



Increased activity of the antioxidants systems modulate the oxidative stress in saliva of toddlers with early childhood caries



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ABSTRACT

Objective: This study aimed to evaluate the oxidative stress levels and the enzymatic and non-enzymatic antioxidant systems in saliva of toddlers with severe early childhood caries (S-ECC).

Design: Unstimulated saliva samples were collected at the morning from 0 to 3 year-old S-ECC (n = 30) or caries-free (CF) children (n = 30/group) for evaluation of oxidative stress (OS) and total antioxidant capacity (TAC), which were measured by the ferric reducing antioxidant power (FRAP) assay, as well as to assess the activity of enzymatic (superoxide dismutase, SOD) and non-enzymatic (uric acid, UA) antioxidant systems, respectively. Data were analyzed by Student's *t*-test ($p < 0.05$).

Results: Significantly higher protein levels were observed in saliva of S-ECC children (0.083 mg/mL) than in the CF group (0.070 mg/mL). Oxidative damage was significantly lower in saliva of S-ECC children (0.0019 μ mol/L/mg protein) than in CF children (0.0039 μ mol/L/mg protein), while salivary TAC (61.5 μ mol/L), SOD activity (36.6 UE/mL) and uric acid (7.05 mg/mL) were significantly higher in saliva of S-ECC when compared to the CF group (49.1 μ mol/L, 26.8 UE/mL and 5.02 mg/mL, respectively for TAC, SOD and UA).

Conclusion: Oxidative stress levels were significantly lower in saliva of S-ECC children, what might be associated with the increased activity of salivary enzymatic (SOD) and non-enzymatic (uric acid) antioxidant systems.

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1. Introduction

Severe early childhood caries (S-ECC) is one of the most common oral diseases in children. S-ECC is defined by the European Academy of Pediatric Dentistry as the presence of one or more primary decayed tooth (cavitated lesions or not), missing (due to caries) or restored tooth surfaces before 71 months of age (Vadiakas, 2008), while the American Academy of Pediatric Dentistry defines as severe S-ECC as any sign of smooth-surface caries in children younger than three years of age (American

Academy of Pediatric Dentistry, 2014). High prevalence rates of S-ECC have been reported in developed and in developing countries, ranging from 46 to 96% in 3–7-year-old children, reaching levels up to 17% in 0–3-year-old children from low-income communities or where the access to dental services is difficult by political, economic and social factors (Grund, Goddon, Schüller, Lehmann, & Heinrich-Weltzien, 2015; Warren et al., 2016).

Biomarkers of oxidative damage are found in saliva as 8-hydroxy-desoxguanosine (8-Hodgkins) and malondialdehyde (MDA). Enzymatic antioxidants – glutathione peroxidase (GPx) and superoxide dismutase (SOD) – can be measured in saliva and other biological fluids, similarly as for the non-enzymatic antioxidant systems, which include uric acid (UA) and glutathione (GSH) and comprise the non-enzymatic total antioxidant capacity (TAC) (Battino, Ferreiro, Gallardo, Newman, & Bullon, 2002). Oxidative

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stress is attributed to an imbalance between free radical production, as reactive oxygen species (ROS), and the activity of enzymatic and non-enzymatic antioxidant systems, which are a powerful defense of body against damages caused by free radicals (Kirschvink, De Moffarts, & Lekeux, 2008; Tunes et al., 2007).

Increased oxidative damage biomarkers have been observed in saliva of individuals presenting periodontal disease or dental caries (Battino et al., 2002; Tóthová, Celecová, & Celec, 2003). Conversely, TAC of stimulated saliva has been shown to be significantly decreased in patients with periodontal disease (Diab-Ladki, Pellat, & Chahine, 2003), and in saliva of patients with peri-implant disease (Liskmann et al., 2007). However, unlike what would be expected, the levels of TAC were significantly higher in saliva of children, adolescents and adults presenting carious lesions in comparison with caries-free subjects (Ahmadi-Motamayel, Goodarzi, Hendi, Kasraei, & Moghimbeigi, 2013; Hegde, Rai, & Padmanabhan, 2009; Kumar, Pandey, & Agrawal, 2011; Mahjoub, Ghasempour, Gharage, Bijani, & Masrourroudsari, 2014; Hegde, Hegde, Ashok, & Shetty, 2013). These results indicate that the relationship between oxidative stress and the antioxidant systems in saliva of individuals with caries activity is not fully understood. In fact, the association between TAC and oxidative damage has not yet been evaluated in S-ECC children. In addition, no data is available on the role of non-enzymatic antioxidant system, such as uric acid, in saliva of patients with caries. Finally, and most importantly, while studies have reported isolated data on the effects of oxidative damage and TAC in S-ECC children, these aspects have not been collectively evaluated in the same group of children, so that the relationship between these variables could not be determined.

