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Nitric oxide modulates levels of salivary *Lactobacilli*

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Abstract

Objective. Nitric Oxide (NO) is one of the most powerful antibacterial compounds. The aim of this study was to determine the association between salivary NO, dental caries and cariogenic bacteria. ***Materials and methods.*** The salivary NO concentration of 257 Korean children was analyzed by the Griess colorimetric reaction method. Salivary mutans streptococci (MS) and *Lactobacilli* (LB) were counted using the Dentocult MS and Dentocult LB kit, respectively. Dental caries status was examined using the WHO criteria. Confounders were age, gender, salivary flow rate and salivary buffer capacity. Analysis of covariance (ANCOVA) was used to evaluate the association among NO, salivary MS level, salivary LB level and dental caries status after adjusting for the effects of confounders. ***Results.*** A significant decrease was found in salivary NO levels as the salivary LB count increased after controlling for confounders ($p = 0.049$). However, the MS level, caries experience and active caries status showed no significant association. ***Conclusion.*** This result indicates that NO production might be a host defense mechanism against the growth of cariogenic bacteria.

Key Words: *cariology, microbiology, preventive dentistry*

Introduction

Dental caries is the most common disease of the oral cavity. Although there has been a steady decrease in the prevalence of coronal caries in Korean children over the last decade, 'high-risk' groups still exist within such populations [1]. Dental caries is an infective condition and the dental plaque/biofilm supports a microecosystem of bacteria that exhibit a variety of physiological characteristics related to their adherence characteristics, aciduric nature and resistance to low pH levels [2]. Because mutans streptococci (MS) are frequently isolated from cavitated caries lesions, induce caries formation in animals fed a sucrose-rich diet and are highly acidogenic and aciduric, these micro-organisms are considered major pathogens responsible for the initiation and progression of dental caries [3]. Additionally, human common salivary protein 1 binds to MS cells and may influence the initial colonization of this pathogenic bacterium onto the tooth surface [4]. However, the presence of other non-MS acidogenic bacteria, such as lactobacilli (LB), could moderate caries outcomes in children [5].

Although nitrate has historically been reported to have deleterious effects in humans, such as infant methemoglobinemia [6], recent evidence suggests a beneficial, antimicrobial role for inorganic nitrate in several systems in humans, including the gastrointestinal tract, oral cavity and skin [7–9]. In the oral cavity, inorganic nitrate is present in saliva in high concentrations. Dietary nitrate, present in large quantities in foods such as leafy green vegetables, is absorbed from the small intestine into the bloodstream. Approximately 25% of this dietary-derived nitrate is secreted into saliva; and ~ 20% of that (5% of the ingested intake) is converted to nitrite in the mouth by commensal bacteria on the tongue [10]. This results in a nitrate concentration in saliva of almost 1500 μM, dependent largely upon dietary nitrate intake. The nitrate concentration of food varies widely according to the quality and preparation of the food; for example, boiling results in the loss of nitrate from most vegetables.

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In the oral cavity, salivary nitrate will come into contact with bacteria that are capable of rapidly reducing nitrate to nitrite as part of their respiration. Areas of low oxygen tension encourage the reduction of nitrate [11]. The ability of the oral cavity to reduce nitrate is known to vary widely between individuals [12]. The product of nitrate respiration by bacteria is nitrite. Bacteria respiring nitrate rapidly extrude nitrite from their periplasm into the surrounding milieu. Thus, saliva in the oral cavity contains almost 1500 μM nitrate and 100 μM nitrite due to bacterial respiration of salivary nitrate. The increase in plasma nitrite seen after a nitrate load is critically dependent on nitrate reduction in the oral cavity by commensal bacteria. The removal of these bacteria with an antibacterial mouthwash attenuates the NO-dependent biological effects [13] and plasma nitrite was 3-fold higher in germ-free mice receiving nitrate compared to placebo [14], supporting the role of nitrate-reducing bacteria in its production.

