The Role of Carbonic Anhydrase Enzyme in Salivary Buffer Activity as a Marker in Caries Risk Assessment

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Abstract

A study was conducted to evaluate the activity of carbonic anhydrase (CA) and components of the buffer system (saliva pH and phosphate and bicarbonate buffer concentrations) in unstimulated and stimulated saliva of children with extremely high and extremely low risk of caries to determine and assess the risk of caries utilizing the saliva biomarker parameters.

Included in the study were 123 children with permanent dentition and a mean age of 13.8 ± 0.8 years. Based on their DMF (D = decay, M = missing, F = filled, which represents the total number of teeth with caries, teeth with filling, and extracted teeth), the patients were divided into two groups: low caries risk group with an average DMF of zero (0) and high caries risk group with an average DMF of 7.7. Both non-stimulated and stimulated saliva samples were collected from patients in each group.

When comparing the activities of CA of the examined groups, the study group with a low risk of caries had significantly higher activity ($p < 0.001$) of this enzyme in both unstimulated and stimulated saliva relative to the high-risk group.

High CA activity and better buffer parameters in saliva anticipated a lower caries incidence in children, suggesting that saliva CA activity is a crucial indicator of caries susceptibility.

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Introduction

Assessing the risk of caries in children is an essential diagnostic parameter in the planning and implementation of appropriate preventive measures. The role of saliva in the etiopathogenesis of caries is vital, and its composition can be crucial for developing this disease.[1] Although the primary saliva buffer system is a carbonate and phosphate buffer, proteins and fluorides also have a significant role in this buffer system. During stimulated salivary secretion, the dominant buffer is a bicarbonate buffer that comprises a combination of bicarbonate and carbonic acid (H2CO3). The concentration of the buffer in unstimulated saliva is 1 mM, whereas stimulating salivation increases its concentration to 60 mM. Bicarbonates mainly occur in the acini of the parotid salivary glands. Therefore, the bicarbonate buffer's most critical function is expressed during food intake, which stimulates salivary secretion and involves the reversible hydration of carbon dioxide as mentioned in the equation: CO2 + H2O <=> HCO3- + H2.[2, 3] Bicarbonate ions diffuse into the dental plaque and neutralize the acidic products of bacteria. It is known that the increase in saliva secretion comes with an increase in bicarbonate levels which are the product of cellular metabolism, so stimulated saliva contains significantly more bicarbonate than unstimulated, which is physiologically justified because the food intake network significantly increases acid production. Under these conditions, the buffering effect of bicarbonate is maximal, and the pH

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value of stimulated saliva depends on their concentrations.[4]

The dominant buffer in unstimulated saliva is the phosphate buffer, which is a combination of primary and secondary phosphate (H2PO42- and HPO43-). The concentration of these phosphates in the resting saliva is 7-8 mM; however, during salivary stimulation, the concentration decreases to 2-3 mM. Due to this concentration of buffer (7 to 8 mM) in unstimulated saliva, a reduction in salivary pH up to 6.1 is noted, which justifies why it becomes slightly acidic. Mild acidity increases the ability of saliva to act even in those conditions where the total volume of unstimulated saliva is 20-30 times less than normal. As a result, the effects of saliva buffer action will be enhanced rather than reduced.[5] The increase in phosphate concentration has an adsorbing effect on salivary glycoproteins. The formed early dental pellicle is decomposed and removed from the tooth surface, and teeth are clean of these deposits. Because of this function, phosphate buffer is in the true sense of the word oral cleaner.[6]

Carbonic anhydrase (CA) is a zinc metalloenzyme whose activity has been known in human saliva for almost 70 years,[7,8] Few studies have examined the physiological function of CA in saliva.[9] Murakami and Sly[10] first isolated this enzyme from human saliva. Immunohistochemical investigations indicate that CA secretion is restricted to the secretory granules of acinar cells in humans, followed by the parotid and submandibular glands.[11] Kadoya et al.[12] pointed out that CA regulates saliva's pH and buffering capacity.[13] However, another group of authors found that these variables do not have a direct relationship with the activity of CA in saliva.[14,15] CA does not appear to be directly involved in regulating saliva pH and its buffering capacity. Also, no correlation was observed between CA concentration and mutant streptococcus levels of lactobacilli.[1] Research by Leinonen et al.[16] indicated the binding of CA VI to the enamel pellicle with enzymatic activity on the tooth surface. CA VI is believed to be present in the enamel pellicle and catalyzes the conversion of bicarbonate and hydrogen ions (produced by microbial activity) in saliva into carbon dioxide and water.