Therefore, this study aimed to evaluate the oxidative stress levels and the enzymatic (SOD) and non-enzymatic (uric acid) antioxidant systems in saliva of children in early childhood (0–3 years old) presenting S-ECC. The study's hypothesis was that oxidative stress levels and enzymatic and non-enzymatic antioxidant systems would be increased in saliva of children with caries lesions.

2. Materials and methods

2.1. Patient selection

The research protocol was approved by the Human Ethics Committee of Araçatuba Dental School, UNESP- Univ. Estadual Paulista (Permission Number CAAE 36416414.5.0000.542). Children at the age range of 0–3 years were selected from public kindergartens in the city of Araçatuba, State of São Paulo, Brazil. An initial meeting was conducted with directors of kindergartens and parents of the children to explain in details the study protocol and to answer to possible questions raised by the attendants. Following, free and informed consent forms were distributed to all parents/caregivers, and those who were unsigned entered as exclusion criteria, along with children presenting systemic diseases. From the signed informed consent forms returned to the researchers, clinical examinations were performed in 100 children, for the determination of dmfs index based on World Health Organization recommendation (WHO, 1997), performed by a calibrated dentist (PVS). Subsequently, sixty children were randomly enrolled in the study (blocking stratification), comprising 30 subjects with severe early childhood caries (S-ECC group) and 30 caries-free (CF group) (Vadiakas, 2008).

2.2. Saliva collection

To minimize possible variation due to circadian rhythm, unstimulated whole saliva was collected between 7:00 am to

8:30 am, 2 h after fasting and oral hygiene with water and toothbrush without fluoride products. All salivary samples were collected by the same investigator within kindergartens during 5 min, using a Salivette[®] (Sarstedt, Germany). Samples were kept on ice during collection and then were centrifuged at 5500g for 10 min as previously described (Cunha-Correia, Neto, Pereira, Aguiar, & Nakamune, 2014). The supernatants were fractionated and kept at -80°C until analysis.

2.3. Determination of total protein concentration

Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951) with bovine serum albumin used as standard. The absorbance was determined at 660 nm. The results were expressed in mg/mL.

2.4. Measurement of malondialdehyde (MDA)

MDA is one of the products of lipid peroxidation evaluated by the method thiobarbituric acid-reactive substances (TBARS), which has been considered a biomarker of oxidative stress. MDA was determined as described by Buege and Aust (Buege and Aust, 1978). Trichloroacetic acid (10% w/v) was added to the saliva samples (125 μL) to precipitate proteins and to acidify the reaction solution. This mixture was then centrifuged (1000g, 3 min) and thiobarbituric acid (TBARS, 0.67% w/v) was added to the reaction medium. The sample was placed in a water bath (100°C , 15 min). The absorbance was read at 535 nm, the molar absorption coefficient used was $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The results are expressed $\mu\text{mol/L/mg}$ protein.

2.5. Salivary total antioxidant capacity

Salivary total antioxidant capacity was assessed by ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). This method is based on reducing the ferric complex tripyridil triazine (Fe^{3+} TPTZ) to form Fe^{2+} in acidic medium. An aliquot of saliva (15 μL) was used and the absorbance was determined at 595 nm, using a standard curve of ferrous sulfate. The results are expressed in $\mu\text{mol/L FeSO}_4$.

2.6. Superoxide dismutase (SOD) activity

SOD activity was determined in saliva by the method of Maklund (1985) based on the inhibition of the pyrogallol autoxidation, using an aliquot of saliva (20 μL) previously diluted in tris (1:10 v/v). Absorbance was detected at 420 nm. The amount of enzyme required to inhibit 50% of the autoxidation of pyrogallol was considered as a unit of enzyme activity. Results are expressed as UE/mL.

2.7. Uric acid

Uric acid was determined in saliva using a commercial kit (Labtest Diagnóstica SA, MG, Brazil) based on enzymatic Trinder method, following the manufacturer's instructions. The results are expressed in mg/ml.

