Longitudinal Evaluation of Salivary Iga-S in Children with Early Childhood Caries Before and After Restorative Treatment

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Background: *Our aim was to compare salivary levels of secretory immunoglobulin A (s-IgA) in children with early childhood caries (ECCG) and those who are caries-free (CFG) and verify these levels in a follow-up period after restorative treatment.* ***Materials and methods:*** *We selected 46 systemically healthy children in the complete primary dentition period, who were allocated into two groups: CFG (n = 23) and ECCG (dmf-s > 0; n = 23). Unstimulated whole saliva was obtained at baseline from both groups and during the follow-up period (7 days, 1, 2 and 3 months) in the ECCG group. The s-IgA was measured using an ELISA assay, and total protein was assessed using the Bradford method. We also evaluated the flow rate (mL/min), Streptococcus mutans and Lactobacillus spp. counting using selective media plaques. The data were submitted to statistical analysis using the software SPSS 20.0 (SPSS Inc, IL, USA) with a confidence interval set at 95%.* ***Results:*** *Salivary s-IgA levels were higher in baseline of ECCG than in CFG ($p<0.05$). No statistically significant differences were observed between s-IgA salivary levels at baseline and the evaluations after dental treatment in ECCG ($p>0.05$). However, we observed two different changes in s-IgA levels among participants: one group presented s-IgA reduction, and the other group demonstrated its maintenance. It was shown that patients from the ECCG group who presented a reduction in s-IgA levels during follow-up also showed a decrease in Streptococcus mutans and Lactobacillus spp. count ($p<0.05$), in contrast to patients who did not present this reduction. The flow rate and total protein were similar between groups ($p>0.05$).* ***Conclusions:*** *The present data support the idea that children with early childhood caries present higher levels of s-IgA in saliva than caries-free children. The restorative dental treatment does not have a significant influence on salivary levels of this immunoglobulin during the follow-up period.*

Keywords: *Saliva; Dental Caries; Immunoglobulin A; Child, Preschool; Streptococcus mutans; Lactobacillus spp.*

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INTRODUCTION

Dental caries is one of the most common chronic diseases in childhood.[1] Although its frequency has decreased around the world in recent decades, there is still a high incidence in specific population groups, such as young children.[2, 3] Early childhood caries (ECC) is characterized by the presence of one or more decayed, missing or filled tooth surfaces due to caries in children aged up to 71 months.[4]

Among the risk factors related to the establishment of dental caries and its progression, socioeconomic factors, microbiota, diet, dental structure characteristics and fluoride consumption are well known.[5] There is limited knowledge of immunological protection factors, and studies on this area are extremely important for an understanding of the disease, thereby allowing the implementation of preventive measures.[6-8] In this context, saliva is a complex biofluid that plays an important role in modulation of the occurrence of dental caries. Among its many components, is the secretory immunoglobulin A (s-IgA), which is considered a specific local acquired defense mechanism in the oral cavity. Among its functions, s-IgA is able to neutralize viruses and bacteria and act in synergy with other innate antibacterial factors.[9] In addition to these functions, it is an antibody that is mainly related to the interference in microbial adherence to epithelial cells and tooth surfaces.[10]

Some studies have investigated the relationship between salivary levels of s-IgA and dental caries. Nevertheless, there are important methodological differences between the studies, and these studies mostly present a cross-sectional study design and a lack of longitudinal evaluations.[11-16] The s-IgA levels in the saliva of patients with caries are found to be higher in some studies,[11, 13, 16] while others have found the opposite results, with lower levels in patients with caries than in those who are caries-free.[12, 14, 15, 17] The present literature does not show strong evidence that elevated levels of this immunoglobulin in saliva protect against cariogenic microorganism colonization or that it is a result of a host immunological response to the microbiota.[18] In order to evaluate whether the s-IgA levels are a result of the immunological response against the disease, the aim of the present study was to investigate the s-IgA levels in whole saliva of ECC children in a follow-up after restorative dental treatment and compare the baseline levels with those of a group of caries-free children.

