Salivary Flow Rate, pH, Buffering Capacity, Total Protein, Oxidative Stress and Antioxidant Capacity in Children with and without Dental Caries

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Objectives: *To measure and compare the levels of salivary flow rate, pH, buffering capacity, total protein, malondialdehyde (MDA) and total antioxidant capacity (TAC) between caries active and caries free children and to study the correlation between the DMFS/dfs score and above salivary parameters in caries active children.* ***Study design:*** *50 caries active (DMFS/dfs ≥ 5) and 50 caries free (DMFS/dfs = 0) children aged between 6 to 12 years were included in the study. From all the children, unstimulated, mid-morning saliva samples were collected and salivary flow rate was calculated. Salivary pH, buffering capacity, total protein, MDA and TAC were measured.* ***Results:*** *The mean levels of salivary flow rate, pH, buffering capacity were significantly decreased ($p < 0.05$) and total protein, MDA and TAC were significantly increased ($p < 0.05$) in caries active children when compared to caries free controls. There was a proportionate decrease ($p < 0.05$) in salivary flow rate, pH and buffering capacity and proportionate increase ($p > 0.05$) in salivary total protein, MDA and TAC as DMFS/dfs score increased in caries active children.* ***Conclusions:*** *Significant alteration in the levels of salivary flow rate, pH, total proteins, MDA and TAC and their correlation with DMFS/dfs score in caries active children suggest, the levels of these physico-chemical properties of saliva can act as strong indicators of caries status in children.*

Key words: *Flow rate, pH, Buffering capacity, Malondialdehyde, Total antioxidant capacity, Caries active, DMFS/dfs score.*

INTRODUCTION

Dental caries at an early age is an important problem, with a worldwide incidence ranging from 3-45%.[1] Dental caries is an infectious and communicable disease and multiple factors influence its initiation and progression.[2] Saliva plays a crucial role in oral homeostasis, as it modulates the ecosystem within the oral cavity. Identification of Quantitative and/or qualitative alterations in saliva can help in the diagnosis of caries.[3]

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After the intake of food stuffs containing sugar, the pH in plaque will drop due to the fermentation of carbohydrates by the cariogenic bacteria which can result in demineralization of the tooth if the actual pH remains below the critical pH for a longer time. Flushing and neutralizing effects of salivary flow commonly referred to as "salivary clearance" or "oral clearance capacity" are probably the most important caries-preventive functions of saliva.[4] The buffering capacity of saliva which mainly depends on bicarbonate concentration is another important factor, which plays a role in the maintenance of salivary pH, and in dental remineralization.[3]

Saliva contains many proteins such as lysozyme, lactoferrin and salivary peroxidase which are known to be involved in the soft tissue repair and prevention of oral infections. These proteins act directly or indirectly through various methods on plaque and bacteria modulating susceptibility of the tooth to dental caries.[5]

Free radicals are the molecules that contain one or more unpaired electrons in their outer orbital and are extremely reactive in nature. The major sources of free radicals and reactive oxygen species (ROS) in the body are, leaks in electron transport chain in mitochondria, enzymes like xanthine oxidase and acyl-CoA oxidase in peroxisomes and NADPH oxidase during the process of respiratory burst and phagocytosis in inflammatory cells.[6] One of the routes by which free radicals can disturb the integrity and functions of cells

is lipid peroxidation (LP). Malondialdehyde (MDA) is a stable end product of peroxidation of membrane lipids and is widely used as an indicator of increased oxidative stress.[7] Antioxidants protect against the potentially harmful effects of free radicals and prevent various pathologic diseases. The most important salivary antioxidants include uric acid, albumin, glutathione, ascorbic acid, salivary peroxiadse, gamma glutamyl transferase, glutathione peroxidase, superoxide dismutase, and lactate dehydrogenase.[8] Total antioxidant capacity (TAC) is the sum of both endogenous and exogenous antioxidant systems. It is suggested that free radicals and antioxidant system appear to act in concert rather than alone, and measurement of any individual antioxidant may be less representative of whole antioxidant status and also more expensive.[9]

Despite of established role of these physico-chemical properties of saliva in the pathogenesis of dental caries, there are very few studies which have investigated their usefulness in caries risk assessment and diagnosis. Hence, we aimed to evaluate the role of salivary flow rate, pH, buffering capacity, total protein, MDA and TAC in caries risk assessment in children.

