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Quantitative Determination of Inorganic Constituents in Saliva and their Relationship with Dental Caries Experience in Children

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Abstract

Background: Saliva is one of the most important factor in regulating oral health, with flow rate and composition changing throughout development and during disease. Saliva can affect incidence of dental caries in four general ways, firstly as a mechanical cleansing, secondly by reducing enamel solubility by means of calcium, phosphate and fluoride, thirdly by buffering and neutralizing the acids produced by cariogenic organisms and finally by anti-bacterial activity. Thus, the present study aimed to assess the levels of salivary immunoglobulin A (IgA), immunoglobulin G (IgG), proteins, calcium, inorganic phosphorous and alkaline phosphatase levels in caries free and caries active children.

Material and methods: Forty school children in the age group of 12-15 years with full complement of permanent dentition except third molars were included by stratified random sampling method. They were divided into two groups of 20 each based on DMFS score, Group I – Caries free (DMFS score=0) and Group II – Caries active (DMFS score ≥ 10). Unstimulated midmorning saliva samples were collected and analyzed colorimetrically and by radial immunodiffusion method for constituents of saliva under study.

Results: The mean salivary IgA levels in children in Group-I (caries free children) was 10.63 ± 2.85 mg/dl which was statistically higher as compared to caries active children in Group-II (8.50 ± 1.43 mg/dl). The mean salivary protein level in children of Group-II was statistically higher at 3.28 ± 0.12 mg/dl as compared to Group-I (2.89 ± 0.11 mg/dl).

Conclusion: An inverse relationship was noticed between the salivary IgA levels and dental caries experience and higher salivary protein levels were associated with high caries experience whereas no significant difference was observed in levels of calcium, inorganic phosphorous, alkaline phosphatase and IgG in saliva samples of children with and without dental caries.

Keywords: Alkaline phosphatase; Dental caries; Immunoglobulin A (IgA); Immunoglobulin G (IgG); Saliva

Introduction

Oral health is an integral part of the general health of an individual. Dental caries is a complex multifactorial disease caused by the interplay between a susceptible host, fermentable substrate, microflora and saliva. Saliva is essential for maintaining the oral equilibrium; and the effects of saliva and its constituents on the oral micro-organisms influence the development of dental caries. Salivary components (immunoglobulins, salivary protein, salivary calcium, and inorganic phosphorous and alkaline phosphatase levels), its flow rate, viscosity, buffering capacity, pH etc plays a major role in initiation, and progression of dental caries [1].

Mutans streptococci (MS), gram-positive micro organism, are implicated as the primary causative agent in the formation of dental caries in humans. Early colonization and growth of mutans streptococci, changes local conditions, e. g. pH, thereby enabling more organisms to further colonize the oral biofilm, forming dental plaque which results in demineralization of tooth structure and consequently dental caries ensues [2]. The infective nature of dental caries suggests that the host immunity regulates caries activity [3,4]. The specific immune defense against mutans streptococci is provided through the Common Mucosal Immune System (CMIS). Immunoglobulin A (IgA) is predominantly released by common mucosal immune system in human body secretions including saliva. Naturally occurring salivary IgA antibodies against different streptococcal antigens are present in saliva and constitute major defensive actions against dental caries [2,5].

The gingival crevicular mechanism involves the humoral and cellular components of the systemic immune system. The gingival crevicular fluid, serve as a source of secretory immunoglobulin G

(IgG) as well as some monomeric IgA contributing towards host defense against dental caries. IgG are capable of opsonizing bacteria for phagocytosis and thus, intervene in the colonization and pathogenic activity of cariogenic microorganisms.

Saliva also contains various inorganic and organic constituents apart from immunoglobulin. A major part of organic component is formed by salivary proteins which play an important role in modulating microbial colonization and formation of enamel pellicle. Salivary proteins bind with calcium and phosphate ions and help to maintain these in a supersaturated state, in respect to enamel and maintain tooth integrity via the common ion effect [6,7].

Alkaline phosphatase, an enzyme present in saliva, is active at pH 9-10 and is important for the process of remineralization. A variation in the level of alkaline phosphatase affects the ionic concentration of phosphate and calcium, which in turn can alter the equilibrium of demineralization and remineralization. The interplay of the various components of saliva and their protective role against dental caries has been of much interest. Thus, the present study further investigated

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and estimated the levels of salivary immunoglobulin A (IgA), immunoglobulin G (IgG), proteins, calcium, inorganic phosphorous and alkaline phosphatase levels in association with presence and absence of dental caries among children.

