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Correlation among *mutans* streptococci counts, dental caries, and IgA to *Streptococcus mutans* in saliva

Correlação entre contagens de estreptococos do grupo mutans*, cárie dentária e IgA anti-*Streptococcus mutans *na saliva*

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ABSTRACT: Two-hundred and forty individuals were studied, divided into five groups as follows: caries-free children, children with caries, children with rampant caries, young adults with and without caries. Whole stimulated saliva was collected and all individuals were investigated for DMFT/dmft according to the WHO criteria and the simplified oral hygiene index (OHI-S). Quantitative analysis of the total aerobic flora and *mutans* streptococci in saliva was performed. Also, the level of salivary anti-*S. mutans* IgA was determined by ELISA. Children with rampant caries showed the highest OHI-S value. The highest total counts of microorganisms were found in the group of children with caries. No statistically significant differences were observed for salivary flow, OHI-S and microorganism counts between the groups of young adults. No correlation between *mutans* streptococci counts and anti-*Streptococcus mutans* IgA levels was observed in the studied groups. A correlation between increased anti-*Streptococcus mutans* IgA levels and caries-free status was observed among young adults but not among children.

DESCRIPTORS: *Streptococcus mutans*; IgA; Saliva.

RESUMO: Duzentos e quarenta indivíduos divididos em cinco grupos foram estudados: crianças livres de cáries, crianças com cáries, crianças com cáries rampantes, adultos jovens com e sem cáries. Saliva total estimulada foi coletada, e todos os indivíduos foram investigados para CPO-D/ceo-d seguindo-se os critérios da OMS e o índice de higiene oral simplificado (IHOS). Foi também realizada a quantificação da microbiota aeróbica total e de estreptococos do grupo *mutans* na saliva. Além disso, o nível de IgA anti-*S. mutans* foi determinado por ELISA. Crianças com cárie rampante apresentaram maior valor de IHOS. A contagem total de microrganismos mais elevada foi encontrada no grupo de crianças com cárie. Nenhuma diferença estatisticamente significativa foi observada em relação a fluxo salivar, IHOS e contagens de microrganismos entre os grupos de adultos jovens. Nenhuma correlação entre contagem de estreptococos do grupo *mutans* e níveis de IgA anti-*Streptococcus mutans* foi observada em nenhum dos grupos estudados. Foi encontrada uma correlação entre níveis mais altos de IgA anti-*S. mutans* e ausência de cáries entre os adultos jovens, mas não entre as crianças.

DESCRITORES: *Streptococcus mutans;* IgA; Saliva.

INTRODUCTION

The infectious nature of dental caries assumes the hypothesis that some form of host immunity can regulate caries activity[2]. Salivary IgA seems to be directly involved in the immunity to dental caries. This immunoglobulin presumably prevents the adherence of cariogenic microorganisms to hard surfaces[7], and may also inhibit the activity of glucosyltransferases[10].

Previous investigations have reported contradictory results in relation to the immunity to dental caries. Some authors reported higher levels of salivary IgA in caries-resistant individuals in relation to caries-susceptible ones, suggesting an effective protective function[4,12,20]. Legler *et al.*[15] (1981) observed higher caries incidence in patients with IgA deficiency. On the other hand, other authors did

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not observe any correlation between caries activity and salivary IgA levels[19,22]. The influence of IgA antibodies on the experimental implantation and elimination of *S. mutans* has also been reported in the related literature[14,18].

Age-dependent variations in IgA concentrations were reported by Tappuni, Challacombe[21] (1995). These authors observed lower concentrations of IgA in predentate children in relation to dentate ones, and the highest values of IgA among adults. Challacombe *et al.*[5] (1995) reported lower levels of IgA among the elderly. On the other hand, Närhi *et al.*[16] (1994) did not observe any alterations related to age. Based on the lack of conclusive information on dental caries immunity, the aim of this study was to analyze the correlation among *mutans* streptococci counts, dental caries experience, and levels of IgA anti-*Streptococcus mutans* in saliva.

