.3–80.3 months. The children attended public nursery schools in Piracicaba town, state of São Paulo, Brazil. Two groups of preschool children were formed: 17 presenting caries (C) and 13 being caries-free (CF). The inclusion criteria were presence of dental caries, and children caries-free. The exclusion criteria of the study were presence of systemic diseases, severe fluorosis, dental hypoplasia, use of braces, antibiotic therapy, and communication or neuromotor difficulties. The two groups presented the following characteristics: C (10 girls and 7 boys, mean age 62.8 ±17.5 months); CF (5 girls and 8 boys, mean age 57.2 ± 7.2 months). All children were reported to use fluoridated water (0.7 ppm F) and F dentifrice. Their teeth were brushed at the daycare centers twice a day using fluoridated dentifrice. The children stayed at the daycare center from 7 a.m. to 5 p.m. and all of them were fed with the same meals.

Calibration of the examiner

An examiner considered all components of the diagnostic criteria of World Health Organization + initial lesion of caries [Assaf et al., 2006] (Apêndice 1). This calibration was assessed by reexaminations of 10 percent of the children with a 1-week-interval period, to avoid dental examiner memorization. The intra-examiner agreement, measured using Kappa calculation, regarding the surface level, was 0.95. Theoretical discussions between examiner and a gold standard were conducted using clinical photographic slides about the use of the criteria and the examination method, including explanations about the diagnosis of early caries lesions.

Caries assessment

The clinical examinations were carried out with a focusable flashlight at nurseries using a mirror and a ball-ended probe to remove debris to enhance visualization and confirm questionable findings. Gauze was employed in order to dry or clean the teeth, favoring the identification of early caries lesions. A portable flashlight was also used to make non-cavitated lesions more easily recorded. All examinations were carried out by a single clinician, following rigorously strict cross-infection control measures. In the present study, the WHO criteria [WHO, 1997] + early caries lesions (ECL) [Assaf et al., 2006,] were used for caries diagnosis. The units of evaluation used in the clinical examinations were dmfs (decayed, missing and filled surfaces).

Dental biofilm pH measurement

The determination of biofilm pH was performed according to the method of Kimoto et al., [2006], in different days of the saliva collection, to avoid a possible effect of circadian rhythm on salivary flow rate and composition [Ferguson and Botchway, 1980]. Biofilm collection was performed at the same time of the day on buccal and lingual surfaces of primary maxilary molars and primary mandibular molars. To determine biofilm acidogenicity, each subject rinsed his mouth with a 20% sucrose solution at room temperature for 1 min, and two biofilm collect were made: the first one was performed immediately before a mouth rinse with a 20% sucrose solution and the second one 5 min after the mouth rinse. Thus, dental biofilm was collected with sterile curettes, placed in microdishes, diluted in 40μL of distilled water and stirred. Each measurement of pH took about 30 s, including 10–15 s for a stable reading to be obtained. The pH was read with a glass electrode (Model 6261-10C, Horiba Ltd., Kyoto, Japan) that had previously been standardized in pH 4.0 and 7.0 standard buffers at the start of each biofilm sample measurement and the response in pH 7.0 buffer checked again at the end (Apêndice 2). Following, the upper biofilm pH variation (upperΔpH) and lower

biofilm pH variation (lower∆pH) - biofilm pH difference in the upper and lower arches respectively between pre-rinse biofilme pH and 5 min after 1 minute mouthrinse of 20% sucrose solution were determined.

Salivary flow rate

One saliva stimulated sample was collected from each subject that chewed a parafilm for 2 minutes and deposited this saliva in a cup, as previously described by Dawes and Kubieniec [2004]. Salivary flow rate was calculated by measuring the total volume of saliva and dividing this by the collection time for each child, obtaining the salivary flow rate in mL/minute [Ericsson and Hardwick, 1978]. After the salivary flow rate calculation, saliva samples were stored in of 1.5 ml centrifuge microtubes, kept in a polystyrene box containing ice and were frozen at -70^0 C for later determination of the activity of CAVI. After the first saliva collection, the pre-school children performed a rinse with 2 mL of a 20% sucrose solution for 1 minute. After 5 min, a second sampling of stimulated whole saliva was carried out to determine the effect of sucrose on the salivary flow rate as well as on the activity of CAVI. All saliva samples were collected in the morning 30 min after breakfast.

