Original Article

Correlation of Carbonic Anhydrase VI Enzyme, Total Proteins, Antioxidant Levels of Saliva and Dental Caries in Caries-Free and Caries-Active Children – A Case–Control Study

Anjana Cherath Vasudevan[1], Nekkanti Sridhar[1], Vinutha Bhat[2], Meghana Bhat[2]

[1]Department of Pediatric and Preventive Dentistry, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, India, [2]Department of Biochemistry, Kasturba Medical College, Manipal Academy of Higher Education, Manipal, Karnataka, India

Abstract

Background and Objectives: Factors in the biofilm influence the induction and advancement of the carious process. This study was done to relate and assess the levels of enzyme carbonic anhydrase VI, total protein, and antioxidants (superoxide dismutase [SOD], catalase, lipid peroxidase, and uric acid) in caries-free and caries-active children. **Methods:** This case–control study comprised 60 children of age group 6–12 years who were evaluated for decayed missing filled teeth (dmft)/DMFT criteria and distributed into two groups: Group 1 – caries active (case) and Group 2 – caries free (control) for saliva collection with 30 participants in each of the above groups. Stimulated saliva was obtained, and the samples were then evaluated using biochemical lab tests. The data were then statistically evaluated using independent *t*-test. **Results:** Catalase in the caries-free group was significantly higher, but the concentration of carbonic anhydrase (CAVI) enzyme, total protein, and other antioxidant enzyme activity was enhanced in caries-active children in which uric acid demonstrated a statistically significant difference with higher levels in caries-active group. **Conclusion:** There is an increased concentration of CAVI enzyme in caries-active group and total protein showed a linear relation with caries activity. Antioxidant parameters such as SOD and lipid peroxidase were increased with caries activity. Uric acid was significantly higher in the caries-active group, whereas catalase showed an indirect relation with dental caries. Significant variations in the levels of these parameters imply that the levels of these components of saliva can act as strong markers of caries status in children.

Keywords: *Antioxidants, carbonic anhydrase enzyme, dental caries, proteins, saliva*

Introduction

Dental caries is the result of disintegration of hard tissues due to the microflora and other factors in the biofilm contributing to the cariogenicity. These elements influence the induction and advancement of the carious process.

Whole saliva which is an alkaline secretion of water containing enzymes, proteins, and inorganic substances adversely affects the oral conditions including the tooth surfaces when produced in minimum quantities compared to normal.[1] The first line of protection against free radical (FR) oxidative stress is assumed to be saliva which is primarily done by the phosphate and bicarbonate systems.[2] Oxidative stress is defined as a disruption in the balance between the synthesis and accumulation of reactive oxygen species (ROS) in cells and tissues, as well as the body's ability to remove these reactive products.[3]

Oxidative stress biological markers are balanced in saliva so levels of these will indicate certain caries-specific oxidation pathways.[4]

This salivary buffering potential is a very important factor for caries protection. The salivary isoenzyme carbonic anhydrase VI (CAVI) formed by parotid and submandibular serous acinar cells catalyzes the reversible reaction of carbon dioxide, thus increasing the buffering potential causing acid neutralization:-

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3.$$ [5]

Acids produced by plaque bacteria are buffered by the bicarbonates inhibiting the demineralization where the CAVI accumulates on the surface of hydroxyapatite crystals enabling acid neutralization.

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Address for correspondence:
Dr. Nekkanti Sridhar, Room No: 6, Department of Pediatric and Preventive Dentistry, Manipal College of Dental Sciences, Manipal Academy of Higher Education, Manipal - 576 104, Karnataka, India.
E-mail: drsri.pedo@gmail.com

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The principal cause of FRs is physiological metabolism. Saliva and its antioxidants are being investigated as redox status regulators of the oral cavity under normal and diseased conditions. Peroxidase, catalase, superoxide dismutase (SOD), and glutathione peroxidase form the major part of the antioxidant system of saliva with minor constituents as uric acid and Vitamin E, C.[6] Earlier studies have shown that with increasing caries conditions, the total antioxidant levels also increased instantaneously.[7] These antioxidants can be enzymatic and nonenzymatic compounds which avoid complications caused by additional oxidants generated by oxidation reactions in the body.

