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Oral Arginine Metabolism May Decrease the Risk for Dental Caries in Children

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ABSTRACT

Arginine metabolism by oral bacteria *via* the arginine deiminase system (ADS) increases the local pH, which can neutralize the effects of acidification from sugar metabolism and reduce the cariogenicity of oral biofilms. To explore the relationship between oral arginine metabolism and dental caries experience in children, we measured ADS activity in oral samples from 100 children and correlated it with their caries status and type of dentition. Supragingival dental plaque was collected from tooth surfaces that were caries-lesion-free (PF) and from dentinal (PD) and enamel (PE) caries lesions. Regardless of children's caries status or type of dentition, PF (378.6) had significantly higher ADS activity compared with PD (208.4; $p < .001$) and PE (194.8; $p = .005$). There was no significant difference in the salivary arginolytic activity among children with different caries status. Mixed-model analysis showed that plaque caries status is significantly associated with ADS activity despite children's age, caries status, and dentition ($p < .001$), with healthy plaque predicting higher ADS activity compared with diseased plaque. Plaque arginine metabolism varies greatly among children and tooth sites, which may affect their susceptibility to caries.

KEY WORDS: oral biolfim, dental plaque, dental caries, bacteria, arginine, risk factor.

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INTRODUCTION

Dental caries is one of the most common infectious and chronic oral diseases of children (Petersen, 2003) and is associated with costly treatment. In recent years, there has been an alarming rise in childhood caries, especially among underserved populations (Dye *et al*., 2010). Despite significant efforts to prevent and treat caries, including the use of fluoride, caries still remains a major public health problem. Importantly, caries can have life-long consequences on the health of children by affecting nutrition, growth, and development (Skeie *et al*., 2006). Early identification of at-risk children and early intervention can significantly reduce the risk for caries development. Thus, there is an urgent need to identify novel and more effective strategies for caries risk assessment and interventions.

As has long been recognized, a caries lesion occurs when acids produced by bacterial glycolysis of dietary carbohydrates cause tooth demineralization. Current evidence suggests that alkali production from the metabolism of salivary substrates, such as arginine or urea, inhibits tooth demineralization by neutralizing glycolytic acids, while positively affecting the ecology of oral biofilms (Shu *et al*., 2007; Nascimento *et al*., 2009; Morou-Bermudez *et al*., 2011). Our previous studies revealed that an increased risk for caries is associated with reduced alkali-producing capacity of bacteria colonizing the adult oral cavity (Shu *et al.*, 2007; Nascimento *et al.*, 2009). A technology containing arginine was demonstrated to have significant effects in inhibiting caries in children (Acevedo *et al.*, 2008). Although the cost-effective use and potential of this technology to reduce caries appears high, the underlying basis for the impact of alkali production in caries pathogenesis has yet to be fully disclosed. Likewise, the capacity of oral bacteria from children with different caries experience to produce alkali from arginine has not been explored.

Oral alkali production *via* arginine is a highly promising but underexplored approach for caries risk assessment and for caries prevention. Arginine is found free in saliva in micromolar concentrations (Van Wuyckhuyse *et al.*, 1995) and is also abundant in salivary peptides and proteins. In oral biofilms, arginine is mainly metabolized by the arginine deiminase system (ADS) of oral bacteria, which yields citrulline, ornithine, CO_2, ATP, and ammonia (Burne and Marquis, 2000). A substantial knowledge base about the physiology and genetics of the ADS in oral bacteria has been established (Marquis *et al*., 1987; Casiano-Colon and Marquis, 1988; Burne *et al.*, 1991; Curran *et al*., 1998; Dong *et al*., 2004; Griswold *et a*l., 2004; Zeng *et al*., 2006; Liu and Burne, 2009), but the role of arginine metabolism in oral ecology and oral health has not been thoroughly studied in humans. The main goal

of this study was to explore the relationship between ADS activity and caries experience in children. In addition, we examined whether the arginolytic capacity of oral bacterial populations could be correlated with the bacterial colonization site and type of dentition.