Determination of the Activity of Carbonic Anhydrase VI in Saliva

After salivary flow rate measurement, the samples were frozen at -70^0 C for later analysis of the CAVI activity. The determination of the CAVI activity was performed by the zymography method [Kotwica et al., 2003]. The determination was performed on saliva, since this isoenzyme can adhere to the acquired pellicle and promote the neutralization of excess acid by catalyzing the reaction $H^+ + HCO^-_3 \leftrightarrow CO_2 + H_2O$ which constitutes the most important buffer in the oral environment [Leinonen, 1999]. It is important to mention that prior to the commencement of the analysis of enzymatic CAVI activity, the technique of Western blotting was performed to make sure that indeed we would be working with the respective isoenzyme. We used anti-CAVI (Sigma Chemical Company-St. Louis Mo., USA).

To analyze the CAVI activity, saliva was kept frozen at -70°C. After being thawed, 200µl of saliva was added to 200µl of Tris buffer for zymography, and from the total of 400 µl of sample only 15µl were placed in each channel of the gel. This material was stirred for 1 minute before being placed on acrylamide gel at 12% / 0.8% bisacrylamide which remained for 21 hours at 30 volts in the refrigerator. After electrophoresis, the gel was washed for 20 minutes in 10% isopropanol diluted in 100 mM Tris, pH 8.2 followed by two washes of 100 mM Tris, pH 8.2. The gel was incubated in bromothymol blue 0.1% in the 100 mM Tris, pH 8.2, for 30 minutes at 4°C. The reaction of CAVI is observed after immersing the gel in distilled deionized water saturated with CO_2. The gels were photographed, and the image obtained from the bands was quantified by Image J® software [Collins, 2007], which calculated the luminescence in the area of the bands and quantified the CAVI activity in numerical value (pixels area) (Apêndice 3). After determining CAVI activity before and after a 20% sucrose mouthrinse, CAVI variation activity (ΔCAVI) was obtained (difference between the post-rinse CAVI activity and pre-rinse CAVI activity).

Statistical analysis

The statistical analysis was performed considering two the groups of children (caries free and with caries) as the dependent variables. The independent variables were: the CAVI activity pre and post-rinse, CAVI variation activity (ΔCAVI), the salivary flow rate, the upper biofilm pH variation (upperΔpH) and lower biofilm pH variation (lowerΔpH). Each variable under study was submitted to Wilcoxon and Mann-Whitney tests. In addition, the correlation between dental caries and all variables under study was assessed by the Spearman correlation test. The level of significance was set at 5%.

Results

Table 1 shows that the two groups of children presented statistically significant differences in the CAVI variation activity (p=0.0047). The pre-rinse CAVI activity was also higher in caries group and probability was close to the significance level (p=0.0516). However, we found no difference between the two groups in the post-rinse CAVI activity (p=0.5165). Also, from table 1 it is clear that the pre-rinse CAVI activity is significantly different from the post-rinse CAVI activity (p=0.0191) in caries group but not in the caries free one (p=0.2213). In respect to SFR and plaque pH, no difference was found between caries and caries-free groups (ISFR p=0.1738, FSRF p= 0.1071, upperΔpH p=0.4513, lowererΔpH p=0.9499, data not shown).

Table 2 shows that after a sucrose rinse, the biofilm pH difference was statistically significant lower in both groups (p=0.0012 and p=0.0037 for the caries and caries free group respectively). Table 2 also reveals that in both groups, the salivary flow rate significantly increased after the sucrose rinse (p=0.0003 and p=0.0037 for caries and caries-free group respectively). The correlations between dmfs and variables characteristics are shown in table 3. The results reviews that the variables that showed a statistically significant correlation with dental caries were child`s age (r=0.456 and p=0.011) and ΔCAVI (r=-0.501and p=0.005).

Discussion

Salivary CAVI, the unique secreted isoenzyme of CA enzyme family, protects dental enamel from caries by acting in the local environment of dental surface [Leinonen et al., 1999]. To investigate its function in the oral cavity, we determined its activity in whole saliva of preschool healthy children. In our study, we performed a quantitative analysis of the activity of salivary CAVI instead of evaluating just the isoenzyme concentration. With respect to the CAVI activity, our

results showed that the pre-rinse with 20% sucrose activity of CAVI as well as its variation was higher in saliva of caries children than in caries-free children. However, we found no difference in CAVI activity between the two groups after sucrose rinse (Table 1).