MATERIALS AND METHOD

The present study was conducted on 50 caries active and 50 age and sex matched caries free children aged between 6 to 12 years reported to the Department of Pedodontics and Preventive Dentistry, Navodaya Dental College and Hospital, Raichur, in association with the Department of Biochemistry, Navodaya Medical College, Raichur, during the period from January 2015 to August 2016. Based on the prevalence of caries in children from previous studies[1] and using following statistical formula, sample size for the present study was determined.

$$n = (1.96)^2 \times p \times (1-p) / d^2$$

Where, n = Sample size; p = Prevalence = 25.5%; d = Margin of error = 15

So, minimum sample size required is 33 cases and same number of controls. The study was approved by the Institutional Ethical Clearance Committee (IRB Approval No.: NDC/RCR RGUHS/ Disst/PG 14-15/2016-17/2245) and written informed consent was obtained from the parents / guardians of all the participating children.

Peadiatric patients aged between 6 – 12 years coming with complaints of food lodgement / discolored teeth / pain were enrolled for the study. The subjects were allowed to sit on a dental chair, where sufficient light was illuminated. One investigator after recording a detailed history and general physical examination findings, conducted oral examination using No. 5 dental mouth mirror and 0.5 mm diameter explorer and assessed DMFS / dfs score. Same investigator did oral examination in all the participants of the study and used WHO caries assessment form[10] for the determination of DMFS / dfs score. Children having at least five decayed tooth surfaces requiring restoration were taken as caries active subjects according to the WHO criteria.[10] Age and sex matched children without any symptoms and DMFS / dfs = 0 were considered as caries free. [DMFS score for permanent teeth = Number of decayed surfaces + Number of missing surfaces + Number of filled surfaces; dfs score for Primary teeth = Number of decayed surfaces + Number of filled surfaces]. Children with systemic or local diseases which affect salivary secretions**,** physically or mentally compromised children**,** Patients who are on medications**,** Children on fluoride supplementation and participants not giving consent were excluded from the study.

Unstimulated mid-morning saliva samples were collected from all the participants by the same investigator. To control the circadian variations, samples were collected between 10 am – 11.30 am. The children were instructed not to eat or drink anything for at least one hour before the collection of saliva sample. Children were asked to rinse their mouth with water thoroughly 10 minutes before collection of saliva to avoid the contamination of food debris. Then they were made to sit on a dental chair. The unstimulated saliva was allowed to accumulate in patient's mouth for 5 minutes & was aspirated directly from the floor of mouth using disposable syringe which was then transferred to a sterile test tube. The children were then asked to spit the saliva in the pre-weighed plastic sterile cylinders for 5 minutes. Saliva collected by spitting method was used for estimation of salivary flow rate, pH and buffering capacity. Aspirated saliva samples were centrifuged at 3000 rpm for 10 minutes at room temperature and used for the estimation of total protein, MDA and TAC.