2.8. Statistical analysis

Data are expressed as mean \pm SD (Standard Deviation). Statistical analysis of the results was performed using independent Student *t*-test (Graph Pad Prism, 5.0 version). Values of $p < 0.05$ were considered as statistically significant.

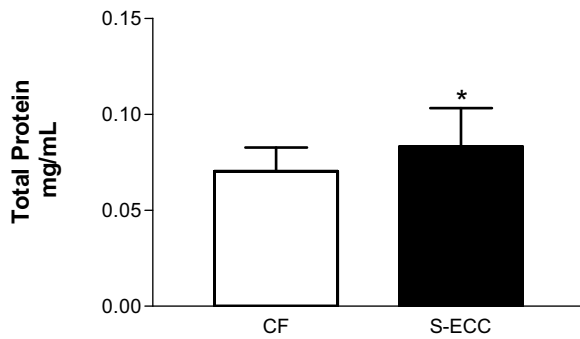


Fig. 1. Total protein (mg/mL) in saliva of caries-free (CF) and severe early childhood caries (S-ECC) groups. Bars represent mean \pm SD. * $p < 0.05$ (Student's *t*-test).

3. Results

Sixty children aged 0–3 years participated in this study, twenty-nine boys and thirty one girls. Within the S-ECC group ($n = 30$), 47% and 53% were boys and girls, respectively, while the corresponding percentages for the CF group ($n = 30$) were 53% and 47%, respectively. Mean number of cavitated surfaces (dmfs) in the S-ECC group was 3.7 ± 3.44 .

3.1. Salivary total protein

Salivary total protein was significantly higher ($p < 0.01$) in the S-ECC group (0.083 ± 0.020 mg/mL) when compared to CF group (0.070 ± 0.012 mg/mL) (Fig. 1).

3.2. Salivary MDA levels

Salivary MDA levels were significantly lower ($p < 0.0001$) in the S-ECC group (0.0019 ± 0.0008 $\mu\text{mol/L/mg}$ protein) than in CF group (0.039 ± 0.0021 $\mu\text{mol/L/mg}$ protein) (Fig. 2).

3.3. TAC in saliva evaluation

TAC in saliva was significantly higher ($p < 0.05$) in S-ECC group (61.5 ± 20.18 $\mu\text{mol/L}$) compared to the CF group (49.1 ± 23.24 $\mu\text{mol/L}$) (Fig. 3).

3.4. Enzymatic salivary antioxidant activity

SOD activity (36.6 ± 22.79 UE/mL) was significantly higher in S-ECC ($p < 0.05$) when compared to the CF group (26.8 ± 9.09 UE/mL) (Fig. 4).

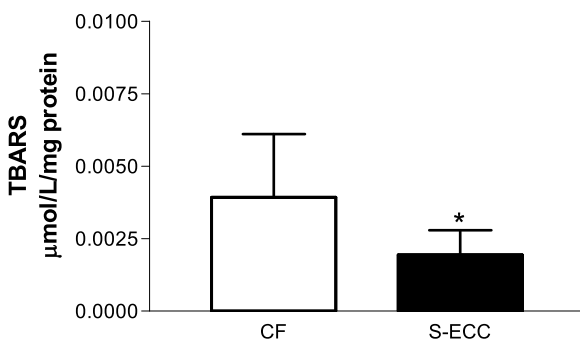


Fig. 2. Concentration of 2-thiobarbituric acid-reactive substances (TBARS, in nmol/mg protein) in saliva caries-free (CF) and severe early childhood caries (S-ECC) groups. Bars represent the mean \pm SD. * $p < 0.05$ (Student's *t*-test).

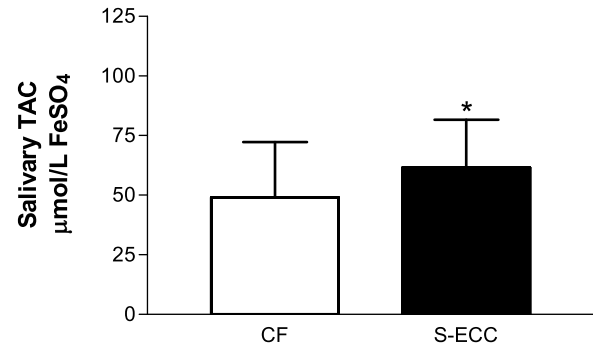


Fig. 3. Total Antioxidant Capacity (TAC, $\mu\text{mol/L FeSO}_4$) in saliva of caries-free (CF) and severe early childhood caries (S-ECC) groups. Bars represent the mean \pm SD. * $p < 0.05$ (Student's *t*-test).