The disproportionation of the superoxide anion FR (O_2-) into molecular oxygen and hydrogen peroxide (H_2O_2) is caused by SOD. It also reduces O_2^-level which destroys the cells at excessive concentration.[8]

Saliva also has several proteins that act on plaque and bacteria by many strategies, decreasing tooth predisposition to dental caries. Lysozyme, lactoferrin, and salivary peroxidase are found to be contributing to restoring soft tissue and inhibiting oral infections. Tulunoglu *et al*. found a positive association in which total protein coupled with total antioxidant potential increased with the progression of caries.[9]

Although many studies have shown the major properties of saliva and their role in the pathogenesis of caries, very few have specifically shown its effectiveness in the caries risk assessment and diagnosis in children. Therefore, the assessment of salivary factors that may enhance the risk of tooth decay will surface the way for making recommendations that would serve the needs of an individual in particular. Existing literature has also not shown the variation of individual antioxidant components, correlating their functions with isoenzymes and proteins in carious lesions. Taking into consideration the complex interplay described above, this study was done to compare and analyze the levels of enzyme carbonic anhydrase VI, total protein, and antioxidants (SOD, catalase, lipid peroxidase and uric acid) in caries-free and caries-active children. The formulated hypothesis for the study is that there exists a definite relationship of enzyme carbonic anhydrase VI, total proteins, and antioxidants (SOD, catalase, lipid peroxidase and uric acid) levels with the caries activity.

Materials and Methods

The present study was undertaken in the Department of Pedodontics and Preventive Dentistry, Manipal College of Dental Sciences, Manipal, Karnataka, in collaboration with the Department of Biochemistry, Kasturba Medical College, Manipal, Karnataka.

The study duration was 2 years which was approved by Kasturba Hospital Ethics Committee (IEC Number 826/2018) and registered with Clinical Trials Registry-India (CTRI registration number: CTRI/2019/01/017135).

The estimation of sample size was completed with the help of Department of Biostatistics, Manipal Academy of Higher Education, and using the formula:

$$\frac{2(Z_{1-\alpha/2}+Z_{1-\beta})^2\,\sigma^2}{d^2}$$

- α =5% is the level of significance
- $Z_{1-\alpha/2} = 1.96$
- $Z_{1-\beta} = 0.84$
- $\sigma = 0.545$
- d (clinically significant difference) = 0.4

Assuming the design effect of the two groups in this case–control study, the required total sample size was 60, with 30 participants each, Group 1 – caries-active group and Group 2 – caries-free group, respectively.

Children aged 6–12 years having enamel caries with decayed missing filled teeth (DMFT)/dmft >5 were included in Group 1 and caries-free children with DMFT/dmft = 0 were included in Group 2. Healthy children with a good record of general health and those who were not taking any medications were selected for the study after obtaining informed consent.

Children having deep dentinal caries and pulpally involved teeth, children with abscess due to dental caries, children with salivary gland disorders, those with special health care needs, recent antibiotics exposure (last 3 months), poor oral hygiene, and presence of periodontal disease were excluded from participating in the study.

Consent forms were issued to be signed along with patient information sheets by the parents of the children who were willing to participate in the research. After obtaining written consent from parents, a clinical examination was carried out**.**

Caries assessment

The DMFT/dmft index given by Henry Klein, Carole Palmer, and Knutson JW was used to estimate the prevalence of caries.[10] After rinsing and drying the teeth with gauze, the diagnosis of dental caries was conducted by visual and tactile examination technique using mouth mirror, No. 23 explorer, and a flashlight. Current nation's recommendations and standards were followed for both infection control and waste disposal.[11] Disposable masks and gloves were used.