The plastic cylinders containing saliva were then weighed & the flow rate was calculated using the below formula.[11]

$$\text{Salivary flow rate} = \frac{\text{Postcollection weight} - \text{Precollection weight}}{\text{Collection period}}$$

$$= \_\_\_\_\_ \text{ gm/min}$$

Salivary pH was estimated by using the digital pH meter (ELICO Ltd., Hyderabad, India.). Calibration is first carried out with a pH 7 buffer, followed by pH 4 buffer (if the sample is expected to be acidic) or pH 9 buffer (if the sample is expected to be basic). Once the pH electrode is calibrated, it is simply immersed in the saliva sample to be measured and the reading is noted. The immersed portion of the electrode is wiped with filter paper before the next sample is immersed.[12]

Salivary buffering capacity was estimated by Ericsson method (1959).[13] Buffer capacity is a measure of the efficiency of a buffer in resisting changes in pH. Conventionally, the buffer capacity (β) is expressed as the amount of strong acid or base, in gram-equivalents, that must be added to 1 litre of the solution to change its pH by one unit. Initial pH of the saliva sample is measured and value is noted as explained above. Then, 0.5 ml of saliva is added to 1.5 ml of 5mmol/l HCl in a centrifuge tube. The mixture is vigorously shaken and then centrifuged for one minute and allowed to stand for 10 min when the final pH is measured by using digital pH meter similarly. Buffer capacity is quantitatively expressed as the ratio of acid added to the change in pH produced (e.g., mEq./pH for x volume). The buffer capacity was calculated as below.[13]

$$\text{Buffer capacity } (\beta) = \Delta B / \Delta pH$$

Where, ΔB = Gram equivalent of strong acid to change pH of 1 liter of buffer solution and ΔpH = pH change caused by the addition of strong acid (Final pH – Initial pH).

Salivary total protein was estimated by Biuret method. Protein in saliva forms a violet coloured complex when reacted with cupric ions in an alkaline medium. The intensity of violet colour is

measured as optical density at 545 nm using a spectrophotometer which is directly proportional to the amount of protein present when compared to a solution with known protein concentration.[14]

Salivary MDA was estimated by Thiobarbituric acid (TBA) method. Auto-oxidation of unsaturated fatty acids results in the formation of semi-stable peroxides, which then undergo a series of reactions to form MDA. MDA reacts with TBA to form pink coloured chromogen. The resulting chromogen is extracted with 4.0ml of n-butyl alcohol and the absorbance of which is measured at 530 nm which is directly proportional to the concentration of MDA.[15]

Salivary TAC was estimated by spectrophotometric method. A standard solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton – type reaction, producing hydroxyl radicals (OH). These reactive oxygen species degrade benzoate, leading to the release of Thiobarbituric Acid Reacting Substances (TBARS). Antioxidants from the saliva cause suppression of the production of TBARS. This reaction is measured spectrophotometrically at 532 nm and the inhibition of colour (red) development is directly proportional to TAC.[16]

Statistical analysis

Data is presented as mean ± SD values. Difference between means of two groups was assessed with the Student 't' test. Correlation between the two variables was assessed by Pearson's correlation coefficient. For all the tests, p-value of 0.05 or less was considered for statistical significance. The statistical analysis was performed with statistical software SPSS 16.1 for Windows (SPSS Inc., Chicago, Illinois).

RESULTS

A total number of 100 children were included in this study. Among them 50 were caries active children and 50 were caries free controls. Among the 50 caries active children 29 were males and 21 were females. Among 50 caries free children 28 were males and 22 were females.

Table 1 shows comparative analysis of various salivary parameters between caries active and caries free children. The levels of salivary flow rate, pH, were decreased and the values of salivary total protein, MDA and TAC were increased in caries active children when compared to caries free controls and these changes were statistically significant ($p < 0.05$). The mean value of salivary buffering capacity was decreased in cases when compared to controls, but the difference was statistically not significant ($p = 0.08$).

Table 2 shows the Pearson's correlation between DMFS / dfs score and various salivary parameters in caries active children. It is evident from the table that, As the DMFS / dfs score increases, the levels of salivary flow rate, pH, buffering capacity decrease. This correlation is statistically significant ($p < 0.05$). As the DMFS / dfs score increases, levels of salivary total protein, MDA and TAC also increase. But this correlation is statistically not significant ($p > 0.05$).