The objective of the current study was to evaluate the activity of carbonic anhydrase and the components of the buffer system (including the pH of saliva and the concentrations of phosphate and bicarbonate buffer) in unstimulated and stimulated saliva samples obtained from children with extremely high and low risk of caries. The purpose of this assessment was to determine if the values of these saliva biomarkers could be used to predict the risk of developing caries.

Materials and methods

This study was performed at the College of Dentistry, Gulf Medical University, following the approval of the ethical committee (Ref. no. IRB/COD/FAC/72/DEC-2022).

The prospective study included 123 children with permanent dentition. The children included in the study were those who attended Thumbay Medical and Dental Specialty Center, Sharjah, UAE, for dental treatment. To prevent any distortion in the results obtained, children with permanent dentition who were in higher grades of primary school were selected. This decision was made to avoid mixed dentition because in such cases, the DMF (D = decay, M = missing, F = filled, representing the total number of teeth with decay, fillings, and extracted teeth) score is largely affected by the caries of deciduous teeth, which can obscure the actual picture of the patient's DMF. The demographic distribution of the patients in the study with data related to sex, age, and type of caries risk (low or high) is presented in Table 1.

Inclusion criteria:

Children with permanent dentition
Participants with DMF=zero for the low-risk group
Participants with DMF=7-8 for the high-risk group

Exclusion criteria:

Children with primary and mixed dentition
Participants with DMF=1-5 which represent a moderate-risk group.

Gender	Total Number of Patients	Low Caries Risk (avg. DMF 0)		High caries risk (avg. DMF 7.7)		Average age
Male	65	26	Total 52	39	Total 71	13.8 ± 0.8
Female	58	26		32		

Table 1. The demographic distribution of the patients in the study with data related to sex, age, and type of caries risk (low or high).

After determining the DMF of permanent teeth through a systematic dental examination of each patient, they were divided into two groups based on their DMF scores. The first group consisted of 52 patients with an average DMF of zero (0), indicating children with a low risk of caries. The second group included 71 patients with an average DMF of 7.7, indicating children at high risk of developing caries. Subsequently, four groups of saliva samples were defined based on this categorization. Saliva samples were collected in the morning from patients in both the unstimulated and stimulated groups using sterile tubes. The unstimulated samples were collected immediately, while the stimulated samples were collected after the subjects had chewed medical paraffin for five minutes to induce extensive secretion of stimulated saliva. Test tubes were closed immediately to prevent the loss of bicarbonate from the samples. It was directly determined in the sample's pH value (pH meter) and bicarbonate concentration by titration with 0.1 M HCl in the range of pH 7 to pH 3, in volumes of 5 µl.[17]

According to the Goldenberg spectrophotometric method, phosphate concentration was determined by a molybdenum reaction modified by Bardow et al.[17,18] A reaction mixture composed of [10% trichloroacetic acid, 1% urea and 3% NaCl] was added to the saliva. After 10 minutes, centrifugation was performed, and supernatants were added to concentrated H2SO4 and 4.5% ammonium molybdate in deionized water. After 20 minutes, the absorbance was measured at 700 nm. The standard curve was made in the range from 0 to 10 µmol / l phosphate.

CA activity was determined by the spectrophotometric method according to Armstrong et al.[19], modified by Polat and Nalbantogl.[20] P-nitrophenyl acetate (Sigma Chemical Co., St. Louis) was used as a substrate, and the unit of enzyme activity was expressed as 1 µmol of p-nitrophenol released in 1 minute at room temperature. Acetazolamide (Sigma Chemical Co., St. Louis) was used as a CA inhibitor.

Statistical analysis was done with MS Excel and SPSS in a Windows 10 operating system, where the results are shown in Table 2.

Results

The prospective study included 123 children with permanent dentition, which were approximately equal in sex representation, and the mean age was 13.8± 0.8 years (stratified sample), as shown in Table 1.

In the group of children with a low risk of caries, the average pH value of stimulated saliva was statistically significantly higher ($p < 0.001$) than the unstimulated saliva values. In comparison, in the group of children with a high risk of caries, the mean pH value of stimulated saliva was significantly lower ($p < 0.01$) compared with unstimulated saliva (Table 2). In the group of children with high caries risk, saliva pH values were statistically significantly lower ($p < 0.001$) compared to the group with low caries risk, both in basal conditions and after stimulation.