3.5. Non-enzymatic salivary antioxidant activity

Salivary uric acid values were significantly increased ($p < 0.0001$) in S-ECC group (7.05 ± 1.40 mg/mL) than in the CF group (5.02 ± 1.61 mg/mL) (Fig. 5).

4. Discussion

In this study, oxidative damage levels were significantly lower in saliva of S-ECC children, while increased activity of salivary enzymatic and non-enzymatic antioxidant systems were increased in this group of children. Therefore, the study's hypothesis was partially accepted. Since no study had assessed oxidative stress and

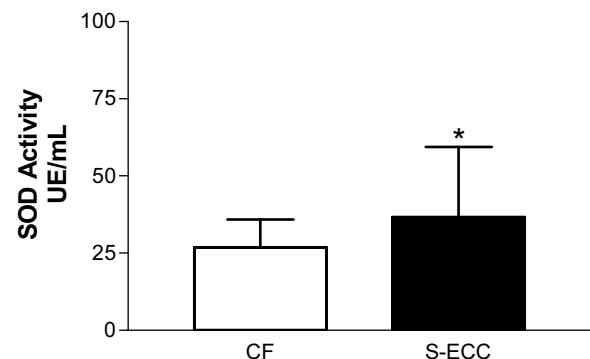


Fig. 4. Superoxide dismutase (SOD) activity (UE/mL) in saliva caries-free (CF) and severe early childhood caries (S-ECC) groups. Bars represent the mean \pm SD. * $p < 0.05$ (Student's *t*-test).

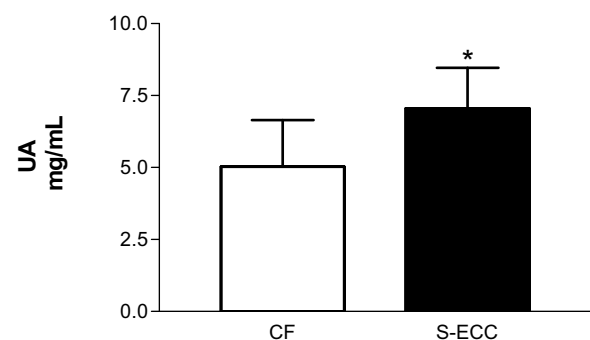


Fig. 5. Uric acid (UA, in mg/mL) in saliva of caries-free (CF) and severe early childhood caries (S-ECC) groups. Bars represent the mean \pm SD. * $p < 0.05$ (Student's *t*-test).

antioxidant system salivary profile in S-ECC children, the present findings bring novel information to the field.

The concentration of salivary total protein was higher in S-ECC children than in CF group. These results are in line with previous data demonstrating increased concentration of total protein in saliva of children and adults diagnosed with pneumonia (Kremer, Kuzminsky, Bentur, & Nagler, 2014), cystic fibrosis (Livnat, Bentur, Kuzminsky, & Nagler, 2010), and in children with severe caries (Hegde et al., 2009). It is possible that the higher protein concentration in saliva of S-ECC children would be a protective and/or an adaptive response of the body against dental caries, given that salivary proteins have a protective role of oral tissues by forming a film of protein against enamel wear, preventing the adherence and growth of microorganisms, promoting the remineralization of enamel by attracting calcium ions, and reducing enamel demineralization in association with salivary calcium and phosphate ions (Amerongen, Bolscher, & Veerman, 2004).

Reduced levels of TBARS were observed in saliva of S-ECC children when compared to CF children, suggesting reduced oxidative damage in the saliva of children presenting caries lesions. These results are in agreement with a previous work conducted with caries-active adults (17–50 years old) (Öztürk et al., 2008; Sarode, Shelar, Sarode, & Bagul, 2012), and patients (16–46 years old) with periodontal disease and gingivitis (Celec et al., 2005; Almerich-Silla et al., 2015). However, variable levels of salivary OS of adults have been reported when associated to oral infectious diseases. No difference in salivary MDA levels (Öztürk et al., 2008) was observed, or higher lipid peroxidation was detected in saliva of adults with active caries than in caries-free group (Rai, Jain, & Anand, 2006). Moreover, the increased OS, which was observed in saliva of patients with oral lichen planus (25–75 years old), squamous cell carcinoma (34–82 years old) or periodontal disease, was associated with a reduced salivary TAC (Agha-Hosseini, Mirzaii-Dizgah, Farmanbar, & Abdollahi, 2012; Baltacioglu et al., 2014; Sculley & Langley-Evans, 2003).