MATERIALS AND METHODS

A total of 240 individuals were analyzed, divided into five groups as follows:

- Children groups: i) caries-free children: 30 individuals, 13 females and 17 males, 3-12 years of age (mean ± standard deviation = 5.53 ± 1.31 years) with dmft/DMFT = 0; ii) children with caries: 50 individuals, 28 females and 22 males, 3-12 years of age (6.42 ± 1.86) with dmft/DMFT > 0 and without active lesions; iii) children with rampant caries: 30 patients, 19 females and 11 males, 3-12 years of age (5.70 ± 1.80) with 10 or more active and non-treated dental caries lesions. The children included in the study were students from schools localized in the city of São José dos Campos, SP, Brazil.
- Young adult groups: i) caries-free young adults: 30 individuals, 18 females and 12 males, 18-25 years of age (18.93 ± 2.12) with DMFT = 0; ii) young adults with dental caries: 100 individuals, 58 females and 42 males, 18-25 years of age (20.37 ± 1.89), with DMFT > 0 and without active lesions. The young adults included in the study were students of the School of Dentistry of São José dos Campos, São Paulo State University (UNESP).

This study was submitted to and approved by the São José dos Campos School of Dentistry Bioethics Committee. All the volunteers or the responsible person (in case of children) were informed about the aim of the study and authorized the clinical examination and saliva sample collection. A single examiner examined all the patients. Dental health data were collected through clinical examination, recording dental caries prevalence by means of the DMFT/dmft index as defined by the WHO[24] (1977). Oral hygiene was recorded according to the simplified oral hygiene index (OHI-S) as defined by Greene, Vermillion[9] (1964).

Saliva collection and salivary flow determination (SF)

For saliva sample collection, sugarless gum (Epoxiglass Indústria e Comércio de Produtos Químicos Ltda., Diadema, Brazil) was given to the patients aiming at the stimulation of salivary flow. Whole saliva was collected in sterile cups (Laborglass, São Paulo, Brazil), discharging the first portion, and the salivary flow determination was performed according to Krasse[13] (1986).

Quantitation of total aerobic microbiota

The total aerobic microbiota of mixed saliva was studied by plating duplicate samples of saliva dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) on blood agar plates (Tryptic soy agar, Difco, Detroit, USA; 5% rabbit blood) and incubating for 2 days at 37°C.

Mutans streptococci counts and identification

In a maximum period of 3 hours after sampling, saliva samples were diluted to 10^{-1}, 10^{-2} and 10^{-3} and *mutans* streptococci counts were performed. For this purpose, the dilutions of saliva were plated on mitis salivarius (Difco, Detroit, USA) bacitracin sucrose agar for 72 h in candle jars (Nigro, São Paulo, Brazil) at 37°C. After this period, the colonies of *mutans* streptococci were counted. Five colonies from each patient were stored for *Streptococcus* species identification, which was performed according to Hardie[11] (1986).

Anti-*Streptococcus mutans* antibody analysis

Saliva samples containing 5.0 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, USA) and 0.002% sodium azide (Merck, Darmstadt, Germany) were stored at –20°C until antibody analysis. *S. mutans* ATCC 35688 grown in tryptic soy broth (Difco, Detroit, USA) for 72 h at 37°C in candle jars was used as source of antigen. The cells killed with thimerosal (Merthiolate™,

Sigma, St. Louis, USA) (0.2 g/l) were harvested by centrifugation and washed three times with 125 mM Tris-HCl (Gibco BRL, New York, USA), pH 6.8. The antigens were extracted from cells by boiling in 125 mM Tris-HCl, pH 6.8, 20 mM 2-mercapethanol (Sigma, St. Louis, USA), 6 M urea (Merck, Darmstadt, Germany) for 5 min. Then, the boiled product was centrifuged at 10,000 g for 30 min at 4°C and the supernatant was dialyzed against three changes of 3 liters of distilled water and lyophilized. Antigen preparation was stored at –20°C until use. Protein amount was determined by the method of Bradford[3] (1976).

ELISA

Antibody levels were measured by solid-phase ELISA, performed in 96-well, flat-bottomed plates (number 3590, Costar, Cambridge, MA, USA). All plates were coated with 50 µl of crude antigen solution (100 µg/ml) dissolved in 0.1 M carbonate buffer (Merck, Darmstadt, Germany) (pH 9.6), incubated for 2 h at 37°C and overnight at 4°C. The wells were blocked with 0.5% gelatin (G) (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS) (Sigma, St. Louis, USA) for 1 h. Then, the plates were washed five times with 0.5% Tween 20 PBS (T-PBS) (Merck, Darmstadt, Germany) and incubated with 50 µl of saliva sample diluted in T-PBS-G at 1:8 for 2 hours at 37°C. After an additional wash step with T-PBS, the wells were filled with goat anti-human immunoglobulin A peroxidase-labelled (Sigma, St. Louis, USA) and incubated for 1 hour at 37°C. Finally, 100 µl/well of *o*-phenylenediamine (Merck, Darmstadt, Germany) in 0.1 M citrate buffer (Merck, Darmstadt, Germany) (pH 5.5) were added at room temperature until a yellow color developed. The reaction was stopped with 2.5 M H_2SO_4 (Merck, Darmstadt, Germany) and the color was measured at 490 nm with a model 3550 reader (Bio Rad Laboratories, Hercules, California). The data were expressed as values of optical density (OD), obtained by the mean of two readings.