MATERIALS & METHODS

One hundred children ages 2 to 14 yrs were recruited. The wide age range used in this study was selected so that we could examine a spectrum of different dentitions, which may influence oral biofilm ecology and therefore its arginolytic capacity. The selection process excluded children who: (i) had been treated with antibiotics within the preceding 3 mos, (ii) were taking any medication, or (iii) had orthodontic appliances. Parent-administered questionnaires were used to collect information about: (a) demographics, (b) oral health practices, (c) dietary habits, and (d) medical and (e) dental histories. Informed consent was obtained from parents or legal guardians of each child under a protocol approved by the Institutional Review Board of the University of Florida Health Science Center.

Children were grouped by caries status: caries-lesion-free (CF) had no reported or clinical evidence of caries experience [decayed, missing, and filled teeth (DMFT) = 0]; caries-active (CA) had at least 2 active, dentinal, cavitated, and unrestored caries lesions (DT ≥ 2, MFT ≥ 0); and caries experienced (CE) had previous experience of caries but absence of caries activity (DT = 0; MFT ≥ 0). For all CE children, the recorded restorations (FT) had been placed at least 6 mos prior to this study. Children were also grouped by type of dentition, as primary, mixed, or permanent dentitions.

A single examiner (M.M.N.) conducted all clinical examinations and determined the children's caries status and type of dentition. Caries lesions were recorded according to the International Caries Detection and Assessment System (ICDAS) criteria, which range from 0 to 6 (Ismail *et al*., 2007). Teeth were examined before and after removal of plaque, as well as before and after being dried with compressed air for 5 sec. The activity of caries lesions was determined by clinical appearance, plaque stagnation, and tactile sensation. The range of ICDAS scores as a function of caries-status group was CF and CE (no activity, ICDAS = 0) and CA (active lesions, ICDAS = 0-6). The threshold for defining the CA group was the presence of at least 2 ICDAS scores of 5 and/or 6 (dentinal, cavitated lesions); however, CA children could also present other types of caries lesions with lower ICDAS scores (enamel and dentin, non-cavitated lesions).

Children were required to refrain from oral hygiene procedures for at least 8 hrs prior to the collection of saliva and plaque, which was performed between 8 and 11 a.m. We collected whole unstimulated saliva by asking the child to expectorate into a sterile plastic tube. We were unable to collect saliva from 11 young children. After saliva collection, supragingival plaque was collected separately from: (i) tooth surfaces that were caries-lesion-free (PF; ICDAS = 0); (ii) active, enamel caries lesions (PE; ICDAS = 1-3); and (iii) active, dentinal caries lesions (PD; ICDAS ≥ 4). PF were collected from all participating children, whereas PD and PE were collected only from CA children. Each plaque sample was obtained by pooling material from at least 2 different tooth sites of similar health condition by means of sterile periodontal curettes, and more than one type of sample could have been collected from the same child. PD was recovered from the internal surfaces of dentinal caries lesions without removing the infected dentin as well as from the surrounding margins. No plaque was collected from superficial root surfaces. Plaque samples contaminated by blood were rejected and not analyzed in this study. The plaque samples were transferred to sterile micro-centrifuge tubes containing 250 μL of 10 mM sodium phosphate buffer (pH 7.0). The oral samples were immediately transported on ice to the laboratory to be analyzed or, if necessary, snap-frozen and stored at -80ºC, which does not adversely affect ADS activity.

Before enzymatic assays were conducted, saliva and plaque samples were dispersed by external sonication for 2 cycles of 15 sec, with cooling on ice during the interval. Plaque samples were then washed once with 10 mm Tris-maleate (pH 7.0) and re-suspended in 500 μL of the same buffer. We measured the arginolytic capacity of saliva and plaque bacteria by monitoring citrulline production from arginine (Liu *et al*., 2008). For the accommodation of small samples of site-specific plaque, the assays used a nano-drop scale on the Biotek Synergy H4 with microspot quantification. ADS activity was normalized to protein content (Nascimento *et al*., 2009) and defined as nmol of citrulline generated [minute x (mg protein)] $^{-1}$.

For descriptive analysis, distributions of percentages and means were calculated when appropriate. We used a *t* test or analysis of variance (ANOVA) to evaluate continuous variables, with the chi-square test used for categorical variables. A linear mixed model was also used for data analysis, with the SAS procedure of PROC MIXED. ADS activity was examined as a function of both level 1 (teeth level) predictors and level 2 (individual level) predictors. The Akaike's Information Criterion (AIC) was used to select a better-fitted model, which indicated a smaller AIC value. All data management and statistical analyses were performed with SAS procedures (SAS 9.1.3, SAS Institute Inc., Cary, NC, USA).