Caries Risk	Type of Saliva	Carbonic Anhydrase (IU/min/ml)	pH	Phosphate (mmol/l)	Bicarbonate (mmol/l)
Low	unstimulated	3,97±0,86	6,66±0,32	5,61±0,3	13,17±0,93
	stimulated	7,26±0,83	6,8±0,22	8,68±2,2	18,35±1,12
High	unstimulated	1,75±0,42	6,21±0,34	4,06±1,2	7,38±0,95
	stimulated	4,41±0,56	6,12±0,39	6,22±0,8	12,36±1,78

Table 2. Carbonic anhydrase activity, pH, phosphate, and bicarbonate in the saliva of children with low and high caries risk.

Phosphate values of unstimulated and stimulated saliva in both examined groups of children indicate a very pronounced increase in concentration after stimulation ($p < 0.001$) (Table 2). In the group of subjects with a low risk of caries, significantly higher phosphate values were observed, both in unstimulated ($p < 0.001$) and in stimulated saliva ($p < 0.001$) compared to the examined group with a high risk of caries.

The results in Table 2 also show that the bicarbonate level in unstimulated saliva in a low caries risk group was significantly lower (13.17 ± 0.93 mmol / l; $p < 0.001$) compared to the values of bicarbonate in stimulated saliva of children of the same study group (18.35 ± 1.12 mmol / l). Bicarbonate values in unstimulated and stimulated saliva of children at high risk for dental caries exhibited a similar trend (p 0.001). The concentration of phosphate in the unstimulated and stimulated saliva of children with a low caries risk was statistically significantly higher (p 0.001) than in the group with a high caries risk.

In the stimulated saliva of children with a low risk of caries, the mean value of CA activity

showed a highly significant increase (p <0.001) compared to basal conditions (Table 2). Also, in the group of subjects at high risk for dental caries, the mean value of CA activity in unstimulated saliva was significantly lower (p 0.001) than in stimulated saliva from the same group of children. When comparing the activities of CA of the examined groups, significant differences were observed in relation to the type of saliva and level of caries risk: the study group with a low risk of caries had significantly higher activity (p <0.001) of this enzyme in both unstimulated and stimulated saliva relative to high caries risk children.

Discussion

The research results confirm the view that the ability of stimulated saliva to reduce local acidity is a significant factor in the reduction of caries.[20] Long-term saliva stimulation leads to a decrease in its buffer capacity because it reduces the total amount of bicarbonate. During long-term stimulation (e.g., chewing gum), bicarbonate values drop to about 15 μmol / l, which depletes its buffering power despite the presence of further stimulation.[21] As the pH value of saliva depends on the presence of specific buffers, whose synthesis in the salivary glands is genetically determined, it is almost impossible to influence the change of physiological pH values, which are individual and prone to mild variations.[21] According to Makawi Y. et al.[22], salivary calcium apatite, phosphate buffer concentration, and pH values are potential biomarkers for estimating the incidence of dental caries risk in children.

The analysis of the obtained results can conclude that the stimulation of salivary secretion in children at high risk of caries leads to a decrease in the pH value of total saliva, which results in a local increase in acidity with the consequent possibility of developing many carious lesions. The values obtained in this study agree with the results of other authors[23], who emphasize the importance of individual variations in pH values of stimulated saliva in the formation and evolution of caries. According to the findings of the cited authors, the higher pH value of stimulated saliva significantly reduces the acidity on the tooth surface, which in turn reduces the release of H+ ions from the enamel surface and, ultimately, the number of newly formed carious lesions. Significantly higher pH values in both unstimulated and stimulated saliva in the group of subjects with low risk of caries indicate that the pH value of saliva has an important role in reducing caries in children and confirms data from the literature that it is one of the important parameters in assessing caries risk. Laputkova et al.[24], on the other hand, stated that there is no conclusive evidence that salivary pH could serve as a potential indicator for the early diagnosis of dental caries risk factors.