Statistical analysis

In our study, descriptive statistic data were presented as means and standard deviation values. Dependent variables were OHI-S, SF, *mutans* streptococci counts and IgA anti-*Streptococcus mutans*. The independent variable was dental caries (caries free, with caries and with rampant caries). Statistical significance of differences among groups was tested by means of one-way ANOVA (parametric and non-parametric approaches). *Post hoc* multiple comparisons were performed according to Tukey's and Dunn's (5%) tests. Statistical significance was defined at $\alpha = 5\%$. The correlation between anti-*S. mutans* IgA levels and *mutans* streptococci salivary concentrations was tested by Spearman's rank order correlation. Statistical analysis was performed using the software Statistix for Windows (2000, version 7.0, analytical software, Tallahasse, USA) and Statistica for Windows (version 5.0, 1995, StatSoft Inc., Oklahoma, USA).

RESULTS

Considering the salivary flow, no differences could be observed among the children groups (ANOVA, $F_{2;107} = 0.25$, $p = 0.781$). For OHI-S, children with rampant caries showed the highest value and significant differences were observed in relation to the other groups (ANOVA, $F_{2;107} = 11.52$, $p = 0.001$ and Tukey's test 5%).

Statistically significant differences were observed for the total aerobic microbiota among the children groups (ANOVA $F_{2;107} = 9.53$; $p = 0.001$ and Tukey's test). The multiple comparison test (Tukey's test 5%) showed that children with caries presented higher counts in relation to the caries-free group. Children with rampant caries presented an intermediary value and significant differences were not found in relation to the other studied groups.

The group of children with caries also presented the highest salivary concentration of *mutans* streptococci with a significant difference in relation to the controls (ANOVA $F_{2;107} = 3.38$; $p = 0.038$ and Tukey's test 5%).

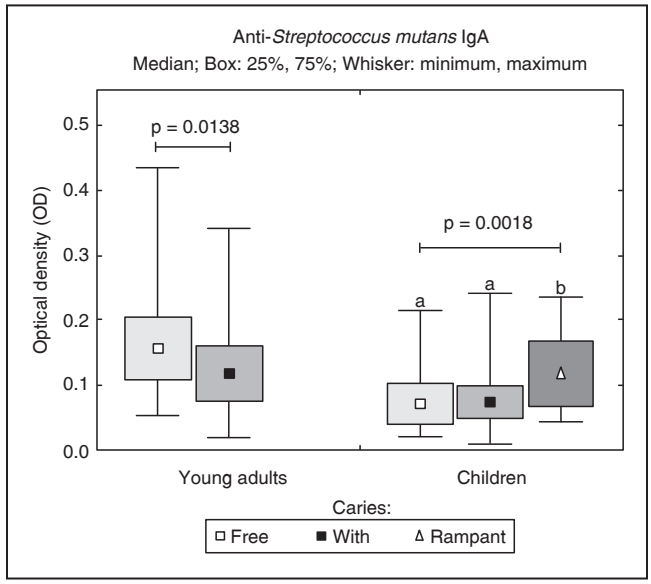
Considering the young adult groups, the Mann-Whitney statistical test detected no significant differences for salivary flow ($p = 0.951$) and OHI-S values ($p = 0.514$). Also, for total aerobic microbiota ($p = 0.918$) and *mutans* streptococci ($p = 0.406$) salivary concentrations, no statistically significant differences could be observed between median values.

Graph 1 shows the box plot for the optical density (OD) values obtained for immunoglobulins against *Streptococcus mutans*. Statistically significant differences were detected by the Mann-Whitney test for anti-*S. mutans* IgA between the young adult groups ($p = 0.013$). Caries-free young

adults presented higher IgA level in relation to young adults with caries.

Considering the children groups and OD values obtained for anti-*Streptococcus mutans* IgA, we can observe that statistically significant differences could be detected among the groups (ANOVA, Kruskal-Wallis, kw = 12.67; df = 2; p = 0.001). The multiple comparison test (Dunn's test 5%) showed that the OD values obtained for children with rampant caries were higher in relation to the other groups (Graph 1).