Regarding the pre-rinse CAVI activity in caries children, our results are not in agreement with Szabó [1974], who found a higher concentration of CAVI in 7-14 year –old caries-free children and Kivela et al. [1999] who demonstrated a low but significant negative correlation between the CAVI concentration in saliva of young adults and DMFT index. However, it should be pointed out that while the methods of analysis employed by the previous authors were able to determine just the concentration of salivary CAVI, in the present study, we used the zymography method [Kotwica et al., 2003] to quantitatively determine the activity of salivary CAVI. This is particularly important since a high concentration of the isoenzyme CAVI in saliva may not necessarily means that all isoenzyme present in the media is active.

The present results also showed that the CAVI variation activity was negatively correlated with caries (Table 3). It is interesting to note that the variation of CAVI showed a significant negative correlation with the pre rinse CAVI activity ($r=-0.671$ $p=0.000$ data not shown). These results suggest that the higher the pre rinse CAVI activity, the lower the CAVI variation and consequently, the higher the caries index. These results are probably related to the fact that in the caries group, the post-rinse CAVI activity was significantly lower than the pre rinse value (Table 1). Other possible explanation for these results is that the caries group children may have had high daily sugar consumption since it is know that this sugar consumption pattern is significantly correlated with early childhood caries [Nobre dos Santos et al., 2002; Parisotto et al., 2010]. In the presence of a sugar rich diet, and acid formation by metabolism of the microbial flora on dental surface, there is a possibility that salivary CAVI being lower after sucrose rinse, the acid neutralization via conversion of salivary bicarbonate and microbe-delivered

hydrogen ions to carbon dioxide and water by CAVI did not completely occurred. In the other side, there was no change in CAVI activity in the caries-free group after sucrose rinse. This is probably related to the fact that in caries-free children, the events of pH drops are less frequent than in caries children and in this way, the CAVI activity would not be so necessary to speed the neutralization of acid during the frequent cariogenic challenges as in caries children by catalyzing the reverse reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO^-_3$ when the proton concentration increases [Kivela et al. 1997b].

Our findings suggest that CAVI may protect the enamel surface by catalyzing the most important buffer system in the oral cavity, thus accelerating the neutralization of acid from the local environment of the tooth surface [Kivela et al., 1999]. Moreover, it has been demonstrated that salivary CAVI may accumulate in enamel pellicle and function as a local pH regulator on enamel surface [Leinonen et al., 1999]. The proposed mechanism is that in the enamel pellicle, CAVI is located at the optimal site to catalyze the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water [Leinonen et al., 1999]. Another mechanism recently suggested is that CAVI in saliva penetrates biofilm and facilitates acid neutralization by salivary bicarbonate. Therefore, biofilm CAVI would contribute to neutralization of biofilm acid, mainly in stimulated saliva whose buffering is mainly performed by bicarbonate and thus would help to prevent dental caries [Kimoto et al., 2006].

Although there was no information about the frequency of acidogenic episodes, biofilm pH in caries as well as in caries free group decreased significantly after five minutes of rinsing with 20 % sucrose solution for 2 minutes. However, the two groups showed no difference in biofilm pH before or after the rinsing with 20 % sucrose solution (Table 2). Additionally, in the present work no correlation was found between biofilm pH and caries ($r=-0.22$ and $p=0.236$; $r=-0.088$ and $p=0.236$ for upper ΔpH and lower ΔpH respectively). Early works suggested a consistent relationship between biofilm acidogenicity factors and

dental caries [Stephan, 1948; Fosdick et al., 1957], however, other studies have cast some doubt on this association [Fejerskov et al., 1992; Dong et al., 1999]. These authors showed that not only the variation of biofilm pH but also the frequency of acidogenic episodes may be more important to dental caries than the degree of acidogenicity during an isolated episode [Dong et al., 1999]. Moreover, no correlation was found between biofilm pH and the post-rinse CAVI activity (r=-0.045 and p=0.814; r=-0.023 and p=0.902 for upper ΔpH and lower ΔpH respectively).This findings may suggest that CA VI is not directly involved in the regulation of the actual biofilm pH. In spite of that, it may speed the removal of acid by catalyzing the reverse reaction $CO_2 + H_2O \leftrightarrow H^+ + HCO^-_3$ when the proton concentration increases [Kivela et al. 1997b]. An interesting alternative is that CAVI may accumulate in enamel pellicle and function as a local pH regulator on enamel surface [Leinonen et al., 1999].