Nitrite in the presence of acid will form nitrous acid, which, being inherently unstable, can be oxidized or reduced to form a range of nitrogen oxides, most notably nitric oxide (NO). NO is a vital component of mammalian host defense, produced in and by cells comprising the innate immune system, most importantly macrophages. NO is unique as an antimicrobial agent as it can inhibit or kill a broad range of micro-organisms [15]. NO-derived reactive molecules such as peroxynitrite [16] can induce oxidative damage of key pathogen machinery.

Accordingly, when salivary nitrite comes into contact with the acid environment around the teeth created by acid-producing bacteria such as MS and LB, a bolus of antimicrobial compounds, including NO, is formed and has bacteriostatic and possibly bacteriocidal effects [17]. Our hypothesis in this study was that, in the presence of large amounts of nitrite in the oral cavity, the growth and possible survival of cariogenic bacteria would be limited, thereby improving caries status. To test our hypothesis, we investigated whether salivary nitric oxide levels are associated with MS, LB and dental caries in Korean children.

Materials and methods

Study design, ethical considerations and subjects

We designed a cross-sectional study to test our hypothesis. The study protocol was approved by the Institutional Review Board of Pusan National University Hospital at Yangsan campus (IRB2009016) and subjects and their guardians/parents were informed about the aim of this study well in advance and signed consent forms.

Community Child Centers (CCCs) in Korea have provided comprehensive childcare including group meal services and various after-school educational programs to children living in poverty since the Children Welfare Act was passed in 2006. Among the CCCs in Busan, two or three CCCs from every district were asked to participate in this study and a total of 13 CCCs were accepted for the survey. A total of 300 children were chosen from among the 13 selected CCCs (13–44 children were sampled per each CCC). Forty-three children were excluded because of (1) no salivary flow, (2) no response or missing data or (3) refusal to participate by the children and/or their caregivers. The final sample for analysis consisted of 257 children who completed the survey. The subjects comprised 122 boys and 135 girls. Their ages ranged from 6–14 years,with a mean and standard deviation of 10.1 ± 2.2 years.

Oral examination

The state of dentition and the level of dental caries in all individuals were determined following the WHO criteria by the same person (Dr Han DH) using the DMFT index. D3 caries into the dentine threshold were considered dental caries. Examinations were performed using disposable instruments: plane mirrors and CPITN probes. No radiographs were taken. The 1-week interval test–re-test intra-examiner reliability had a κ-index of 0.958 based on 42 subjects.

Evaluation of cariogenic bacteria

All subjects were instructed to refrain from eating or drinking for a minimum of 2 h before the saliva samples were collected (i.e. between 15:00–17:00 pm). Individuals rinsed their mouth with water and collected stimulated whole saliva samples using 50 ml ice-chilled Falcon screw cap tubes. Stimulated whole saliva samples were obtained from subjects after they chewed for 5 min on wax blocks from commercial kits. Salivary levels of MS and LB were estimated using the Dentocult SM and Dentocult LB kits (Orion Diagnostica Co. Ltd, Epsom, Finland), respectively. The incubation time for the Dentocult SM kit was 48 h and that for the Dentocult LB kit was 96 h. MS and LB levels were evaluated using the model charts provided with the kits.

Saliva collection and preparation

To evaluate the amount of NO in the saliva, collected stimulated whole saliva was centrifuged at 13,000 rpm for 10 min at 4°C. Then, supernatants were aliquoted into 1.5 ml Eppendorf tubes and frozen at −80°C until use.

NO measurement

Salivary nitrite levels were measured using the Griess colorimetric reaction [18]. The Griess reagent is a

Table I. Characteristics of the study subjects (n = 257).