According to a study by Araujo et al., more severe caries may enhance the activity of the salivary antioxidant system, resulting in less salivary oxidative damage.[25]

In both examined groups, the phosphate values in stimulated saliva were significantly higher, which is in agreement with research by other authors.[26] The presented data support the fact that the ability of stimulated saliva to reduce local acidity is a significant factor in caries reduction.[23] This can be explained by the fact that values in unstimulated saliva are somewhat compromised, by the fact that phosphate buffer is already active in the oral cavity, and that most of the total amount of phosphate has already been consumed by neutralizing the acidity in the mouth and maintaining saliva in the conditions of its individual physiological homeostasis.[27] In contrast to the results in unstimulated saliva, which contains quantitatively partially depleted phosphate buffer, the collection of stimulated saliva gave a sample with a high concentration of phosphate.[17]

Bardow et al.[28] pointed out that the concentration of inorganic phosphates, and bicarbonate concentration in the whole saliva depends on the degree of secretion. Under physiological conditions, at an unstimulated saliva intensity of 0.55 ml/min, the phosphate buffer system represents about half of the total buffer capacity between pH 5 and 7. The contribution of phosphate buffer in stimulated saliva, whose average secretion intensity is 1.66 ml/min, is significantly lower due to the lower concentration of phosphate.[29] According to Kreusser et al.[30], the basal concentration of HPO42- ions increases with increasing salivary secretion, followed by increasing salivary pH (in unstimulated saliva, it is low and increased with saliva secretion).

Our study results are also statistically significant in stimulated saliva with lower values

of bicarbonate buffer in the high caries risk group compared to the low caries risk group, which is in accordance with data from the literature.[24] If it is known that bicarbonate buffer is the primary buffer of stimulated saliva, high values in stimulated saliva in the low-risk group of caries certainly favor the fact that the high capacity of this buffer is a significant factor contributing to the reduction of acidity in the oral cavity.[3] As the secretion of stimulated saliva in physiological conditions is mainly caused by food intake, the role of this buffer is critical just after food intake, during which there is a reaction of neutralization of food residues. Analysis of the results concludes that stimulation of saliva secretion in this study group causes the opposite effect in terms of lowering the pH of the whole saliva, which undoubtedly leads to a local increase in acidity with the consequent development of many carious lesions. The values obtained in the study agree with the results, which emphasize the importance of individual variations in the pH value of stimulated saliva in the occurrence and evolution of caries.[23]

The presented results indicate that in both unstimulated and stimulated saliva, the activity of CA was substantially higher in children with a low caries risk compared to those with a high caries risk. Based on the results obtained, high CA activity positively correlates with the declining incidence of caries in children. These results agree with data from the literature.[30] The obtained results indicate significantly higher activity of CA in the stimulated saliva of both groups, which confirms that the activity of this enzyme is very pronounced during the period of stimulated salivation when the need for acid neutralization is greatest.[30] Unfortunately, it is known that the intensity of carbonic anhydrase (CA) secretion in the oral cavity increases CA activity, and this is influenced by hereditary parameters and the pH value of saliva. It is not possible to directly control the amount of CA in saliva.

There is a positive concentration correlation between CA and salivary secretion. There is also a negative correlation of CA with DMFs index. Recent research indicates that CA in the saliva is essential in protecting teeth from caries in adults. Research by Leinonen et al.[16] shows that CA binds to the enamel pellicle and maintains its enzymatic activity on the tooth surface. In the pellicle, CA is located in optimal places where it catalyzes the conversion of salivary bicarbonates and hydrogen ions of microbial origin to carbon dioxide and water. This way fulfills its presumed role in accelerating the elimination of acids on the tooth surface.

Higher pH values in stimulated and unstimulated saliva in the group of low caries-risk subjects indicate the importance of this parameter in the prevalence of caries. The high pH value of the stimulated saliva group with low caries risk plays a significant role in neutralizing the local acidic environment of the oral cavity. The basal concentration of HPO42- ions increases with increasing salivary secretion, followed by increasing salivary pH.

Our study agrees with other studies[31,32], which emphasize that caries risk assessment involves evaluating an individual's risk for developing caries, which includes assessing factors such as oral hygiene, diet, saliva flow, and buffering capacity. Understanding the relationship between pH, salivary buffering capacity, and demineralization/remineralization can help dentists and hygienists identify individuals at higher risk for caries and develop appropriate prevention strategies, such as recommending remineralization treatments or increasing fluoride exposure.

Conclusions

In children with high CA activity and higher values of the buffer system parameters in saliva, a lower incidence of caries can be anticipated, suggesting that the activity of CA in children's saliva is an essential indicator of an individual's propensity to develop caries. Also, the activity of this enzyme and the values of the examined parameters of the buffer system in saliva can be important biomarkers in risk assessment caries in children.

Declaration of Interest

The authors report no conflict of interest.

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