Following clinical examination, proformas with indices were filled and the children were categorized into 2 groups: Group 1 caries-active and Group 2 caries-free population.

Collection of saliva samples

Samples were obtained between 10 and 11:30am during the morning hours. The participants were instructed not

to eat or drink anything 1 h earlier to sample collection. Each child was asked to chew a paraffin wax block of 1 cm diameter for 6 min**.** The initial pooled saliva was discarded and the child was asked to expectorate 6 ml of the pooled saliva into collection funnel placed in a sterile graduated jar during the next 5 min which was then shifted to sterile Eppendorf tubes, which were transported to the laboratory within 30 min of collection**.**

Estimation of carbonic anhydrase VI enzyme

The assessment of the CAVI concentration in saliva was done using an ELISA kit for CAVI (ELISA kit E-EL-H0474 96 tests, Elabscience Biotechnology Inc., USA) in accordance with the manufacturer's guidelines [Figures 1 and 2].

Estimation of catalase

Three milliliter of H_2O_2 PBS solution was mixed with 50 µL of saliva and reading at zero time (absorbance at 240 nm) using cuvette was taken.

Estimation of superoxide dismutase

One thousand eight hundred and fifty microliter of sodium carbonate buffer was mixed with 50 uL of saliva and 100 uL of Adrenaline was included directly in the cuvette after putting in ultraviolet mode. The absorbance at 480 nm (A_0–A_{60}) (kinetic method) was taken.

Estimation of lipid peroxidase

A mixture of 0.5 ml of saliva, 2.5 ml of thiobarbituric acid, trichloroacetic acid, and butylated hydroxyl toluene was heated at 90°C for 10 min. The mixture was centrifuged for 5 min at 2000 rpm and absorbance was taken at 525 nm. Lipid peroxidation was expressed as nanomoles (nM) of malonaldehyde form per ml of saliva.

Estimation of uric acid

The uric acid concentration test in saliva was performed using the uric acid reagent kit following the instructions from the manufacturer.

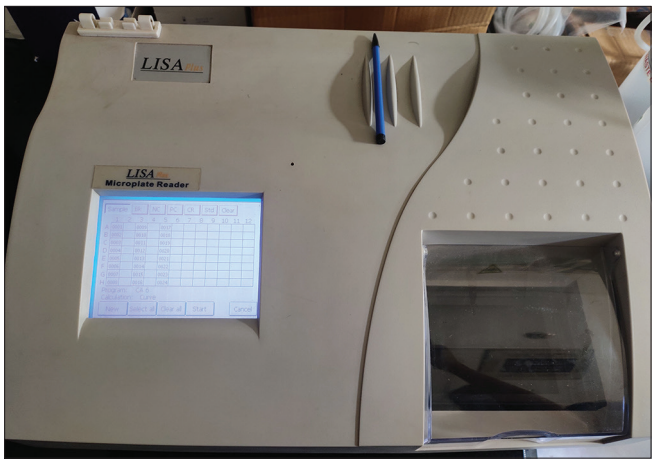

Figure 1: ELISA microplate reader

Uricase generated allantoin and H_2O_2 from uric acid. Through the catalytic action of peroxidase, the H_2O_2 produced further reacted with a phenolic compound and 4 aminoantipyrine to form a red colored quinoneimine dye complex. The color intensity was directly proportional to the amount of uric acid contained in the sample.

Estimation of total protein

Proteins along with copper ions formed an alkaline solution of the violet–blue color complex. The absorbance of the color was directly proportional to the overall protein content in the specimen.

$$\text{Protein} + Cu^{++} \xrightarrow{\text{Alkaline pH}} \text{BlueViolet complex}$$

Statistical analysis

Data were analyzed using the statistical package SPSS 22.0 (SPSS Inc., Chicago, IL, USA) and the level of significance was set at $P < 0.05$. Descriptive statistics was performed to assess the mean and standard deviation of the respective groups. Normality of the data was assessed using Shapiro–Wilkinson test. Inferential statistics to find out the difference between the groups was done using independent -test.

