Salivary Urease and ADS Enzymatic Activity as Endogenous Protection against Dental Caries in Children

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*The **aim** of this cross sectional study was to evaluate the ureolytic and arginolytic activities of saliva in children and associate them with their caries status. Study design: 65, 8 year old children, were randomly selected. The ureolytic and arginolytic activity of non stimulated saliva was studied and associated with DMFT and dmft index. Saliva of children were sampled under fasting conditions; Children refrained from any oral hygiene procedures during the 12 hours that preceded the sample collection. Caries activity was scored and divided in 3 groups: Group **A:** Index zero: without lesions; **Group B:** Moderate Index: 1 to 3 enamel caries lesions; and Group **C:** High Index: more than 4 dentin caries lesions. **Results**: DMFT scores were moderate: 0.4(±0.79) and dmft: 2.78(±2.45). Results expressed in* **µ***mol/min/mg/protein, for urease activity were statistically significant (p=0.048): Group A= 0.69 (±0.7); Group B= 0.45 (±0.43); and Group C= 0.39 (±0.55). The arginine deiminase activity was not statistically significant (p=0.16): Group A= 2.53 (±1.42), Group B= 2.31 (±1.57) and Group C= 1.97 (±2.0). **Conclusion**: Higher levels of ureolytic (statistically significant) and arginolytic activity (trend) in saliva were associated with lower DMFT/dmft scores in 8 year old children. There was a higher production of ammonia from the arginine deiminase system than the urease enzyme in saliva (p>0.05).*

Key words: children, caries, DMFT/dmft, urea, arginina, urease, ADS.

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INTRODUCTION

Despite the development of preventive dentistry, tooth decay is still a major public health problem. It is the most common chronic disease in the world population,[1] affecting 60 to 90% of children.[2] In Chile, caries has dramatically increased to 98% in the 35 to 44 years population, reaching close to 100% of individuals among 65 to 74 years.[3] Caries is prevalent among 96% of adults and among 99.5% of individuals older than 65 years in the U.S.[4] Caries is the main reason for dental loss and dental pain. Both conditions associated with impaired performance in school for children and absence at work for adult patients,[2, 5] ultimately decreasing quality of life.[2]

The transition from health to dental caries disease, is characterized by changes in oral biofilms composition and metabolism, [6, 7] A drop in the pH in the oral environment, could promote the proliferation of acidogenic and aciduric microorganisms, such as *Streptococcus mutans*, *Lactobacillus*, *Veillonella* among others. The acidogenic microbiota can produce rapid fermentation of carbohydrates reaching a point where significant tooth demineralization occurs. Repeated acidification cycles, promote the establishment of an acidogenic microbiota, which has been prevalent in individuals with increased susceptibility to dental caries.[8-12] The changes from a caries free to a caries active child has been associated with the reduced potential for generating alkali in the oral environment, combined with increased acidogenic microbiota. For years, caries disease has been studied for its acidogenic nature, and only in the last decades, it has been proposed the increase in alkali production as strategy to reach oral pH homeostasis and prevent dental caries. [13-15]

One of the alkali components is urea, and is found naturally in the saliva of healthy humans, at levels similar to serum 3-10mM. It is rapidly hydrolyzed by the enzyme urease that converts it in ammonia and CO_2. Another component is arginine, a semi essential amino acid, that it is also present in the oral environment, in concentrations of about 50 µM in its free form. Arginine Deiminase System (ADS) transforms it in another peptide called ornithine, ammonia, CO_2 and ATP. The groups of bacteria involved in this activity are *Streptococcus sanguis, Streptococcus parasanguinis Streptococcus gordonii, Streptococcus salivarius Actinomyces naeslundii* and others. [13, 16, 17]

The production of alkali in mouth is associated with two routes; Hydrolysis of urea by urease enzyme, and hydrolysis of arginine by arginase deimino system (ADS).[18]The expression of these enzymes by bacteria is highly regulated in response to environmental factors such as availability of substrates, presence of carbohydrates and pH. [19] Low ureolytic and ADS activity from bacteria has been observed in subjects with high-carbohydrate diets, additionally, it is known that pH levels of 4 or lower inactivate the enzyme, as does temperatures below 7 º C and above 60°C.[13, 20]

The urea is a nitrogenous substrate that can produce alkali rapidly enough to buffer salivary acids and thereby contribute to a pH rise, however, subjects with low levels of urease, exhibit reduced capacity for compensating the glycolytic activity which lowers the pH and is associated with low levels of ureolytic activity from bacteria, producing up to 300% less enzymes in individuals with active caries. [13, 21]