Regarding salivary flow rate, we found no statistical difference between the caries and the caries-free group (Table 2). This result is in line with Farsi et al., [2008]. Additionally, a significant increase in salivary flow rate was observed after sucrose rinse in both groups. This result agrees with previous studies showing that the salivary flow rate increases after sucrose rinse [Dawes and Kubieniec, 2004]. Our findings suggest that future investigation should be conducted to search for a possible association between CAVI activity and salivary flow rate and pH since previous studies investigated only the salivary CAVI concentration and found a weak positive correlation with salivary flow rate [Kivela et al., 1997a] and no significant correlation with saliva pH [Kivela et al., 1997b, Kivela et al, 1999; Kivela et al., 2003].

In summary, our data showed that that variation of salivary CA VI activity and child's age are associated with dental caries in primary dentition.

Acknowledgements

We thank the São Vicente de Paulo Nursery Piracicaba-SP/Brazil for collaborating with this research, as well as all children who took part in this study. We also thank FAPESP (2008/02412-6 and 2008/10064-8) and CAPES for the financial support.

Table 1. Means ± SD of salivary CAVI activity and variation CAVI in the two groups of children

Variables	Caries Mean ± SD		Caries-free Mean ± SD		*p* value
Pre-rinse CAVI	42752.11**a**	32476.62	19130.79**a**	16911.68	0.0516
Post-rinse CAVI	30828.87**b**	25937.81	21962.15**a**	17124.77	0.5165
∆ CAVI	-11923.24	17022.03	2831.36	9831.13	0.0047

Pre-rinse CAVI (isoenzyme CAVI activity before rinse of 20% sucrose); Post-rinse (isoenzyme CAVI activity 5 min after 2 min rinse of 20% sucrose);. *p* values derived from Wilcoxon and Mann-Whitney tests. Groups whose means followed by distinct letter differ statistically ($p<0.05$).

Table 2. Means ± SD of clinical parameters in the two groups of children

Groups	Variables (Mean ± SD)		*p* value
Caries	**ISFR**	**FSFR**	
	1.31±0.98	2.36±1.28	0.0003
	UpperΔpH	**LowerΔpH**	
	1.75 ± 0.68	1.21±0.51	0.0012
Caries-Free	**ISFR**	**FSFR**	
	0.71 ± 0.53	1.54±0.94	0.0019
	UpperΔpH	**LowerΔpH**	
	2.01 ± 0.93	1.36± 0.89	0.0037

ISFR (salivary flow rate before mouthrinse of 20% sucrose); FSFR (salivary flow rate 5 min after 2 min mouthrinse of 20% sucrose); upperΔpH (biofilm pH difference in the upper arch- resting pH and 5 min after 2 min mouthrinse of 20% sucrose); lowerΔpH (biofilm pH difference in the lower arch- resting pH and 5 min after 2 min mouthrinse of 20% sucrose); *p* values derived from Wilcoxon test.

Table 3. Spearman correlation coefficients (r) and probabilities of statistical significance (p) between dental caries and the analyzed variables

Variables	dmfs	
	R	p
ISFR	0.315	0.90
FSFR	0.325	0.080
UpperΔpH	-0.223	0.236
LowerΔpH	-0.088	0.643
Age	0.456	0.011*
Pre-rinse CAVI	0.344	0.063
Post-rinse CAVI	0.052	0.784
ΔCAVI	-0.501	0.005*

* statistically significant (α=0.05); n=30.

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3. CONCLUSÃO GERAL

Assim, os resultados sugerem que a variação da atividade da ACVI e a idade das crianças está associada com a cárie dentária em pré-escolares, já que houve uma correlação negativa da variação entre a atividade da ACVI e a cárie; e uma correlação positiva entre a idade das crianças e a cárie.