Results

This study was conducted to compare and evaluate the levels of enzyme carbonic anhydrase VI, total protein, and antioxidants (SOD, catalase, lipid peroxidase, and uric acid) in caries-free and caries-active children.

Carbonic anhydrase VI enzyme concentration in saliva

The concentration of CAVI isoenzyme was higher in the caries active group (1925.54 ± 1398.57) compared to caries free group (1444.17 ± 1039.81). This difference was not statistically significant ($P = 0.135$) [Table 1].

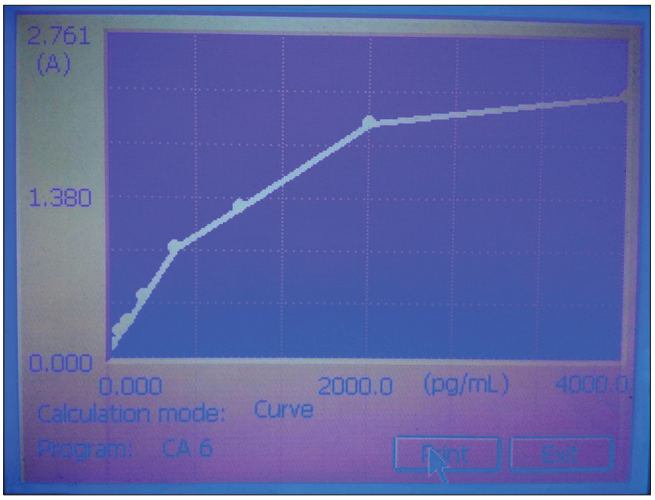

Figure 2: Standard graph for human carbonic anhydrase VI concentration (pg/ml)

Concentration of total protein in saliva

The concentration of total protein was higher in the caries active group (1.723 ± 1.943) compared to caries free group (1.429 ± 1.284). This difference was not statistically significant (P = 0.492) [Table 2].

Lipid peroxidase activity in saliva

The activity of lipid peroxidase was higher in the caries active group (0.0089 ± 0.0171) compared to caries free group (0.01423 ± 0.01344). This difference was not statistically significant (P = 0.184) [Table 3].

Concentration of uric acid in saliva

The concentration of uric acid was higher in the caries active group (1.498 ± 0.664) compared to caries free group (0.97 ± 0.164). This difference was statistically significant (P = 0.001) [Table 4].

Superoxide dismutase activity in saliva

The activity of SOD was higher in the caries active group (0.027 ± 0.021) compared to caries free group (0.015 ± 0.029). This difference was not statistically significant (P = 0.07) [Table 5].

Catalase activity in saliva

The activity of Catalase was significantly higher in the caries-free group (−0.0071 ± 0.0162) compared to caries-active group (−0.0214 ± 0.0073). This difference was statistically significant (P = 0.001) [Table 6].

Discussion

The present study was conducted taking into consideration the lack of evidence-based studies on evaluation of levels of oxidative stress biomarkers and bicarbonate buffering isoenzyme of saliva in carious activity. The acidity of beverages and foods is neutralized by bacterial activity by bicarbonate, phosphate, and protein buffer systems, thus preventing the colonization of pathogenic microorganisms.[12] In our sample, it was found that the CAVI levels in the caries-active group were higher than in the caries-free group, although the difference was not statistically significant. Concerning the interaction between CAVI concentration and dental caries, conflicting outcomes were reported by previous investigations. No substantial difference in CAVI concentrations was observed in a report by Oztürk *et al*.[13] when comparing caries and young adults without caries. The research conducted by Kivelä *et al*.,[14] on the other hand, shows that a decreased concentration of CAVI is correlated with an elevated index of caries. In accordance with this study, Szabó[15] found that saliva in 7–14-year-old caries-free children had a greater CAVI concentration than in children with active caries.