Variables		DMFT			DT		
		No (n = 133)	Yes (n = 124)	p^*	No (n = 180)	Yes (n = 77)	p^*
Age (years), mean ± SD		9.50 ± 2.20	10.75 ± 2.13	<0.001	9.81 ± 2.25	10.78 ± 2.12	0.001
Gender	Male	71 (53.4)	51 (41.1)	0.049	91 (50.6)	31 (40.3)	0.130
	Female	62 (46.6)	73 (59.9)		89 (49.4)	46 (59.7)	
Salivary flow rate (ml/min), mean ± SD		1.38 ± 1.27	1.66 ± 1.90	0.157	1.45 ± 1.45	1.66 ± 1.94	0.348
Salivary buffering capacity	Low	18 (13.5)	17 (13.7)	0.314	22 (12.2)	13 (16.9)	0.407
	Medium	36 (27.1)	44 (35.5)		54 (30.0)	26 (33.8)	
	High	79 (59.4)	63 (50.8)		104 (57.8)	38 (49.4)	
Salivary MS level	Low	43 (32.3)	33 (26.6)	0.014	57 (31.7)	19 (24.7)	0.005
	Medium	50 (37.6)	32 (25.8)		65 (36.1)	17 (22.1)	
	High	40 (30.1)	59 (47.6)		58 (32.2)	41 (53.2)	
Salivary LB level	Low	61 (45.9)	43 (34.7)	0.028	77 (42.8)	27 (35.1)	0.075
	Medium	51 (38.3)	45 (36.3)		70 (38.9)	26 (33.8)	
	High	21 (15.8)	36 (29.0)		33 (18.3)	24 (31.2)	

* Calculated using a chi-square test for categorical variables (gender, salivary buffering capacity, salivary MS level and salivary LB level) and an independent t-test for continuous variables (age and salivary flow rate).
Italics denotes significant at $p < 0.05$.

1:1 mixture of 1% sulfanilamide in 5% orthophosphoric acid and 0.1% N-naphthylethylene diamine dichloride in distilled water (v/v). This reagent reacts with nitrite and produces a purple azo dye end-product that can be measured spectrophotometrically based on the maximum absorbance at 570 nm. Triplicate samples of saliva (50 µL) were transferred to a 96-well ELISA plate and an equal volume of the Griess reagent was added to each well. To obtain standard curves, triplicates of sodium nitrite ($NaNO_2$) in PBS (pH 7.2) at concentrations of 100, 50, 25, 12.6, 6.25, 3.12, 1.56 and 0.78 µM were also included. After 10 min, the optical density was measured using an ELISA plate reader (Sunrise^{TM}, Männedorf, Switzerland) with a 570-nm filter.

Assessment of confounders

Age, gender, salivary flow rate and salivary buffer capacity were selected as confounders. Age and gender were obtained by interview. Salivary flow rate was measured as volume per min. Salivary buffering capacity was evaluated using Dentobuff strips (Orion Diagnostica Co. Ltd., Epsom, Finland).

Statistical analysis

The dependent variable was the level of NO in the saliva. Independent variables were the salivary MS level, salivary LB level and dental caries status. Scores for salivary MS level, salivary LB level and salivary buffering capacity were evaluated using the charts provided with the Dentocult SM kit, Dentocult LB kit and Dentobuff. Scores were categorized as 0 = low, 1 = medium or 2 ~ 3 = high. Subject characteristics were described using frequency distributions for the categorical variables and means with standard deviations (SD) for the continuous variables. The Chi-square test was used to assess the significance of differences in categorical variables, while the t-test was used to assess the significance of differences in continuous variables according to the presence of DMFT and DT. Analysis of covariance (ANCOVA) was used to evaluate the association among NO, salivary MS level, salivary LB level and dental caries status after adjusting for the effects of confounders (age, gender, salivary flow rate, salivary buffering capacity).

Results

The number of children who had DMFT differed significantly according to age, gender, salivary MS level and salivary LB level. The number of subjects who had active caries varied significantly according to age and salivary MS level (Table I). Older children had more DMFT and DT. Girls had more DMFT. Those subjects with higher salivary MS levels had higher DMFT and DT and children who had higher salivary LB levels showed higher DMFT (Table I).