The increased levels of ammonia from urease activity can produce a significant increase in neutralizing pH the oral environment, helping to balance the acids generated from the glycolytic activity. These are the reasons that determined the hypothesis that the activity of urease and ADS are associated with a decrease in caries activity.[17]

The aim of this cross-sectional and randomized study was to assess the level of oral alkali production in saliva of 8 years old children, and associate it with their caries status. We hypothesized that children with higher levels of urease and ADS enzyme activity would present lower DMFT/dmft index. (Decayed, Missing, Filled in Permanent teeth/ in deciduous teeth)

MATERIALS AND METHOD

This cross-sectional study, gathered information on 65, 8 year old children belonging to low and lower middle class socioeconomic levels from the metropolitan region of Santiago, Chile. The study is ascribed to project FONIS SA12i20205 and was approved by the Human Research Ethics Committee of the University of Chile´s Dental School, under protocol ACTA W:012/13, and accordance with the ethical standards of the Helsinki declaration for human experimentation (revised 2000).

Studies universe and sample design

Of the 32 counties in Santiago, five counties of northern Santiago of low and lower middle socio economic levels were identified. Three counties were selected randomly (with a random function, Microsoft Excel 2007): *Huechuraba, Recoleta* and *Independencia*. The number of 8 year old children (population projection) was determined from the database of the National Institute of Population Statistics, then, together with the Department of Education from the three counties, public elementary schools were identified, by a simple draw. The sample size was calculated assuming alpha = 0.05 (two-sided) alternative power = 0.08 and p = 0.75, with the computer program Stata v11 (Test Ho: p = 0.5, where p is the proportion in the population). The estimated required sample size was: 58 children. Assuming the difficulties to be encountered in localizing the selected children and then applying exclusion criteria, the total number of contacted children was increased to 65.

The inclusion criteria for the sample were: children had to be 8-years of age, both gender, with and without caries lesions and must have had 12-hours of fast and absence of oral hygiene.

The exclusion criteria for the sample were children with decreased salivary flow (less than 0.5 ml / min in girls and 0.7 ml / min in boys), intake of antibiotics, systemic or inhaled steroids or chlorhexidine during the last three months, to be suffering from systemic diseases such as diabetes, hypertension, autoimmune or immunodeficiency and using toothpaste containing arginine. Children with periodontal disease were also excluded, since the biofilm involved in the pathogenesis of periodontal disease is characterized by a lower proportion of acidogenic bacteria and abundance in ureolytic bacteria producing urease and ADS metabolizing nitrogenous substrates from saliva (urea, uric acid, creatinine and amino acids), releasing ammonia that also liberates carbon dioxide increasing biofilms pH.[22]

Children refrained from any oral hygiene procedures in at least 12 hours[13] prior to sample collection. Three ml of non stimulated saliva was collected in a sterile plastic tube (Falcon 2070, Becton Dickinson and Company, Franklin Lakes, NJ, USA) through expectoration; and were kept at 4°C until transported to the laboratory. Samples were stored at -80ºC (Sanyo Electric Co Ltd, Osaka, Japan) until the day of analysis. The thawing of the samples was done at room temperature and they unfroze once they reached 4ºC. The samples were then dispersed by external sonification (Transsonic 460/H, Elma GmbH & Co KG, Singer, Germany) in two cycles of 30 seconds, being refrigerated with ice during intervals.

All parents signed the consent form for their children to participate in the study. A single clinician made the calibration exercises in WHO methodology (Kappa 0.77) and was responsible for all clinical examinations.

Once the 65 children were examined and medical records were complete, the number of decayed, filled, and extracted teeth was determined in primary teeth (dmft index) and permanent teeth (DMFT index). After obtaining both indexes each child (DMFT and dmft) was assigned into the following groups:

A: Index zero: the sum of indices equal to 0, with no caries, filled teeth or indication of extraction for reason of caries.

B: Moderate Index*:* The presence at least 3 teeth with enamel caries was established.

C: High Index*:* The presence of at least 4 teeth with dentinal caries was set.

Laboratory procedure for measuring Urease and ADS activity.

Samples analyses were performed at the Chemistry Laboratory of the School of Dentistry, University of Chile. After thawing in aliquot of saliva, a Tris-maleate buffer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and an exact amount of 25 µmoles of urea (Sigma-Aldrich) was added as substrate for enzymatic action.