Salivary TAC in S-ECC group was significantly higher than in CF group in the present study, in line with previous reports in children (3–5 years old) from North Iran (Mahjoup et al., 2014). In addition, a strong correlation between the number of decayed teeth and increased salivary TAC in children (3–5 years old) of India has been reported (WHO, 1997). An indirect correlation between reduced salivary pH and increased salivary TAC was observed in children (3–5 years old) (Muchandi et al., 2015) from India. Together, these results corroborated the suggestion that high salivary TAC is a biomarker of caries in children (Tulunoglu, Demirtas, & Tulunoglu, 2006), adolescents (Ahmadi-Motamayel et al., 2013) and adults (Hegde et al., 2009). As recently suggested (Tóthova, Kamodyová, Cervenka, & Celec, 2015), salivary markers of oxidative stress have the potential to be used for screening and monitoring oral diseases, including dental caries. The evaluation of these markers in saliva could be an adjuvant tool for identification of patients with poor adherence to dental visits. Furthermore, the study of such biomarkers could bring useful information for the development of novel therapies for the prevention and/or treatment of dental caries. Such markers, however, should be validated in future studies conducted with a larger number of subjects and taking into account varying levels of caries.

Based on the considerations above, we assessed whether TAC would involve the action of antioxidants systems in saliva of children presenting ECC, by evaluating the concentration of uric acid, which has been assessed in saliva of patients as a non-enzymatic antioxidant biomarker. It has been suggested that the increase in uric acid levels in saliva may influence TAC levels (Kumar et al., 2011) since uric acid is responsible for around than 70% of salivary antioxidant capacity (Nagler, Klein, Zarzhevsk, Drigues, & Reznick, 2002). While uric acid was assessed in saliva of

patients with cystic fibrosis (Livnat et al., 2010), chronic periodontitis (Miricescu et al., 2014) and in patients with cleft lip (Aizenbud, Peri-Front, & Nagler, 2008), to the best of our knowledge uric acid had not been previously assessed in saliva of S-ECC children. In the present study, significantly higher uric acid antioxidant response was observed in saliva of S-ECC group, which contributed to the increase in salivary TAC levels of these children. A significant increase in SOD activity in saliva of the S-ECC group was observed in the present study, which might also have contributed to the lower oxidative damage in these children. The evaluation of saliva of caries active adults (25–50 years) also demonstrated increased levels of SOD and its cofactors, copper and zinc (Hegde, Hegde, Ashok, & Shetty, 2014). Previous studies observed higher SOD activity in patients with cleft lip and with cystic fibrosis (Livnat et al., 2010). SOD activity, which has been considered as the first line of enzymatic antioxidant defense in the organism, catalyzes the dismutation of superoxide ion (an oxygen free radical) into oxygen and hydrogen peroxide (Varvara, Traini, Esposito, Caputi, & Perinetti, 2005).

Since saliva can be used for long-term monitoring oral diseases and considering the lack of conclusive studies about oxidative stress in saliva of caries active subjects (Tóthova et al., 2015), our results suggest a salivary profile of ECC children: increased salivary proteins, reduced oxidative damage, increased TAC or FRAP and uric acid, and increased SOD activity. The reasons for the increased non-enzymatic or enzymatic antioxidant status in saliva of caries-active subjects, however, remain unclear. Given that their function is to protect oral tissues against the deleterious effects of endogenous or exogenous oxygen or nitrogen reactive species, our results suggest that the increased UA and SOD activity might be a compensatory mechanism to reduce oxidative damage.

Based on the results of the present study, we can conclude that oxidative damage is reduced in saliva of S-ECC children as a consequence of the increased non-enzymatic and enzymatic antioxidant systems.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Ethical approval

The Research Protocol was approved by the Human Ethics Committee of Araçatuba Dental School, UNESP – Univ Estadual Paulista (Permission Number CAAE 36416414.5.0000.542).

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