Materials and Methods

The quantitative determination of constituents in saliva and its relationship with dental caries experience among forty school children selected by stratified random sampling were assessed in this study. Ethical approval was obtained from the ethics committee and permission was also taken from the school authorities. A written explanatory note was sent to the parents regarding the objectives of the study and written consent was received from them. A total of 150 children between 12-15 years of age, were initially screened from a Municipal school in Central Mumbai, by the principal investigator. Prior to commencement of oral examination relevant medical and dental history was elicited from the parents. The fluoride level in drinking water of different parts of Mumbai was estimated using selective fluoride ion electrode (Orion) and was found to be within the range of 0.15-0.48 ppm.

Inclusion criteria

- Children in the age group of 12-15 years
- Good general health
- No history of intake of antibiotics or any preventive treatment for past 6 months
- Regular attendance in school
- Permanent residents of Mumbai

Exclusion criteria

- Medically compromised children or children with physical limitations
- Children undergoing orthodontic treatment
- Children with moderate – severe gingivitis or any significant soft tissue pathology

The children were examined in their classrooms under natural day light, comfortably seated on ordinary chairs. A thorough oral and soft tissue examination was done with mouth mirror and CPI probe. The dental caries status was assessed and recorded as per WHO criteria (1997) [8]. The examination revealed 100 caries-active and 50 caries-free children. Based on the inclusion and exclusion criteria of the study, 82 children were selected, 49 caries-active and 33 caries-free children of which 48 were boys and 34 were girls.

The selected children were further divided into 2 groups - Group I – Caries free children (DMFS score=0) which included 30 boys and 19 girls and Group II – Caries active children (DMFS score \geq 10) with 18 boys and 15 girls. Within each group, the boys and girls were allotted separate sequential numbers. Through randomized draw of lots done by one of the child, a sample size of 10 boys and 10 girls were obtained (total 20 children) for each group, in the study.

Saliva collection

The unstimulated mid-morning whole saliva samples were collected. The saliva samples were taken one and half hour after school had commenced so that sufficient time had elapsed after breakfast. The children were then asked to allow saliva to drool from the oral cavity into the sterile, labeled disposable containers, to determine the salivary

flow rate expressed as ml/min. A total of 5 ml saliva was collected from each child and transported immediately in a thermostat container for further analysis.

Estimation of immunoglobulin A (IgA) and immunoglobulin G (IgG) in saliva

Immunoglobulin A and Immunoglobulin G levels in saliva were estimated by Single Radial Immunodiffusion method described by Mancini et al. [9] It is based on the principle that a quantitative relation exists between the amount of antigen placed in well of agar antibody plate and the resulting ring of precipitation.

Two ml of each salivary sample was centrifuged at 4000 rpm for 20 min, to remove the particulates [10]. 5 μ l of the supernatant was then placed in each well of the immunodiffusion plate (Diffuplate™ Bioscientifica S. A, batch no. 1017-IgA and 1013-IgG) using a micropipette (CE Biosystems) with disposable tips. After 30 minutes, 5 μ l of the supernatant was added to each well; and the plates were incubated at room temperature for 48 hours. Antigen-antibody precipitate formation was observed in agar in the form of concentric ring around the antigen well, and measured with a Tripartigen ruler (Diffuplate™ Bioscientifica S. A) after 48 hours.

Estimation of salivary proteins

The level of proteins in saliva was estimated by the procedure described by Lowry et al. [11] The saliva samples were treated with alkaline copper sulphate (CuSO_4) and Folin Ciocalteu reagent. The color change was noted colorimetrically (A. E ERMA INC) at 660 nm.

Estimation of salivary calcium

Salivary calcium was measured using Trinder's method [12]. The sample was treated with calcium reagent and the precipitate was mixed with EDTA and treated with ferric nitrate. The reddish brown color complex was measured colorimetrically (AE ERMA INC) at 470 nm. The intensity and color is directly proportional to the calcium content of saliva.

Estimation of salivary inorganic phosphorous

The level of salivary inorganic phosphorus was measured by Fiske and Subarrow method [13]. The inorganic phosphorous in a protein free filtrate reacts with molybdic acid to form a hexavalent phosphomolybdic acid which is further reduced to 1,2,4-aminonaphthol sulphonic acid to give blue colored complex, and the intensities were read at 660 nm using a colorimeter (AE ERMA INC).

Estimation of salivary alkaline phosphatase

The level of alkaline phosphatase was measured by the King-Armstrong method using disodium phenyl phosphate as a substrate [14]. The reddish brown color was read colorimetrically (AE ERMA INC) at 530 nm.

Statistical Analysis

The collected data was tabulated and statistically analyzed by:

- A) Unpaired t-test
- B) Chi-square test

Results

A total of forty children, 20 in group I (DMFS=0), and 20 in group II (DMFS \geq 10) were selected by stratified random sampling from Municipal school of Mumbai Central area.