RESULTS

The demographic characteristics and DMFT scores of the children who participated in the study are presented in Table 1. The distribution of the children's caries status by type of dentition was: primary (19 CF, 10 CA, and 2 CE), mixed (24 CF, 20 CA, and 7 CE), and permanent (9 CF, 8 CA, and 1 CE). The mean ages for children at the primary, mixed, and permanent dentitions were 3.5 (± 1.2; range, 2-6 yrs), 8.7 (± 2.0; range, 5-13 yrs), and 12.6 (± 0.9; range, 11-14 yrs) yrs, respectively.

The ADS activity levels of oral samples collected from children of different caries status and dentitions are presented in Table 2. ADS activity of saliva samples ranged from 1.1 to 372.1 units (mg protein)$^{-1}$, and no differences were observed in saliva ADS activity among the groups. In general, ADS activity of plaque samples ranging from 0.1 to 968.5 units (mg protein)$^{-1}$ was higher than that of saliva samples. Specifically, the range of ADS activities for the PF samples was 0.1 to 968.5, PD was 34.5 to 753.8, and PE was 34.9 to 747.4 units (mg protein)$^{-1}$ of samples. The mean ADS activity of plaque collected from CF

Table 1. Demographic and Clinical Characteristics of the Study Population

Dentition	Primary			Mixed			Permanent			
Caries Status	Caries-lesion-free	Caries-active	Caries Experienced	Caries-lesion-free	Caries-active	Caries Experienced	Caries-lesion-free	Caries-active	Caries Experienced	Total
Number of Decayed Missing and Filled Teeth (DMFT)	0	4.9 (±2.7)	2 (±0)	0	2.8 (±1.6)	1.6 (±0.8)	0	4.9 (±1.8)	1 (±0)	1.6 (±2.3)
Age	2.9 (± 0.9)	4.2 (± 1.1)	5 (± 0)	8.5 (± 2.0)	9.1 (± 1.8)	8.4 (± 2.1)	12.5 (± 1.1)	12.6 (± 0.8)	13 (± 0)	7.8 (± 3.6)
Gender										
Male	11 (21)	3 (6)	1 (2)	14 (27)	11 (21)	4 (8)	2 (4)	5 (9)	1 (2)	52 (100)
Female	8 (17)	7 (15)	1 (2)	10 (21)	9 (19)	3 (6)	7 (14)	3 (6)	0	48 (100)
Ethnicity										
Hispanic	1 (17)	2 (32)	1 (17)	1 (17)	1 (17)	0	0	0	0	6 (100)
Not Hisp.	18 (19)	8 (9)	1 (1)	23 (24)	19 (20)	7 (7)	9 (10)	8 (9)	1 (1)	94 (100)
Race										
White	12 (21)	3 (5)	2 (3)	17 (30)	9 (16)	6 (11)	7 (12)	1 (2)	0	57 (100)
Black	6 (18)	4 (12)	0	6 (18)	8 (24)	1 (3)	2 (6)	7 (21)	0	34 (100)
Asian	1 (20)	1 (20)	0	0	2 (40)	0	0	0	1 (20)	5 (100)
Other	0	2 (50)	0	1 (25)	1 (25)	0	0	0	0	4 (100)

Age is shown in years. DMFT and age are shown as [Mean (±SD)]; SD, standard deviation. Gender, ethnicity, and race are shown as [N (%)]. Percentages are within rows for each demographic characteristic. Other race: Pacific Islander, Native American, Indian, Alaska Native, Native Hawaiian, and others.

Table 2. Activity Levels of the Arginine Deiminase System of Saliva and Site-specific Dental Plaque Collected from the Children