After controlling for age, gender, salivary flow rate and salivary buffering capacity, the salivary MS level had no significant association with the concentration of salivary NO level (mean ± standard error [SE]: 58.94 ± 15.79 µmol/L for a low salivary MS level, 109.75 ± 14.96 µmol/L for a medium salivary MS level and 74.23 ± 13.70 µmol/L for a high salivary MS level, p = 0.055) (Figure 1). The adjusted concentration of salivary NO was shown to have a significant inverse

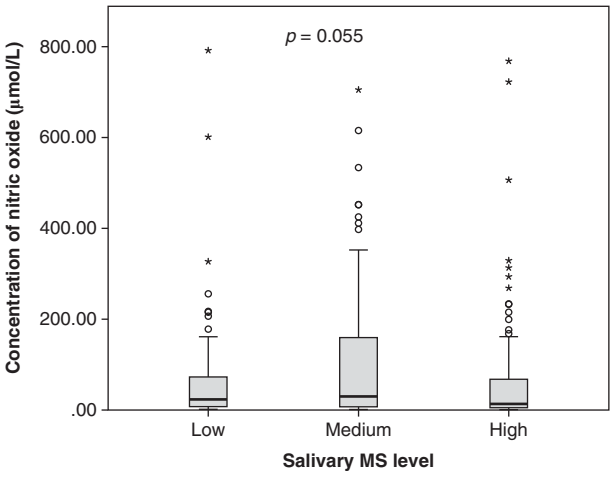

Figure 1. Distribution of NO levels according to salivary MS levels (low corresponds to a score of 0 using the chart provided with the Dentocult SM kit, medium corresponds to a score of 1 and high corresponds to a score of 2 ~ 3, $n = 257$). In the box-plots, the lower and upper margins of the boxes indicate the 25th and 75th percentiles, respectively, and the middle lines are medians. The circles are mild outliers and the asterisks are extreme outliers. p-values were obtained from analysis of covariance (ANCOVA) adjusted for age, gender, salivary flow rate and salivary buffering capacity.

correlation with the salivary LB level (mean ± SE: 104.64 ± 13.30 µmol/L for a low salivary LB level, 72.87 ± 13.84 µmol/L for a medium salivary LB level and 51.74 ± 18.05 µmol/L for a high salivary LB level, $p = 0.049$) (Figure 2). However, dental caries indices such as DMFT and DT did not show a significant association with the salivary NO level (mean ± SE: 67.81 ± 12.07 µmol/L for DMFT = 0, 109.91 ± 17.92 µmol/L for DMFT = 1 and 81.01 ± 17.22 µmol/L for DMFT ≥ 2, $p = 0.165$; 81.08 ± 10.24 µmol/L for DT = 0, 96.82 ± 20.28 µmol/L for DT = 1 and 53.17 ± 25.99 µmol/L for DMFT ≥ 2, $p = 0.424$) (Figures 3 and 4).

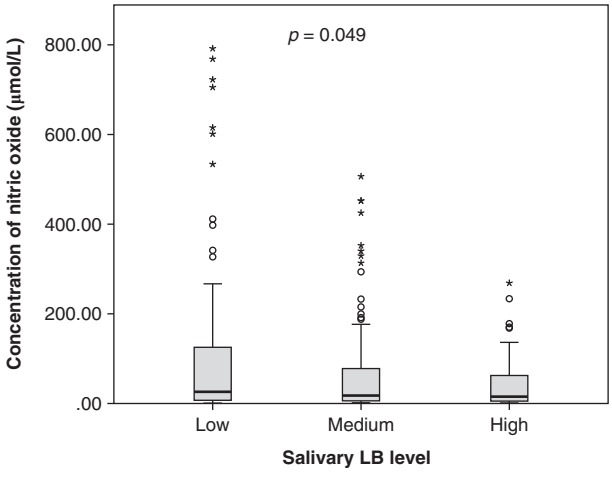

Figure 2. Distribution of NO levels according to the salivary LB level (low corresponds to a score of 0 using the chart provided with the Dentocult LB kit, medium corresponds to a score of 1 and high corresponds to a score of 2 ~ 3, $n = 257$). In the box-plots, the lower and upper margins of the boxes indicate the 25th and 75th percentiles, respectively, and the middle lines are medians. The circles are mild outliers and the asterisks are extreme outliers. p-values were obtained from analysis of covariance (ANCOVA) adjusted for age, gender, salivary flow rate and salivary buffering capacity.