Ammonia (produced by urealysis or the arginine deiminase system) of saliva samples were determined spectrophotometrically (Thermo Spectronic Unicam UV-530 UV-Visible, Rochester, NY, USA). For that, an ammonia calibration curve was created by Nessler's curve, where different known ammonia concentrations were mixed with Nessler's Reagent (Sigma-Aldrich) generating a yellow coloration that was measured by the spectrophotometer, creating a curve of ammonia concentration v/s absorbance. The equation of the curve was obtained and used to determine ammonia concentration (mM) of processed samples. Absorbance of saliva samples was measured using Nessler's reactive at a wavelength of 395 nm.

As saliva samples of every patient are different in content and therefore difficult to standardize, it is necessary to express ammonia and its concentration produced by proteins presented in a known volume. So, ammonia produced by urease or arginine deiminase was divided by protein concentration. For this, proteins presented in the samples were determined spectrophotometrically. A protein calibration curve was created, by Bradford's curve, where a different known protein (albumin from bovine serum, Sigma-Aldrich, USA) (Sigma-Aldrich) concentration was mixed with Bradford Reagent (Sigma-Aldrich) generating a blue coloration that was measured by the spectrophotometer, creating a curve of protein concentration v/s absorbance. The equation of the curve was obtained and used to determine protein concentration of processed samples. Absorbance was measured at a wavelength of 595 nm.

After centrifugation, the first step was using the pellet of the sample containing the enzymes present in the saliva. The urea and arginine substrates were added to this pellet in order to make them react for a certain amount time. After the reaction, there was a second centrifugation using the supernatant of the reaction where the produced ammonia would be found, discarding the pellet contain.

To determine the mM of ammonia (NH_4) produced, the equation obtained from the NH_4 calibration curve was used.

Statistical analysis

Descriptive analysis of the variables was carried out to characterize the sample. Data was analyzed by Kolmogorov-Smirnov test to determine the normality of the data. The comparison between groups was performed with Kruskal-Wallis and Mann Whitney test at 0.95 of confidence by Statistical Package for Social Sciences (SPSS Inc., Released 2009, Statistics for Windows, v18.0, Chicago, IL, USA).

RESULTS

From a total of 65 examined children n=36 (55.3%) were male and n=29 (44.7%) were female.

The characterization of the sample, expressed as average values were: DMFT Index: 0.4 (±0.79); permanent teeth with caries lesions: 0.28(±0.67); permanent filled teeth: 0.12(±0.38); permanent teeth lost 0.00; dmft Index: 2.78(±2.45); decay in primary teeth: 2.22(±2.29); filled primary teeth: 0.48(±0.92); Indicating teeth extraction for primary teeth: 0.12(±0.92) (Table 1)

Table 1: Clinical Characteristics of the mixed dentition study population. Age is shown in years. DMFT and age is shown in years. DMFT and age are shown as [Mean (±SD)].

Caries Score DMFT/dmft	Zero Index	Moderate Index	High Index	Total
n=	15	31	19	65
Number of	0	56	106	162
Decayed	0	7	1	8
Missing	0	33	4	37
Filled Teeth				
AGE	8	8	8	8
Gender				
Male	7	17	12	n=36 (55.3%)
Female	8	14	7	n=29 (44.7%)

The urease activity for DMFT/dmft in 8 years children showed significant differences for urease activity (p=0.048), and for ADS showed trend but not significant (p=0.162) (Table 2).

Table 2: Saliva activity levels of Urease (U) and Arginine Deiminase System (ADS), separated by group of mixed dentition children (n=65). The U and ADS activity was expressed as µmol min^{-1}mg $prot.^{-1}$, [Mean ± SD].

Caries Score DMFT/dmft	Zero Index	Moderate Index	High Index	p-value
UREASE	0.69	0.45	0.39	0.048
ADS	±0.7	±0.43	±0.55	0.162
	2.53	2.31	1.97	
	±1.42	±1.57	±2.0	

The urease and ADS activities increased in children with no caries and it decreased in children with active carious lesions.

DISCUSSION

This population-based study analyzed the activity of two enzymes, urease and ADS as ammonia producing sources and assessed their association with the level of dental caries lesions, in 8 year old children, of low and lower middle class socioeconomic levels. 8 years old children were selected because previous Chilean epidemiological studies, shows that this group have 30% of caries free children.