MATERIALS AND METHOD

After submission and approval by the local Ethical Committee in Research (approval protocol #242-14), we selected children aged between 24 and 71 months who were in the complete primary dentition period from those who attended the dental examination in the Pediatric Dental Clinic of the Federal University of Rio de Janeiro, in Rio de Janeiro, Brazil. It was selected 12 children with 2 years of age, 15 with 3 years, 7 with 4 years and 12 with 5 years, totalizing a sample of 46 participants. The exclusion criteria comprised the use of any systemic antibiotics in the previous 3 months, the presence of systemic disease, spontaneous gingival bleeding or lesions in the oral mucosa and patients who had already undergone restorative dental treatment, were using any orthodontic appliance or had a need for pulp treatment or extraction. The selected children were allocated into two groups according to the decayed-missing-filled surface (dmf-s) index,[19] which was assessed by two calibrated examiners (Kappa = 0.87). The tooth examination was performed in the dental chair, under an artificial light source using a disposable mouth mirror and explorer and the dental records were registered. The early childhood caries group (ECCG; n = 23) included children with at least one decayed surface (dmf-s > 0). The caries-free group (CFG; n = 23) was formed by children who had never had any history of dental caries (dmf-s = 0) and who also had no white spots.

In cases where the patients presented difficulties related to their behavior, especially the younger ones, the parents or legal guardians were asked to help to make the protective restriction of their movements, allowing the clinical procedures (dental examination, saliva collection and dental treatment, if necessary) could be performed.

Saliva Collection and Restorative Dental Treatment

Salivary samples were collected and the required time was set for salivary flow rate calculation (mL/min).[20] The collection of salivary samples in younger children was performed with them sitting in their guardian's lap. One milliliter of unstimulated, whole saliva was passively collected from the floor of the mouth using an automatic pipette and placed into a plastic, universal tube. Saliva sample from all children were taken at the same time (8:00 am to 10:00 am) to avoid fluctuation in the results due to the circadian saliva cycle. All participants received oral hygiene kits, and their legal guardians were instructed about oral hygiene and health dietary habits. ECCG patients received restorative dental treatment with composite resin to standardize the dental material, according to the manufacturer's instructions, and four follow-up saliva sample collections were performed: 7 days, 1 month, 2 months and 3 months after dental treatment. Children who presented any of the exclusion criteria during the follow-up period were excluded from the study.

Salivary Microbiological Analysis

Within a period of 2 hours after collection, the saliva samples were diluted in sterilized 0.85% NaCl to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, and 50 µL of each dilution was plated in duplicate on 10 mL of Mitis Salivarius Agar (Difco, Detroit, USA) with bacitracin for quantification of *Streptococcus mutans* and Rogosa (Difco, Detroit, USA) for the *Lactobacillus spp*. The plates were incubated in candle jars at 37 °C and after 48 hours, the microorganism colonies were counted. The results were expressed as colony-forming units per milliliter (CFU/mL). The remaining samples were stored at −80°C until immunological analysis.

Quantification of s-IgA

Saliva samples were analyzed using an indirect competitive immunoassay commercial kit for the quantitative measurement of s-IgA (SALIMETRICS Science Pro, Carlsbad, CA, USA), following the manufacturer's recommendations.

All kit components and saliva samples were brought to room temperature before use. The samples were homogenized by shaking and centrifuge at 1500 g for 15 minutes. Then, 25 µL of each sample was diluted in 100 µL of s-IgA diluent in appropriate tubes and incubated with 50 µL of diluted antibody-enzyme conjugate for 90 minutes at room temperature. After this, 50 µL from each tube was added to the microtiter plate and subsequently incubated at room temperature with continuous mixing at 400 rpm in a microprocessor shaker table (Quimis®, São Paulo, SP, Brazil) for 90 minutes. Then, each well was washed with a diluted wash buffer, and 50

µL of substrate tetramethylbenzidine (TMB) solution was added. The plate was mixed for 5 minutes at 500 rpm and incubated in the dark at room temperature for 40 minutes. After that, 50 µL of a stop solution was added in wells, the plate was mixed for more 3 minutes at 500 rpm and the optical density was read in a plate reader (SpectraMax Paradigm, Molecular Devices®, CA, USA) at 450 nm. A standard curve was obtained and salivary s-IgA was calculated and expressed in µg/mL.

Quantification of Total Protein

The total protein concentration in salivary samples was determined using bovine serum albumin for calibration. Varying concentrations of protein standards were prepared in the same buffer as the unknown samples. The standard curve was created by using a serial dilution of 1 mg/ml BSA protein standard. From each standard concentration, 5µL of protein standard was added to 150µL of Coomassie Brilliant Blue G-250, following the Bradford method (Sigma Aldrich, MS, USA).[21] The samples were incubated at room temperature for 45 minutes. To determine the protein concentration of the unknown samples, 5µL of protein standard was added to 150µL of Coomassie Brilliant Blue G-250, following the Bradford method (Sigma Aldrich, MS, USA) and the absorbance values were added to the standard curve. The samples were analyzed in duplicate with the use of Coomassie Brilliant Blue G-250, following the Bradford method. The optical density was read at 630 nm on a spectrophotometer (SpectraMax Paradigm, Molecular Devices®, CA, USA), and the results were expressed in µg/mL.