Picco *et al*.[16] concluded that carbonic anhydrase VI is seen to be more active in biofilm matrix of schoolchildren with caries to lead to biofilm acid neutralization. The levels of CAVI in dental biofilm in caries-active children in our current study suggest that their biofilm pH decrease tends to activate the bicarbonate buffer since these individuals are also susceptible to cariogenic challenges, especially sucrose. Our results support this finding because earlier studies have shown that salivary pH in caries-active children is slightly

Table 1: Intergroup comparison of carbonic anhydrase VI enzyme concentration

	Group 1		Group 2
Mean±SD	1925.54±1398.57		1444.17±1039.81
Highest value	4424.35		3162.97
Lowest value	68.739		193.758
P		0.135	
T		1.513	

SD: Standard deviation

Table 2: Intergroup comparison of total protein concentration

	Group 1		Group 2
Mean±SD	1.723±1.943		1.429±1.284
Highest value	8.06		5.41
Lowest value	0.08		0.26
P		0.492	
T		0.691	

SD: Standard deviation

Table 3: Intergroup comparison of lipid peroxidase activity

	Group 1		Group 2
Mean±SD	−0.0089±0.0171		−0.01423±0.01344
Highest value	0.026		0.019
Lowest value	−0.026		−0.028
P		0.184	
T		1.535	

SD: Standard deviation

Table 4: Intergroup comparison of uric acid concentration

	Group 1		Group 2
Mean±SD	1.498±0.664		0.97±0.164
Highest value	4.06		1.16
Lowest value	0.49		0.472
P		0.001*	
T		4.25	

*P<0.05 is statistically significant (Independent t test). SD: Standard deviation

Table 5: Intergroup comparison of superoxide dismutase activity

	Group 1		Group 2
Mean±SD	0.027±0.021		0.015±0.029
Highest value	0.045		0.045
Lowest value	−0.036		−0.08
P		0.07	
T		1.835	

SD: Standard deviation

Table 6: Intergroup comparison of catalase activity

	Group 1	Group 2
Mean±SD	−0.0214±0.0073	−0.0071±0.0162
Highest value	−0.005	−0.007
Lowest value	−0.04	0.016
P	0.001*	
T	4.516	

*P<0.05 is statistically significant (Independent t test). SD: Standard deviation

lower than in children without caries. Oztürk *et al*. (2008)[13] showed that the rise in isoenzyme concentration after a decline in pH may suggest that during cariogenic episodes, more CAVI is available to be triggered.

Salivary protein has been identified as having a double-edged function that, depending on position and activity, that the microorganism colonizes, plays a protective mechanism and leads to mucin development that acts against the formation of caries and protects the tooth from desiccation.[17]

Shetty *et al*.[18] stated that by neutralizing the acid production by Streptococci, the proline-rich protein reduces the occurrence of caries. Salivary albumin serves as a marker of the nature of the underlying disease and has an inhibitory effect on dental caries by entering enamel pores by preventing enamel demineralization.[19]

When we compared the total protein content in both the groups, we found that there was increased total protein in caries-active group relative to the caries-free group which was not statistically significant. This is in accordance with some previous studies done by de Farias and Bezerra[20] and Bhalla *et al*.[21] where no substantial difference was found between the two groups in the total protein content. Statistically significant rise in total protein levels has been seen in caries-active group in studies conducted by Pyati *et al*.,[12] Silva *et al*.,[22] Pandey *et al*.,[23] Preethi *et al*.,[24] Prabhakar *et al*.,[25] and Tulunoglu *et al*.[9]

Increased total salivary protein levels in children with caries may be due to a rise in many antimicrobial components such as lysozyme, lactoferrin, antioxidants such as salivary peroxidase, SOD, glutathione, and many biological factors that are all protein in nature involved in soft tissue repair.[24] The conflicting outcomes of few studies may be due to the fact that total salivary proteins may have both protective and harmful properties.