The results showed greater enzymatic activity of urease and ADS in saliva was found in children with no caries experience (zero caries index group [DMFT + dmft]). Urease activity was 177% higher than the enzyme activity present in the high caries rate group, while ADS activity was 22% higher in the zero caries group, but without any statistical differences. Compared with Reyes et al. study,[23] the differences observed in the present study was lower, both study were made with the same methodology, were ADS activity in saliva was 448% higher in caries free patients, it could be explained because oral imbalances in children incipient stages, not produce caries cavitated lesions yet, compared with adult over 18 years old, that had no previous history of caries.

The different activity of urease between the three groups in the present study was statistically significant ($p<0.05$). Similar findings were made by Morou-Bermudez et al.(2011) in a longitudinal cohort study in saliva of children from Puerto Rico.[19]

When comparing the enzymatic activity of ADS between the different groups studied, there were no statistically differences ($p=0.28$). Similar results were obtained by Nascimento et al (2009) with the ADS activity in oral biofilm.[17] However, Gordan et al (2009)[13] founds that the activity of ADS in biofilm was three times higher in subjects free of caries. The different results could be related with different oral balance or with patients behavior (diet), it is necessary to consider that one of the factors that could negatively affect this measurement could be pre-sampling sugar intakes by children, since consumption of sugar produces low pH, which depress the activity of this system.[19] Carbohydrate intake produces low levels of urease and it is also causes a low proportion of ureolytic bacteria. Frequent sugar consumption, such as sucrose, not only leads to an increase in cariogenic bacteria, but also decreases the beneficial production of ammonia and bacterial species that protect the oral cavity.[20, 24, 25]

Although the values of ADS from this study are not statistically significant, it shows a trend to decrease in the moderate caries group and even more in the high caries group. Higher levels of ADS enzyme in saliva could be biologically meaningful, if they are able to impact the acid-base balance in the mouth so would arginine activity. Following the same line, in an in vitro study, where levels of urease can be manipulated and produced by the recombinant S. mutans, it was observed that small increases in the activity of this enzyme originated significant decreases in the acidification of the environment. [26, 27] Therefore, generation of alkali in the oral environment by both enzymatic systems, urease and ADS, creates a protective atmosphere of healthy microbiota that support and inhibits cariogenic microbiota in children.

The hydrolysis of urea by bacterial urease enzymes generates ammonia and CO_2 and it is considered the major pathway for alkali production in the oral cavity. It has, therefore, been hypothesized that the production of ammonia via ureolysis in the oral cavity may be an important factor inhibiting the emergence of a cariogenic flora and the development of caries. Indeed, a link between elevated urea levels in the saliva patients undergoing renal dialysis and caries resistance has been noted.[28, 29]

Using arginine deiminase, by arginolytic bacteria such as *S. sanguinis*, arginine is metabolized to ammonia which, in turn, can neutralize plaque acids and stabilize the residual biofilm present on susceptible tooth surfaces.[30, 31]

The pH of individuals resistant to caries is higher than those susceptible to caries. This pH increase is correlated with high levels of ammonia. Those findings suggest that the increased risk of caries is associated with a reduced ability to produce alkali in microbial populations colonizing the oral cavity[13]. Therefore, the production of ammonia from urea and arginine metabolism has been identified as the mechanism by which oral bacteria is protected against acid discrimination and maintains relatively neutral pH environments that can suppress or compensate the activity of cariogenic microbiota, in association with bio-energetic advantages through arginine metabolism that generates adenosine triphosphate.[13]

We agree with the study that measured the activity of urease in children under fasting conditions, finding that there is increased activity of urease in children free of caries. Therefore, the increase in urease activity could be associated with lower levels of caries in children.[19]

Recently, metagenome observations shows, that the environmental changes of the oral cavity significantly affect the number of microbial species that live in the mouth. This was demonstrated through comparative analysis of oral metagenome where caries free patients presented 1,015 different microbial species, while patients with incipient enamel carious lesion (white spot) significantly reduced the amount to 193 species. Additionally, was observed 600% increases of *sp mutans* populations in patients with carious lesions, from 0.12% to 0.72% of all species in the oral microbial community.[32] This genomic information confirms that low levels of a bacterium are able to relate to important changes in all oral microbiota and in the oral environment.