Statistical Analysis

The data were analyzed using SPSS 20.0 (SPSS Inc, IL, USA). Descriptive statistics were obtained, and the Shapiro-Wilk test of normality was applied ($p < 0.05$). The data were submitted to Mann-Whitney and Wilcoxon tests ($p < 0.05$).

RESULTS

The descriptive analysis of the sample showed that the demographic data and the reported daily use of fluoride toothpaste, sugar consumption and the use of a nocturnal nursing bottle did not differ statistically between the groups. The participants of ECCG presented an average age of 3.0 ± 1.0 years (varying from 2 to 5 years), 14 children (60.9%) were girls and the mean dmf-s was 10.2, varying from 1 to 32 affected surfaces. In CFG, the average age was 3.7 ± 1.2 years (varying from 2 to 5 years). Until two months after dental treatment, 4 dropouts and exclusions occurred, while 10 children were excluded from the study after three months follow-up, with 13 participants remaining at the end of the study. The main reasons were the consumption of antibiotics and the occurrence of new caries.

A similar flow rate was observed between ECCG (0.172 ± 0.1 mL) and CFG (0.185 ± 0.1 mL) at baseline and also among all evaluations in ECCG during the follow-up. The salivary concentration of total protein did not differ either between the ECCG (38.14± 24.89 µg/mL) or CFG (39.83 ± 23.19 µg/mL), nor did it differ in follow-up samples from ECC children.

Table 1 shows the salivary levels of s-IgA, *Streptococcus mutans* and *Lactobacillus spp.* in CFG and in ECCG at all evaluated timepoints. The subgroup of ECC with dmf-s ≥ 10 showed higher levels of s-IgA (55.20 ± 59.86 µg/mL) than the group with dmf-s < 10 (40.65 ± 22.20 µg/mL), but this difference was not statistically significant.

The s-IgA behavior was varied and complex, showing a wide variation in salivary levels between the participants. The individual trend of s-IgA levels of 16 representative patients from the ECC group, from baseline until the 3 month follow-up after treatment, is shown in Figures 1 and 2. Each line represents one child before and after dental treatment during the follow-up periods. Two groups could be observed; the first consisted of the ECC group which showed a significant reduction ($p < 0.05$) in the s-IgA levels (Figure 1) in all follow-up evaluations after dental treatment. This group also exhibited increased levels of oral microorganisms at baseline and a decrease in *Streptococcus mutans* count, demonstrated by each bar line that represents a child before and after dental treatment during the follow-up periods (Figure 3) and a reduction ($p < 0.05$) in *Lactobacillus spp*. levels after restorative treatment (Table 1). The other subgroup consisted of ECC children that did not show a clear reduction ($p > 0.05$) in s-IgA levels in the follow-up period (Figure 2) and did not show a reduction in *Streptococcus mutans* levels (Figure 4); however, this subgroup showed a reduction ($p < 0.05$) in *Lactobacillus spp*. levels after restorative treatment (Table 1).

Figure 1. Subgroup of children that showed a reduction in s-IgA levels during the follow-up after restorative dental treatment (7 days, 1 month, 2 months and 3 months). Each line represents one child before the treatment and after the follow-up periods.

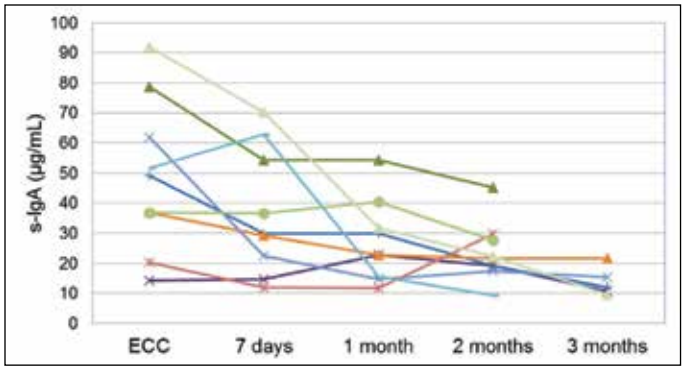


Figure 2. Subgroup of children that did not demonstrate reduction in s-IgA levels during the follow up period after restorative dental treatment. (7 days, 1 month, 2 months and 3 months). Each line represents one child before the treatment and after the follow-up periods.