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5. APÊNDICES

APÊNDICE 1

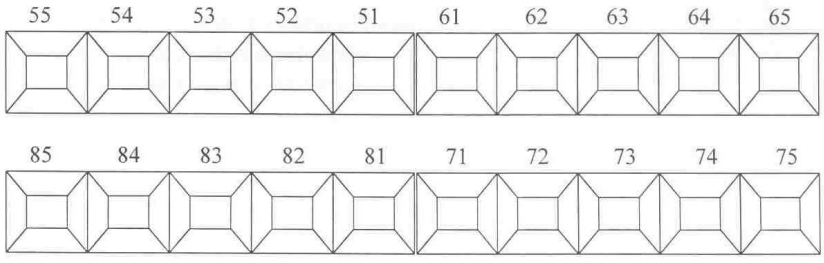
FICHA CLÍNICA

Nome:_____Nº da Ficha:_____

Creche:_____Data exame:_____

Data nasc.:_____Idade (meses):_____Sexo: (F) (M) Raça: (B) (N) (P)

A	Hígido
W	mancha branca ativa
B	cariado com lesão crônica
BW	cariado com lesão ativa
C	cariado com lesão crônica de cárie
D	restaurado sem lesao de cãrie
CW	cariado com lesão ativa de cárie
DW	restaurado com mancha branca
4	perdida devido à cárie
5	perdido por outra razão
F	selante de fissura;
FW	selante de fissura com mancha branca
7	Coroa
T	trauma (fratura)



Biofilme clinicamente visível:_____

0: ausência

1:presença

APÊNDICE 2

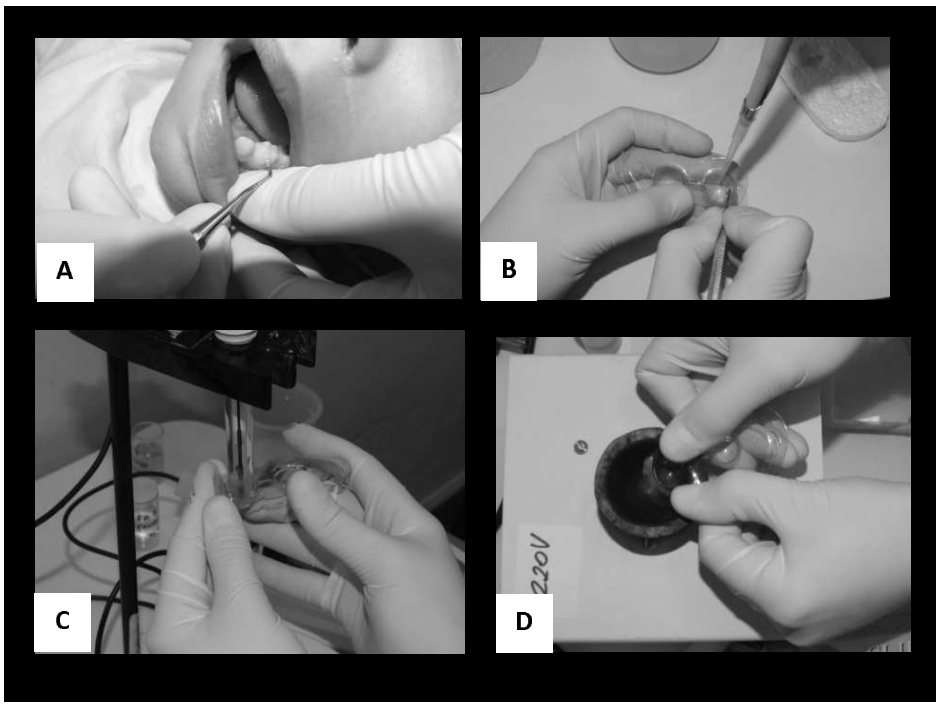


Figura 1. *pH do biofilme* (a) coleta do biofilme com curetas esterilizadas, (b) diluição em água destilada, (c) agitação e (d) medição do pH com eletrodo.