The adaptation to pH changing is a significant driving power that defines dental microbial community of the caries lesions. The acid tolerance of *S. mutans* and another aciduric species is based in part on the activity of the membrane-bound Streptococcal spp. As other like S. Gordinii, Veillonella spp, they possess an arginine deiminase system (ADS) allowing the hydrolysis of arginina to ornithine, ammonia and CO_2 plus ATP.[14] This metabolic system for deacidifying activities has been associated with reduced caries development.[18, 33, 34] Additional indirect evidence, that oral metabolism of urea may enhance caries resistance, was observed in a study involving patients with chronic renal failure. These patients rarely develop caries despite a carbohydrate-rich diet. They are also able to produce 10- to 50-fold greater salivary urea levels than healthy subjects.[29, 35, 36].

Clinical arginine technology became available in toothpastes (CaviStat, Ortek Lab, USA) nine years ago. In Venezuela it showed an inhibition of caries onset and caries progression. It was concluded that this technology, which was simple and economical, substantially reduced one of the most prevalent diseases in children.[37, 38] Lately, after the finalization of this study, a new toothpaste (Colgate-Palmolive, USA) with arginine (1.5%), insoluble calcium and fluoride (1450 ppm) claims that it clinically provides more caries prevention, given the capacity of biofilm to metabolize arginine to ammonia. [39, 40],[41] Clinical observation evaluated by quantitative light-induced fluorescence (QLF) observed that this new dentifrice provides significantly greater anticaries advantage than a dentifrice containing fluoride alone.[42] Additionally, in root caries this toothpaste provided statistically significantly superior efficacy ($p<0.001$) in arresting and reversing active root caries lesions in adults compared to matched positive control dentifrice containing fluoride alone. The same results were observed in children with crown caries lesions.[43,44] Considering that the action mechanisms of fluorides and arginine are different, and do not overlap, they act complementary in preventive dentistry.

Oral biofilms are complex ecosystems with hundreds of metabolically and physiologically varied species and there is considerable competition for nutrients among themselves. The alkali production by bacteria can certainly affect the balance between the remineralization and demineralization of the tooth and also aid to prevent the emergence of a cariostatic microflora,[45, 46] facing the acidification

of dental biofilm that favors the materialization of an acidogenic and aciduric microflora including *mutans streptococci and Lactobacillus spp*., and others which ferment carbohydrates fast and lower the pH to values that accelerate tooth demineralization. [47-49]

To understand the origin and activity of enzymes it is necessary to connect with genetics and biochemical studies that show that urease is a nickel-containing oligomeric enzyme and the activity requires at least seven gene products that are usually encoded in operons.[50-52] The urease expression of oral bacterial is synchronized by multiple contributions, the presence of urea is limited by a nitrogenous source that can induce gene transcription, in other bacteria, urease expression is repressed at neutral pH values, but under acidic circumstances the urease genes become activated, urease gene expression can also be sensitive to carbohydrate availability and growth rate.[53, 54]

Another explanation for these findings might be associated with patients taking more proteins derived from arginine in the diet, this information needs to be investigated further in future studies.

While the genes encoding the ADS are commonly arranged in an operon, the gene order varies among species.[55, 56]

ADS is also subject to regulation by environmental stimuli, and the modes and mechanism of control vary between species, for example ADS expression is induced by arginina and low pH, or the operon is sensitive to carbon catabolite repression and is down regulated by elevated oxygen levels, additionally *A gordonii with A naeslundii* favor an enhanced ADS activity by co-aggregation that activate arginina biosynthesis and ADS expression.[57-59]

Despite this evidence, further studies are necessary to determine the best levels of urease and/or ADS that have the ability to prevent caries lesions. And what is the capability of maintaining this stability over time or how they are related with other risk factors in dental caries.

Future studies should consider observation of other populations such as *S gordinii, A naeslundii, S salivarius, S. parasanguinis, and S sanguis,* alkali-producing organisms, in order to determine the changes in the flora of the patients with different levels of disease, before and after arginine or urea applications for better understanding of this biological process.

Finally, the most important evidence of this study is the suggestion that the natural endogenous alkalinogenic potential of the oral environment is associated with caries free children. High levels of alkali activity in saliva, could be related with neutralizing acids and possibly stabilizing the oral microbiota, favoring the conditions of maintaining oral health and the possibility to be used as a strategy against dental caries. Urea and arginine are natural components of the oral environment and can complement the use of fluorides, by observing the basis and dynamics of acid-based pH changes in the mouth.[14, 15, 18, 19, 60]

CONCLUSIONS

Higher levels of urease (significant) and arginine deiminase system (trend) in saliva of 8 years old children were associated with lower rates of MDFT/dmft. There was a higher production of ammonia from the arginine deiminase system than from the urease enzyme. ($p>0.05$)

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