Dentition	Primary			Mixed			Permanent		
Caries Status	Caries-lesion-free	Caries-active	Caries Experienced	Caries-lesion-free	Caries-active	Caries Experienced	Caries-lesion-free	Caries-active	Caries Experienced
Plaque Caries-lesion-free	329.8 ± 234.6 (25)	301.7 ± 234.5 (11)	368.1 ± 362.9 (3)	403.1 ± 257.5 (42)	397.7 ± 251.9 (20)	258.2 ± 187.0 (11)	381.9 ± 256.1 (17)	540.2 ± 215.7 (10)	421.6 ± 0.0 (2)
Plaque Enamel Caries	-	61.1 (1)	-	-	232.8 ± 208.0 (12)	-	-	141.2 ± 52.9 (6)	-
Plaque Dentinal Caries	-	247.1 ± 220.6 (8)	-	-	220.7 ± 143.0 (18)	-	-	113.9 ± 63.3 (7)	-
Saliva	56.0 ± 39.6 (12)	66.2 ± 65.6 (8)	191.8 ± 122.1 (2)	67.2 ± 30.1 (22)	49.7 ± 64.5 (20)	91.9 ± 102.0 (7)	77.7 ± 106.3 (9)	46.0 ± 27.9 (8)	22.4 (1)

The arginine deiminase system (ADS) activity was expressed as nmol of citrulline generated [minute x (mg of protein)] $^{-1}$. The ADS activity levels of plaque and saliva samples are shown as [Mean ± SD (N)]; SD, standard deviation; N, number of samples.

children (377.1 ± 252.6) of all types of dentition was higher than, but not statistically different from, that of CE (299.3 ± 230.6) and CA (291.7 ± 233.3) children. Among the children with mixed dentition, the mean ADS activity of all plaque from CF (403.1 ± 257.5) was higher than that of CA (294.4 ± 224.2) children, with a marginally significant difference ($p = .059$) between these two groups (Appendix Fig.).

Regardless of caries status or type of dentition, PF (377.1 ± 253.3) samples had significantly higher levels of ADS activity compared with PD (208.4 ± 160.1; $p < .001$) and PE (194.8 ± 176.0; $p < .001$) samples. Although not statistically significant, the distribution of ADS activity by plaque site and type of dentition showed increased ADS activity in PF samples with age, or from primary to permanent dentitions (Fig.). The mean ADS activity of PF (primary = 324.9 ± 247.5; mixed = 379.8 ± 251.9) was significantly higher than that of PD (primary = 247.1 ± 220.6; mixed = 220.7 ± 143.0) and PE (primary = 61.1 ± 0; mixed = 232.8 ± 208.0) for the primary (PF *vs*. PD, $p = .0001$; PF *vs*. PE, $p = .0005$) and mixed (PF *vs*. PD, $p = .003$; PF *vs*. PE, $p = .034$) dentitions. There were comparable levels of ADS activity between PD and PE samples in the mixed and permanent dentitions.

Mixed-model analysis by using plaque caries status and children's age, with dentition and caries status as predictors of ADS activity, demonstrated that plaque caries status is significantly correlated with ADS activity, despite the other factors ($p < .001$), with healthy plaque (PF) predicting higher levels of ADS activity compared with diseased plaque (PD and PE).

DISCUSSION

The most significant finding of this study is that the capacity of plaque bacteria to metabolize arginine varies greatly among children and tooth sites of different caries status, which may profoundly affect the resistance or susceptibility of the hosts to dental caries. Specifically, plaque bacteria from tooth surfaces that were caries-lesion-free had higher ADS activity than those from caries lesions. Importantly, our findings highlight that arginolysis may be a novel and fundamental risk assessment criterion and support the concept that increasing the arginolytic potential of children's dental plaque may be an effective caries intervention strategy. In view of growing efforts to understand the relationship of the composition of the oral microbiome to health and disease, and previous observations that bacterial profiles change with the stages of childhood caries and also differ between the primary and permanent dentitions (Aas *et al*., 2008; Crielaard *et al.*, 2011), our findings provide compelling support for the continued investigation of oral alkali production as a novel approach to the control of caries.