APÊNDICE 3

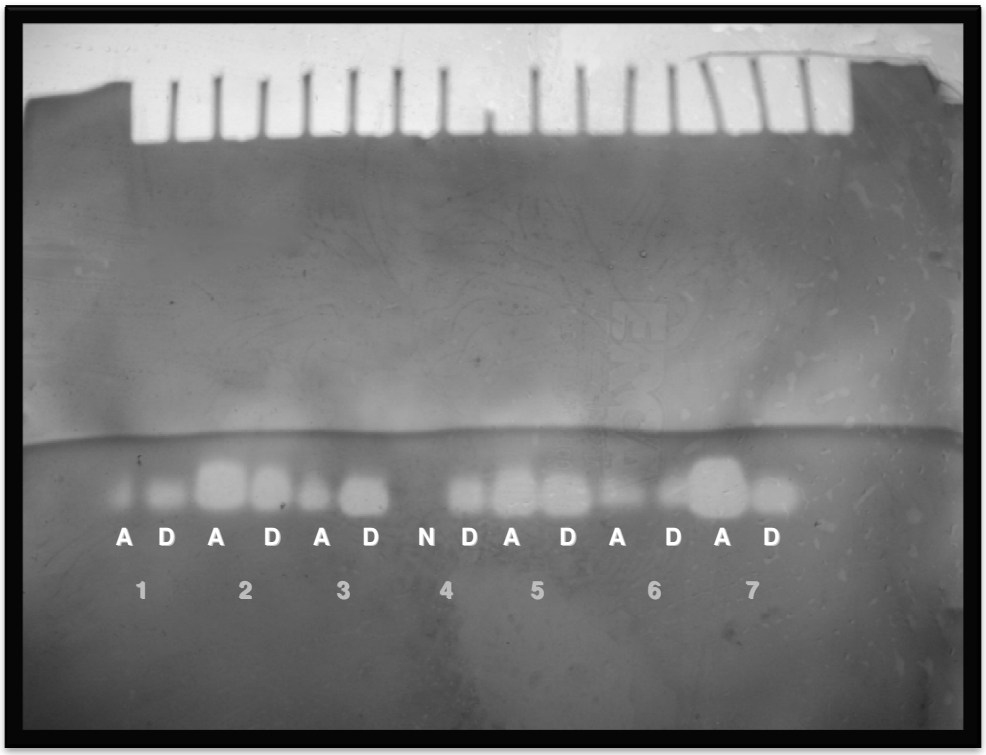


Figura 2. Gel de acrilamida demonstrando a atividade da anidrase carbônica VI antes (A) e depois (D) do bochecho com sacarose a 20%.

N= banda que não acendeu (voluntário 4).

Voluntários 1,2,3,4,5,6 e 7.

6. ANEXOS

ANEXO1

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

ANEXO 2

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Estudo da atividade da anidrase carbônica VI e sua associação com os parâmetros bioquímicos e pH do biofilme de pré-escolares com cárie precoce da infância**", protocolo nº **090/2008**, dos pesquisadores **FERNANDA FRASSETO e MARINÊS NOBRE DOS SANTOS UCHÔA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 02/10/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Study of the carbonic anhydrase VI activity and its association with biochemical parameters and pH of dental biofilm in pre-school children with early childhood caries**", register number **090/2008**, of **FERNANDA FRASSETO and MARINÊS NOBRE DOS SANTOS UCHÔA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 02/10/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 3

-----Mensagem Original----- From: david.beighton@kcl.ac.uk

Sent: Monday, February 21, 2011 6:02 AM

To: nobre@fop.unicamp.br

Subject: Ms. No. 201102011, Caries Research

MS: 201102011

Dear Prof. Nobre dos Santos,

Thank you for submitting your manuscript entitled "Relationship Among Salivary Carbonic Anhydrase VI Activity and Flow Rate, Biofilm pH and Caries in Primary Dentition " to "Caries Research". It will now be submitted to review and we shall inform you as soon as possible of the decision reached by the editorial board. The manuscript reference number is 201102011. Please use this number on all correspondence about the manuscript, which should be sent to the "Caries Research" editorial office at the address listed below.

For information regarding the status of your manuscript and for future submissions you can access this system by logging into the journal's online peer review system as follows:

http://www.karger.com/cre

Logon Name: nobredossantos

Password: flor2

With kind regards,

David.

Prof. David Beighton

(Editor-in-Chief, Caries Research)

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