Chronic illnesses are characterized by the development of oxidative stress induced by FRs or ROS. To restrict disease progression, salivary antioxidants can interact with levels of FR/ROS. The onset of the ailment is in phase if imbalances between antioxidants and FR/ROS are certain.[26]

When the antioxidant parameters were individually analyzed and compared between the two groups, SOD, lipid peroxidase, and uric acid were increased in the caries-active group in which only uric acid showed a statistically significant increase**.** Another interesting finding was that catalase showed a statistically significant increase in the caries-free group.

Kumar *et al*.,[27] indicated that the rise in saliva uric acid levels may affect total antioxidant levels as uric acid accounts for about 70% of salivary antioxidant capacity, explaining the substantial rise in uric acid levels observed in the present study.

The results of the study conducted by Jurczak *et al*.[28] indicated high levels of antioxidants in saliva which have shown to increase substantially in children in line with salivary cariogenic bacterial profiles and development of caries.

AlAnazi *et al*.[29] concluded from their study that total antioxidant potential of saliva in children with extensive dental caries substantially decreases after treatment of carious lesions.

Araujo *et al*.[30] also demonstrated that the enzymatic antioxidant activity of SOD and nonenzymatic antioxidant activity of Uric acid boosted with the severity of caries, thus lowering salivary oxidative harm. It was presumed that the greater severity of caries enhances the function of the salivary antioxidant system, with a subsequent decrease in salivary oxidative stress.

The increase in levels of lipid peroxidase in caries-active group in the current study is in accordance with a former study conducted by Pyati *et al*.[12] which showed a statistically significant increase in malondialdehyde (MDA) levels in caries-active children.

The phagocytic activity is triggered by dental caries which leads to increased generation of FRs and consequent lipid peroxidation and MDA formation. These reactive oxidants aim at destroying invading microorganisms. Neutrophils and other phagocytes develop O_2-(superoxide) at the expense of NADPH by one-electron oxygen reduction. Almost all of the oxygen reacts with itself to form H_2O_2.[30]

Although previous researches have studied similar parameters, there were contradicting results regarding the levels of CAVI enzymes in caries activity and also there is a lack of evidence-based studies on evaluation of levels of oxidative stress biomarkers and bicarbonate buffering isoenzyme of saliva in carious activity.

From our results, it can be interpreted that among the antioxidants, catalase does not have an adaptive response to defense against excessive reactive species in pathogenesis of dental caries. Very few studies have been done correlating individual antioxidant parameters with dental caries.[22]

Further investigation is needed to evaluate the function of catalase specifically in dental caries as our groups were not analyzed according to gender which might have caused the variation which was evident in the previous study.

Previous studies have concluded that, as a result of the improved non-enzymatic and enzymatic antioxidant systems, oxidative damage to saliva in children with extreme caries is minimized.[22]

Based on the findings of the current research, the alternate hypothesis that "There exists a definite relationship of enzyme Carbonic anhydrase VI, Total proteins and antioxidants (SOD, catalase, lipid peroxidase, and uric acid) levels with the caries activity" was accepted.

For the screening and control of oral infections, including dental caries, salivary indicators of oxidative stress may be used. The assessment of these saliva markers might constitute an adjuvant tool for recognizing patients with low adherence to dental appointments. In addition, the analysis of these biomarkers may provide useful evidence to develop new remedies for the prevention and/or management of dental caries. However, such markers should be validated in forthcoming studies conducted with a higher wide range of subjects and taking into account differing levels of caries.

Conclusion

The following conclusions were drawn from the study:

1. There is an increased concentration of CAVI enzyme in caries-active group
2. Total protein showed a linear relation with caries activity
3. Antioxidant parameters such as SOD and lipid peroxidase were increased with caries activity. Uric acid was significantly higher in the caries-active group, whereas catalase showed an indirect relation with dental caries.

Significant variations in the levels of CAVI enzyme, total proteins, and antioxidant parameters in caries-active children imply that the levels of these components of saliva can act as strong markers of caries status in children

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Conflicts of interest

There are no conflicts of interest.

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