Spearman's rank order correlation test indicated no significant correlation between *mutans* streptococci counts and salivary IgA levels in all the studied groups (caries-free children, r = 0.20, p = 0.28; children with caries, r = 0.03, p = 0.81; children with rampant caries, r = –0.02, p = 0.93; caries-free young adults, r = 0.04, p = 0.84; young adults with caries, r = 0.02, p = 0.84).



GRAPH 1 - Box plot for optical density (OD) values obtained for anti-*Streptococcus mutans* IgA and studied groups. Mann-Whitney's test (young adult groups) and Kruskal-Wallis/Dunn's test (children groups) results obtained for the comparison among the groups. Different lowercase letters represent statistical significant difference by Dunn's test.

Mutans streptococci were isolated from all the saliva samples examined. The most frequently isolated species was *S. mutans* (94.53%). *S. sobrinus* isolates (5.47%) were also found (Table 1).

DISCUSSION

Similar values of OHI-S were observed between the groups of children with and without caries. The same was observed between the groups of young adults. This result reinforces the multifactorial characteristics of dental caries etiology. Moreover, we must consider the specific plaque hypothesis and the role of cariogenic microorganisms in the dental caries process[6]. According to this hypothesis, the composition of the dental biofilm is more important than its quantity for this disease's etiology. The group of children with rampant caries presented the highest OHI-S. The presence of caries lesions can lead to higher dental plaque retention and also more difficulty in the accomplishment of oral hygiene. In the group of children with rampant caries, *mutans* streptococci counts were similar in relation to the other groups. Among these patients, it is possible that lactobacilli but not *mutans* streptococci counts were raised, since these microorganisms are related to the progression of caries lesions[1,8].

Analyzing the data obtained for salivary flow evaluation we could observe that the mean values obtained are almost identical among the groups of children (caries free - 1.59 ml/min, with caries - 1.50 ml/min and with rampant caries - 1.58 ml/min). This is in accordance with the findings of Tukia-Kulmala, Tenovuo[23] (1993), who showed a value of 1.56 ml/min. Among the groups of young adults, the mean values were also similar (caries free - 2.03 ml/min and with caries - 2.09 ml/min).

The levels of IgA in saliva were significantly higher in children with rampant caries in relation

TABLE 1 - Number and percentage of *mutans* streptococci species isolated from each studied group.

Species of *Streptococcus*	Children n (%)			Young adults n (%)		Total
	Caries-free	With caries	With rampant caries	Caries-free	With caries	
S. mutans	256 (94.1)	104 (92.9)	97 (95.1)	54 (97.7)	163 (95.9)	674 (94.53)
S. sobrinus	8 (2.9)	7 (6.1)	-	-	5 (2.9)	39 (5.47)
Total	272	112	102	57	170	713

to the other children groups. Similar results were observed by Naspitz *et al.*[17] (1999). On the other hand, these results differ from the observations of Cämling *et al.*[4] (1987) and Bolton, Hlava[2] (1992). These authors related higher IgA anti-*Streptococcus mutans* antibodies in caries-resistant children in relation to caries-susceptible ones. These results are consistent with the view that children with rampant caries are not immunologically compromised, and that inadequate diet, host and microbiota-related factors may be mainly responsible for their clinical condition.

On the other hand, considering the young adult groups, the highest anti-*Streptococcus mutans* IgA level was observed among caries-free individuals. The difference of immunological response in children and young adult groups may be related to the age-dependent variations in IgA concentrations as reported by Tappuni, Challacombe[21] (1995). These authors observed lower concentrations of IgA in children in relation to adults. Our results lead us to question whether the immunological response to *S. mutans*, becoming more effective with age, could reach protective levels at the age of 18-25 years (young adults). If median values of anti-*S. mutans* levels are compared, the values observed for children with and without caries are similar (0.07), suggesting no protective effect. On the other hand, the group of caries-free young adults presented higher IgA levels in relation to the group of adults with caries (median values = 0.14 and 0.11, respectively).

No correlation could be observed between the counts of *mutans* streptococci in the saliva and levels of IgA. These results are in accordance with those of Cämling *et al.*[4] (1987). These authors stated that IgA to *Streptococcus mutans* did not reflect the quantity of *mutans* streptococci at the moment of saliva collection.

CONCLUSION

Our results suggest that the level of IgA to *Streptococcus mutans* in saliva did not reflect the salivary *mutans* streptococci concentrations.

A correlation between increased anti-*Streptococcus mutans* IgA levels and caries-free status was observed among young adults but not among children. IgA response was eminent when children were really infected by *mutans* streptococci, resulting in rampant caries. This may suggest a response reflecting the infectious nature of severe dental caries in contrast to the presence of small lesions.

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