Table 1: Comparison of mean levels of DMFS / dfs score and salivary flow rate, pH, buffering capacity, total protein, MDA and TAC between caries active and caries free children

	Caries active children (n = 50)	Caries free children (n = 50)	'p' value
DMFS/ dfs Score (Mean ± SD)	6.26 ± 0.90	0.0 ± 0.0	0.0001
Flow rate (ml/min) (Mean ± SD)	0.23 ± 0.09	0.33 ± 0.18	0.0006
pH (Mean ± SD)	6.64 ± 0.54	7.47 ± 0.45	0.0001
Buffering capacity (Meq/ pH/ml) (Mean ± SD)	3.70 ± 0.60	4.02 ± 1.12	0.08
Total protein (gm/dl) (Mean ± SD)	0.41 ± 0.15	0.34 ± 0.12	0.017
Malondialdehyde (nmol/ ml) (Mean ± SD)	4.89 ± 2.03	3.95 ± 1.31	0.007
Total antioxidant capacity (mmol/L) (Mean ± SD)	0.44 ± 0.17	0.35 ± 0.16	0.006

'p' value ≤ 0.05 = Significant

Table 2: Correlation between DMFS / dfs score and salivary flow rate, pH, buffering capacity, total protein, MDA and TAC and in caries active children

Variable	DMFS / dfs Score	
	'r' value	'p' value
Flow rate (ml/min)	-0.717	0.001
pH	-0.467	0.001
Buffering capacity (Meq/pH/ml)	-0.378	0.004
Total protein (gm/dl)	0.265	0.064
Malondialdehyde (nmol/ml)	0.223	0.120
Total antioxidant capacity (mmol/L)	0.129	0.372

'r' value = Correlation co-efficient; 'p' value ≤ 0.05 = Significant

DISCUSSION

In the present study, there was a statistically significant ($p = 0.0006$) decrease in the salivary flow rate among the caries active children when compared to the caries free children. There was also significant negative correlation between salivary flow rate and DMFS/dfs score ($r = -0.717$, $p = 0.001$). These findings on salivary flow rate were in accordance with the other previously conducted studies,[5,17-19] who also found decreased salivary flow rate to be a strong indicator of an increased risk of caries in children.

An important function of salivary flow is to dilute the bacterial substrate, mainly different sugars introduced into the oral cavity.[4] Flow of saliva across the oral surface creates the force which easily dislodges any microbes adhering to soft epithelial tissue of the oral cavity. Higher flow rate also mean faster delivery of protective salivary components (such as bicarbonate) to the site, thus limiting the fall in pH, demineralization and the caries process.[20] On the contrary, few earlier studies[21,22] found no significant correlation between caries activity and salivary flow rate. These contradictory results may be because of the methodological diversity such as the time point during the day at which the saliva was collected and the measurement of the saliva flow rate, sialometry.[23]

In our study, we also noted a significant decrease in salivary pH ($p = 0.0001$) and statistically non-significant decrease in salivary buffering capacity ($p = 0.08$) among caries active children when compared to caries free children. Both salivary pH ($r = -0.467$, $p = 0.001$) and buffering capacity ($r = -0.378$; $p = 0.004$) showed significant negative correlation with the DMFS/dfs score. These findings are similar to many studies conducted earlier.[5,17,18,22,26] In contrast, few studies[21,22,24,25] reported no relationship between the incidence of dental caries and pH and buffering capacity of saliva.