This study also provided the first analysis of the arginolytic potential of bacteria collected from site-specific supragingival plaque. We observed an extremely high degree of variability in ADS activity among plaque samples, in some cases greater than 10,000-fold. In *in vitro* studies, a five-fold decrease in alkali-generating potential was shown to markedly diminish the pH-moderating potential of oral bacteria (Clancy and Burne, 1997; Clancy *et al.*, 2000). Plaque bacteria from tooth surfaces without caries lesions showed the widest range of ADS activities. Even though the bacteria from these "healthy" plaque samples showed an overall greater capacity to metabolize arginine compared with plaque bacteria of caries lesions, high levels of ADS activity were also detected in some samples of carious plaque. Notably, human supragingival plaque harbors a highly diverse bacterial community, and the microbial composition of healthy plaque differs substantially from that of carious plaque (Aas *et al*., 2008). Recent work by our group provides evidence that bacterial heterogeneity in arginolytic capacity is related to intra- and inter-species variation in the regulation of the ADS, likely associated with evolution of adaptive strategies for acid tolerance and nutrient limitation (manuscript submitted). Our research findings to date are consistent with the "ecological plaque hypothesis" (Marsh, 2006), supporting that cariogenesis by oral biofilms is a complex polymicrobial process and disclosing that cariogenic biofilms have both acidogenic and alkalinogenic potentials, as do "healthy" biofilms. Therefore, bacterial metabolism resulting in caries lesions is closely intertwined with the balance between acid and alkali production, and multiple bacterial species with acidogenic or alkalinogenic traits can influence the disease process.

In agreement with our previous study in adults (Nascimento *et al.*, 2009), the ADS activity of plaque samples was generally higher than that produced by salivary samples in children. Higher ureolytic activity in plaque compared with saliva was also observed in a longitudinal study with children (Morou-Bermudez *et al.*, 2011). In fact, the oral microbiota of saliva has been shown to be different from that of plaque in children independent of the presence or absence of caries (Ling *et al*., 2010). In the present study, no differences in salivary ADS activity were detected among the children's caries groups. This observation is also in agreement with the findings of our previous study, in which the use of qPCR did not reveal a statistically significant association between the salivary proportions of 2 recognized arginolytic species, *S. sanguinis* and *S. gordonii*, and the caries status of adults (Nascimento *et al*., 2009). It is possible that salivary ADS levels cannot be used as a predictor of caries risk for children.

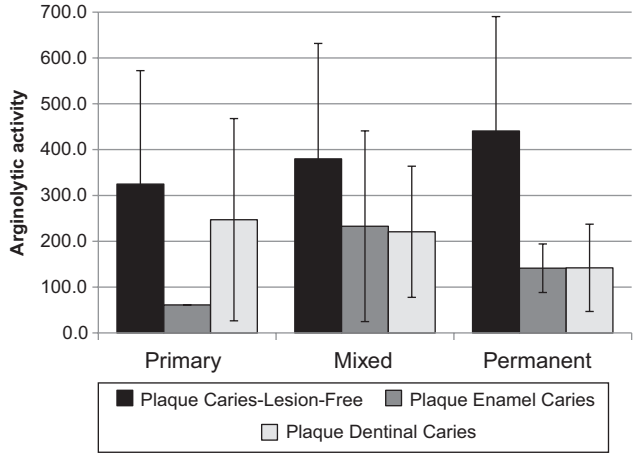

Figure. Mean activity levels of the arginine deiminase system of site-specific plaque from children with different types of dentition. Arginolytic activity is represented by the activity of the arginine deiminase system (ADS) and expressed as nmol of citrulline generated [minute x (mg of protein)]$^{-1}$.

In spite of the abundance of *in vitro* data showing an important role for alkali production in inhibiting caries—and in spite of microbiological and microbiome studies showing associations of base-producing organisms with dental health (Becker *et al.*, 2002; Aas *et al.*, 2008; Gross *et al*., 2010; Crielaard *et al*., 2011)—this study directly addressed the question whether the change in the microbial flora that is seen when caries develops is also associated with a change in the alkali-producing capacity of oral biofilms. It also provides indirect evidence that enhancing arginolytic potential could have the effect of arresting and possibly reversing the caries process. It is even more critical to evaluate these processes in children because of the significant void in our knowledge of the ontogeny and interactions of the oral microbiome in these populations, the higher risk of children for caries, and the potential for rapid translation to caries risk assessment and interventions. Future studies will focus on the physiological and genetic characterization of the oral microbiome of caries-active and caries-lesion-free children, which will greatly assist in defining the microbiological and molecular basis for the heterogeneity in arginolytic capacity of oral biofilms. Similarly, additional clinical studies may be useful to: (i) confirm that the supplementation of arginine to plaque bacteria is effective against caries; (ii) ensure that arginine does not diminish the impact of fluoride in biofilms; and (iii) optimize formulations for caries control. Other areas worthy of investigation include exploring probiotic applications to enhance oral arginolysis and prevent caries. Collectively, this information will facilitate the rationale design of strategies that rely on alkali production for caries risk assessment and interventions.

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