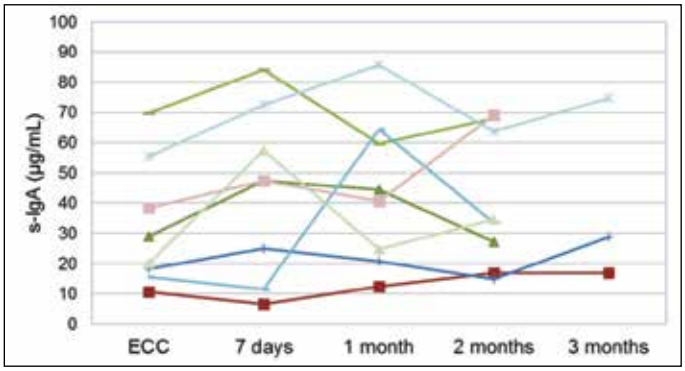


Figure 3. Streptococcus mutans count (CFU/mL in Log10 scale) shows a clear reduction after a 3 month follow-up. Each bar represents a child from the ECC subgroup that presented an s-IgA reduction at baseline and after the follow-up period.

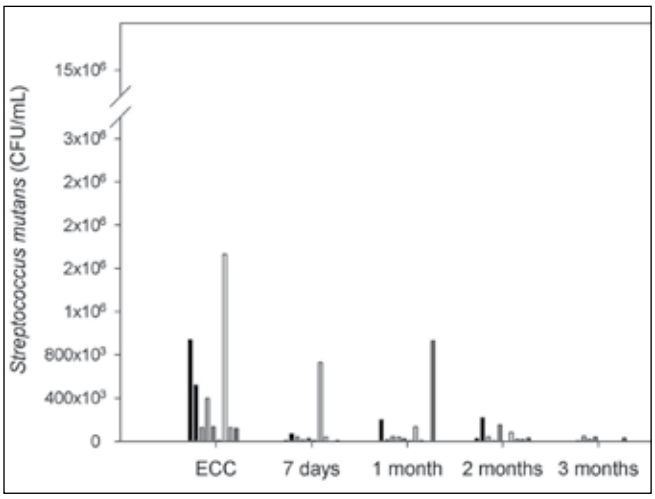
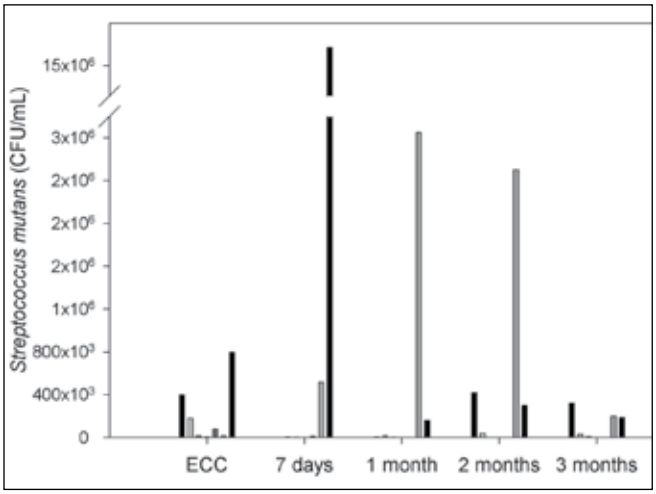


Figure 4. Streptococcus mutans count (CFU/mL in Log10 scale) showing no reduction after the 3 month follow-up. Each bar represents a child from the ECC subgroup that did not present s-IgA reduction at baseline and after the follow-up period.



DISCUSSION

The current research shows different behaviors of s-IgA levels in saliva. One subgroup of ECC children presented the maintenance of higher IgA levels, even after dental treatment. The cross-sectional studies in the literature that usually analyze the average of s-IgA values demonstrates an inter-individual variation in salivary s-IgA. In the present study, a high standard deviation was also found, especially in the baseline and after 3 months of follow-up. For that reason, it was opted to present the s-IgA levels for each child and analyze their behavior during the follow-up period. This may occur for different reasons, such as microorganism fluctuations rates, dmf-t and other factors.[11, 13, 16, 22, 23] Thus, it is believed that elevated levels of s-IgA in saliva are the result of stimulation of the local immune system by a carious process that is already present. This is supported by the fact that the secretion of salivary s-IgA is induced by the direct contact of oral antigens, for example, cariogenic microorganisms, on lymphoid tissue present in the salivary gland ducts.[9]

One limitation of this study is the dropouts. One of the most important difficulties when conducting longitudinal studies, especially in the pediatric population, is the occurrence of dropouts and exclusions. In the current study, the dropouts and exclusions were low after two months of follow-up. However, at the end of three months, half of the children were excluded from the study due to the need to begin antibiotic treatment or because of the occurrence of new caries during the follow-up period. Therefore, the validity and reliability of the findings of the present preliminary study is limited, due to the dropouts and the wide age distribution of the sample.