Bicarbonate, phosphate and protein buffer systems neutralize the acidity from drinks and foods due to bacterial activity, thereby preventing the colonization of pathogenic microorganisms. The reasons for the conflicting results for pH and buffering capacity in caries found in some other studies[21,22,24,25] may be due to the difference in the methodology or extrinsic factors such as dietary and oral hygiene habit, as well as intrinsic factor such as bicarbonate content.[27] It has been well established that, the dissolution of enamel occurs when the pH falls below critical pH, i.e. 5.5,[28] so the mean pH value obtained in our study (6.64 ± 0.54) is not adequate to cause demineralization of the tooth. Hence, it can be speculated that other factors like micro flora, diet, and retention of food might have dominated the buffering capacity to initiate caries, which is a multifactorial disease.[18]

The mean salivary total protein level in caries active group in our study was found to be significantly higher as compared to caries free controls ($p = 0.017$) but there was no statistically significant correlation between salivary total protein with DMFS/dfs score ($r = 0.265$, $p = 0.064$) among the caries active children. The association between the dental caries and the total salivary protein has been studied by several researchers and the results are mixed. Some of the previous studies[5,17,21] found similar results as ours, whereas, few other studies[29,30] found no significant difference in the total protein content between the two groups.

Increase in the salivary total protein levels in caries active children may be due to increase in many antibacterial components such as lysozyme, lactoferrin, antioxidants such as salivary peroxidase, catalase, superoxide dismutase, glutathione and many biological systems involved in soft tissue repair which are all protein in nature.[5] The contradictory results of few studies may be because of the fact that, total salivary proteins may have both protective and detrimental properties. Functions of salivary proteins may depend on the molecule's location or site of action. Some proteins such as antimicrobial and pH modulating proteins play a protective role in the oral cavity, while adhesins and agglutinins play a detrimental role by increasing the colonization of microorganisms.[31]

In the present study, mean levels of salivary MDA are significantly increased ($p = 0.007$) in caries active children when compared to caries free children. Although the salivary MDA levels increased as the DMFS/dfs score increased, this positive correlation was not statistically significant ($r = 0.223$; $p = 0.120$). Our findings are in accordance with the other studies conducted earlier.[7,32] On the contrary, one of the earlier studies[33] reported that there were no differences in salivary MDA levels in caries group compared to controls. However, they stated that, increased salivary MDA levels in periodontal and some systemic diseases, such as diabetes, osteoporosis, etc. are well known.

Dental caries triggers the phagocytic activity leading to increased free radical production and consequent lipid peroxidation and formation of MDA. These reactive oxidants are manufactured for the purpose of killing invading microorganisms. Neutrophils and other phagocytes manufacture O_2^- (superoxide) by the one-electron reduction of oxygen at the expense of NADPH. Most of the O_2^- reacts with itself to form H_2O_2 (hydrogen peroxide). OH^- (hydroxyl radical), produced by the reduction of H_2O_2 by Fe^{++} or Cu^+; $ONOO^-$ (peroxynitrite), formed by the reaction between O_2^- and NO^-; and many others.[34,35]

In the present study, the salivary TAC levels are increased significantly in caries active children when compared to caries free children ($p = 0.006$). Also, TAC was positively correlated to DMFS/dfs score ($r = 0.129$), but the correlation is not statistically significant ($p = 0.372$). These findings are in accordance with the previous studies.[1,2,17,21,36]

The presence of an infectious challenge in the form of caries or poor oral hygiene and oxidative stress as observed in our study group could be the factors for the comparatively increased levels of TAC of saliva.[17] The higher TAC levels in caries active children in the present study can be specifically attributed to triggered function of the salivary antioxidant enzymes particularly salivary peroxidase which constitutes one of the major salivary antioxidant systems. In the present study, increased salivary antioxidants such as enzymes and glutathione (both are protein in nature) is indicated by the increased salivary total protein. Salivary peroxidase catalyses the peroxidation of the thiocyanate ion (SCN-) to generate oxidation products (more stable OSCN-) that inhibit the growth and metabolism of many microorganisms thereby inhibiting caries or at least slowing down the progress of caries.[17]

CONCLUSION

Significant alteration in the levels of salivary flow rate, pH, total proteins, MDA and TAC and their correlation with DMFS/dfs score in caries active children suggest, the levels of these physico-chemical properties of saliva can act as strong indicators of caries status in children.

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