The mean salivary IgA levels of children in Group-I was 10.63 ± 2.85 mg/dl, which was significantly higher as compared to children in Group-II with 8.50 ± 1.43 mg/dl (t-value = 2.600, p-value = 0.015). The mean salivary IgG levels of children in Group-I was 1.04 ± 0.31 mg/dl as compared to Group-II with 0.87 ± 0.14 mg/dl, which was statistically insignificant with t-value = 1.793, p-value = 0.085 (Table 1).

The mean salivary protein levels in children in Group-I was 2.89 ± 0.11 mg/ml while in Group-II, it was significantly higher i.e., 3.28 ± 0.12 mg/dl with t-value = -10.766, p-value = 0.015 (Table 1).

The levels of salivary calcium in Group-I was 6.81 ± 0.72 mg/dl, while in Group-II, it was 6.39 ± 0.59 mg/dl (t-value = 1.966, p-value = 0.057). Inorganic phosphorous levels for children in Group-I was 17.45 ± 1.34 mg/dl, and levels for Group-II children was 16.74 ± 1.02 mg/dl (t-value = 1.896, p-value = 0.066). The levels of alkaline phosphatase in Group-I was 2.41 ± 0.47 KA units, and Group-II showed 2.70 ± 0.46 KA units (t-value = -1.965, p-value = 0.057). These results were statistically insignificant (Table 1).

The intra-group comparison of salivary IgG levels in girls (Group I) was significantly higher than boys (t-value = -2.327, p-value = 0.038) but was not statistical significant for IgA (t-value = 1.802, p-value = 0.093) and protein levels (t-value = 1.802, p-value = 0.293). The difference between boys and girls in Group I with respect to salivary calcium (t-value = -0.708, p-value = 0.488), inorganic phosphorous (t-value = 0.989, p-value = 0.336) and alkaline phosphatase (t-value = 0.811, p-value = 0.335) was statistically not significant (Table 2). In Group II, the mean salivary IgA (t-value = 1.866, p-value = 0.085), IgG (t-value = 2.191, p-value = 0.051), protein levels (t-value = 0.000, p-value = 1.000), salivary calcium (t-value = -0.846, p-value = 0.409), inorganic phosphorous (t-value = 0.592, p-value = 0.561) and alkaline phosphatase (t-value = -0.198, p-value = 0.845) was not statistical significant between boys and girls (Table 2).

Discussion

Oral cavity is a distinctive ecosystem, which performs a wide range of functions, harbours a plethora of microorganisms and is unique in accommodating exposed mineralized tissues. The saliva bathes this ecosystem and possesses a large number of components, plays a major role in the etiopathogenesis of dental caries [15,16].

The present study included school children in the age group of 12-15 years, as both cell mediated and humoral immune system are known to be fully functional at this age group [17]. The unstimulated

Variables		Group	
		I	II
IgA (mg/dl)	Mean	10.63	8.50
	SD	2.85	1.43
IgG (mg/dl)	Mean	1.04	0.87
	SD	0.31	0.14
Protein (mg/ml)	Mean	2.89	3.28
	SD	0.11	0.12
Calcium (mg/dl)	Mean	6.81	6.369
	SD	0.72	0.59
Inorganic Phosphorous (mg/dl)	Mean	17.45	16.74
	SD	1.34	1.02
Alkaline Phosphatase (KA units)	Mean	2.41	2.70
	SD	0.47	0.46

Table 1: Comparison of immunoglobulin A (IgA) and immunoglobulin G (IgG), salivary protein, salivary calcium, inorganic phosphorous and alkaline phosphatase levels between Group I and Group II.

Variables		Group I		Group II	
		Boys	Girls	Boys	Girls
IgA (mg/dl)	Mean	11.82	9.43	9.18	7.91
	SD	3.43	1.53	0.83	1.63
IgG (mg/dl)	Mean	0.87	1.20	0.94	0.79
	SD	0.20	0.31	0.15	0.09
Protein (mg/ml)	Mean	2.91	2.86	3.28	3.28
	SD	0.11	0.11	0.14	0.10
Calcium (mg/dl)	Mean	6.69	6.92	6.28	6.51
	SD	0.80	0.66	0.66	0.53
Inorganic Phosphorous (mg/dl)	Mean	17.74	17.15	16.87	16.60
	SD	1.18	1.47	0.86	1.20
Alkaline Phosphatase (KA units)	Mean	2.49	2.32	2.68	2.72
	SD	0.45	0.50	0.53	0.41

Table 2: Intra-group comparison of immunoglobulin A (IgA), immunoglobulin G (IgG), salivary protein, salivary calcium, inorganic phosphorous and alkaline phosphatase levels in Group I and Group II according to gender.

midmorning whole saliva samples were collected at least two hours after breakfast as this period has been reported to have less diurnal variations in the flow rate and composition of saliva. A total of 5 ml of saliva was collected from each child and transported immediately for the estimation of salivary constituents under study. Prolonged storage of saliva samples should be avoided as it leads to variable loss of protein including immunoglobulins [10,11].