Studies that have assessed s-IgA levels in children with dental caries the literature present high inter-individual variability; in most cases, the s-IgA levels are expressed in mean and present high standard deviation.[11-16] Moreover, the lack of longitudinal studies and the large methodological differences between the research studies in this field were noted.[18] In the present research, the sample size and dropouts that happened during the follow-up evaluations may have occurred due to the restrictions in eligibility criteria to include the participants in the study in order to prevent possible bias in this research.

Cariogenic biofilm comprises a micro-ecosystem with physiological properties that favor colonization, such as adhesion and resistance to low pH levels.[24] *Streptococcus mutans* is an important

Table 1. Salivary s-IgA, Streptococcus mutans and Lactobacillus sp. levels of CF and ECC children before and after restorative treatment.

Group	s-IgA (µg/mL)	s-IgAp-value	Streptococcus mutans (CFU/mL)	Streptococcus mutansp-value	Lactobacillus spp. (CFU/mL)	Lactobacillus spp. p-value
ECC	46.89(± 41.94)	0.58a	3.0 × 105(± 4.1 × 105)	< 0.001a	1.1 × 104(± 2.7 × 104)	< 0.001a
7 day follow-up	40.87(± 30.68)	0.17b	9.1 × 104(± 1.9 × 104)	0.04b	7.9 × 102(± 1.3 × 103)	< 0.001b
1 month follow-up	33.70(± 20.25)	0.20c	2.4 × 105(± 6.4 × 105)	0.04c	8.6 × 102(± 2.6 × 103)	< 0.001c
2 month follow-up	31.46(± 18.90)	0.18d	2.0 × 105(± 5.6 × 105)	0.04d	5.6 × 102(± 1.9 × 103)	< 0.001d
3 month follow-up	32.94(± 32.16)	0.93e	6.1 × 104(± 9.5 × 104)	0.49e	2.8 × 102(± 5.6 × 102)	0.04e
Caries-free	25.40(± 15.44)	0.03f	2.9 × 105(± 5.4 × 105)	0.15f	1.1 × 101(± 5.0 × 101)	< 0.001f

Note: [a]Comparison between ECC and the 7 day follow-up; [b]Comparison between ECC and the 1 month follow-up; [c]Comparison between ECC and the 2 month follow-up; [d]Comparison between ECC and the 3 month follow-up; [e]Comparison between caries-free and the 3 month follow-up; [f]Comparison between ECC and caries-fr

microorganism involved in the initial process of dental caries, and its salivary levels have been shown to be a strong risk indicator for ECC.[25] Great inter-individual variability was observed in salivary levels of s-IgA and *Streptococcus mutans*. However, some participants from ECCG showed a pattern of reduction of these levels after the follow-up period, appearing to show a directly proportional relationship between them. *Lactobacillus spp*. is associated with the progression of caries, and its level was reduced after dental treatment, in all ECC children.

Our data showed a slight decrease in mean s-IgA levels from baseline until the follow-up 2 months later. Other longitudinal studies reported opposite results, presenting an increase of s-IgA levels in the follow-up evaluation both for total s-IgA and *Streptococcus mutans* protein antigens.[22] However, this study made a unique sample collection 1 year after the baseline period, and both caries-active and caries-free groups presented s-IgA higher values in the follow-up than at baseline. This may have also occurred because the participants were in the mixed dentition period, and the inflammatory local changes could have influenced the observed results.[22] This explanation is not applicable to our findings since it was included only children in primary dentition. The need for more longitudinal studies is evident, in order to determine whether the increased levels of s-IgA act as a protective response or as a consequence of the imbalance caused by the established disease.

Previous studies only compared data between groups with and without dental caries. In contrast, we opted to analyze the s-IgA levels individually at all evaluation points. Although all participants were submitted to dental treatment and had their cavities filled, we also observed that children with ECC who presented a reduction pattern in s-IgA levels after dental treatment also exhibited a decrease in the *Streptococcus mutans* count during this period. In addition, those children who did not have reduced s-IgA levels also presented varying levels of *Streptococcus. mutans*. It seems that the immunological system is directly associated with microorganism levels, especially *Streptococcus mutans*. The analysis proposed here may be key to explaining the large inter-individual variations observed, since there were wide differences between the levels of cariogenic microorganisms and the immunological response, this may interfere more than the clinical presence or absence of dental caries.

CONCLUSION

The present data support the idea that children with early childhood caries present higher levels of s-IgA in saliva than caries-free children. The restorative dental treatment does not have a significant influence on salivary levels of this immunoglobulin during the follow-up period.

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