In the present study, the mean salivary IgA level in children in Group-I {Caries free (DMFS=0)} was significantly higher than Group-II {Caries active (DMFS ≥ 10)}, suggesting a possible protective role of IgA in prevention of dental caries. Lehner et al. [10] reported that subjects with caries had decreased IgA concentrations, as compared to those with no detectable caries and proposed it could be due to the deficient transport mechanism, stimulation of immune system via pulp, deficient local immunoglobulin synthesis and molecular size of IgA. Challacombe in a study also reported a significant inverse relationship between the IgA secretion rate and dental caries and stated that the raised IgA and IgG levels in serum reflect past caries experience [15]. Rose et al. [18] compared the IgA levels of whole saliva and parotid saliva of caries susceptible and caries-resistant children aged 7-11 years using enzyme linked immunosorbant assay and concluded that whole saliva and not parotid saliva in caries resistant children had a significantly higher IgA levels as compared to caries prone group.

However, Camling et al. [19] have reported a negative correlation between the degree of caries activity and salivary IgA concentration. Bhatia et al. [20] concluded that higher levels of salivary IgA exist in caries susceptible group, which could be due to either a cumulative antigenic or recent antigenic stimulation, with higher caries experience as compared to caries resistant group. Some authors also reported that the saliva of caries-free subjects includes significant IgA antibody against antigen I/II of Streptococcus mutans, indicating a protective mechanism [21,22]. However, microorganisms may protect themselves from host immune attack by forming biofilms and decreasing expression of antigen I/II. The finding of Cogulu et al. [23] tends to support the hypothesis that higher levels of salivary IgA may provide protection against dental caries.

The mean salivary IgG levels in the present study were higher in Group I as compared to Group II; however, the difference was statistically insignificant. Lehner et al [10] and Everhart et al. [24] reported that salivary IgG does not seem to play any role in prevention of dental caries.

Bagherian et al. [25] reported that the high concentration of

salivary immunoglobulin in children with early childhood caries may be associated with an increased antigenic load, leading to high production of antibodies. According to a study by Kirtaniya et al. [26], the increase in serum IgG, IgA and total antibody titer showed a direct correlation with the increase in number of carious lesions.

The various organic and inorganic constituents of saliva help in maintaining the integrity of teeth and other oral structures. Salivary proteins possess antimicrobial, lubricative and digestive properties and play an important role in modulating the microbial colonization of teeth and soft tissues [6]. The salivary protein levels were estimated by the method using Folin phenol reagent. The Folin-Ciocalteu reagent contains phosphomolybdic acid. The reduction of Mo^{6+} and Mo^{4+} of phosphomolybdic acid by tyrosine and tryptophan present in the salivary proteins gives a blue colored complex which is read colorimetrically at 660 nm [11].

The mean salivary protein level in the present study in caries active group i.e. Group-II was higher as compared to Group-I which is in accordance with Tulungolu et al. [27] and Kargul et al. [28] who observed an increase in the salivary protein concentration with increased caries activity.

The role of saliva in remineralization of enamel and calculus formation is dependent upon the saturation of saliva with respect to calcium and phosphorous. The salivary alkaline phosphatase is one of the factors governing the calcium and phosphorous levels in saliva [29]. The mean salivary calcium and inorganic phosphorous levels in caries free group (Group-I) were higher as compared to caries active group (Group-II) and this difference was statistically not significant which are in accordance with the result of Gandhi and Damle [29], Marray and Shaw [30], Afshar et al. [31], Cornejo et al. [32], Masamurak et al. [33]. These studies investigated the relationship between alkaline phosphatase and inorganic phosphorous with decayed, filled surface and observed that the level of alkaline phosphatase and inorganic phosphorous in rampant caries children was higher than the caries free children.

The level of alkaline phosphatase for caries active (Group-II) was higher as compared to caries free (Group-I) in the present study. Kumar et al. [34], Pandey et al. [35], Bai et al. [36], Vijayaprasad et al. [37] and Mahjoub et al. [38] have also reported a positive correlation between salivary alkaline phosphatase activities with dental caries. In the present study, mean of inorganic phosphorous and alkaline phosphatase in caries free group was higher than the other groups, but this difference was not significant after statistical analysis.

Conclusion

Saliva's buffering capability, ability to wash the tooth surface, antibacterial activities, and to control demineralization, and perhaps other mechanisms all contribute to its essential role in preventing caries. The present study shows decreased levels of salivary immunoglobulin A and high concentration of salivary protein in children with increased caries experience which is indicative of the protective role of salivary constituents in caries free children.

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