Discussion

MS and LB inhabit caries lesions and are released in the saliva. Collection of salivary constituents is a simple, non-invasive procedure that can be performed by an individual without a high level of technical expertise. In addition, saliva samples can be easily frozen and sent to laboratories for analysis of markers such as NO. In particular, the Griess reaction is a practical diagnostic tool because it is a simple, highly specific and extremely sensitive method for measuring micro-molar concentrations of nitrite. Hence, we measured salivary NO production using the Griess reaction to assess the link among cariogenic bacteria, dental caries and oxidative stress.

We found that concentrations of NO were significantly lower in children with higher levels of salivary LB, which indicates that nitric oxide synthesis is decreased in the presence of aciduric bacteria such as species in the genus *Lactobacillus*.

The mechanism by which nitrite inhibits bacterial growth is of considerable interest and has been studied for over a decade [19]. Bacteriostatic compounds derived from nitrite include nitrous acid, peroxynitrite, nitrosothiols and N-nitroso compounds. Nitric oxide formed via nitrous acid reacts with iron sulfur proteins and interferes with energy metabolism. NO is a normal metabolite for some bacteria. The well-known foodborne pathogens *Clostridium* and *Listeria*, to which NO is toxic, generally have low concentrations of the enzymes involved in nitrite metabolism, e.g. nitrite reductase [20].

Increased production of NO in patients with dental caries and during plaque deposition has been reported previously [21,22]. The incidence of caries in individuals with high oral levels of NO was not reduced, indicating that high NO is not able to inhibit plaque cariogenicity. The production of NO has been postulated to be an early event in host defense against pathogens. Salivary streptococci were reported to generate NO during incubation with nitrite [23] and a recent study also reported that pathogenic *S. mutans* generate NO [24].

To the best of our knowledge, this study is the first to demonstrate a linear relationship between salivary NO levels and salivary LB levels. Our data suggest that high levels of salivary NO are associated with a reduction in salivary LB levels. MS was identified early on as a major player in active caries and has been the primary subject of many caries research studies ever since. LB was also classified as cariogenic based on their ability to ferment carbohydrates into acids similar to MS [25]. The MS group of bacteria includes *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*)

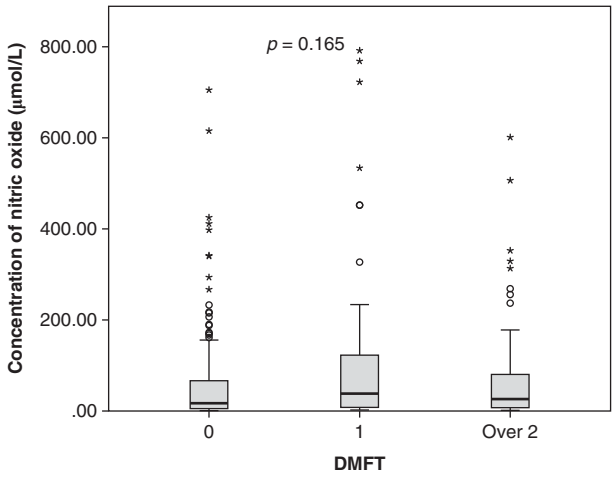

Figure 3. Distribution of NO levels according to DMFT ($n = 257$). In the box-plots, the lower and upper margins of the boxes indicate the 25th and 75th percentiles, respectively, and the middle lines are medians. The circles are mild outliers and the asterisks are extreme outliers. *p*-values were obtained from analysis of covariance (ANCOVA) adjusted for age, gender, salivary flow rate and salivary buffering capacity.

reside within the oral biofilm on the tooth surface and their virulence factors have been well characterized. The key virulence factors are acidogenicity, acid tolerance and synthesis of water-insoluble glucan from sucrose [26,27]. Meanwhile, LB are highly aciduric and are often isolated from established carious lesions [28]. In contrast to the diversity of species able to function at an initial pH of 5.5, the diversity was dramatically reduced after 36 h during incubation at an initial pH of 4.5. Members of the genus LB became dominant [29]. Large numbers of LB have been detected in carious dentin [30]. Nitrate in saliva may enhance oral immunity via the production of

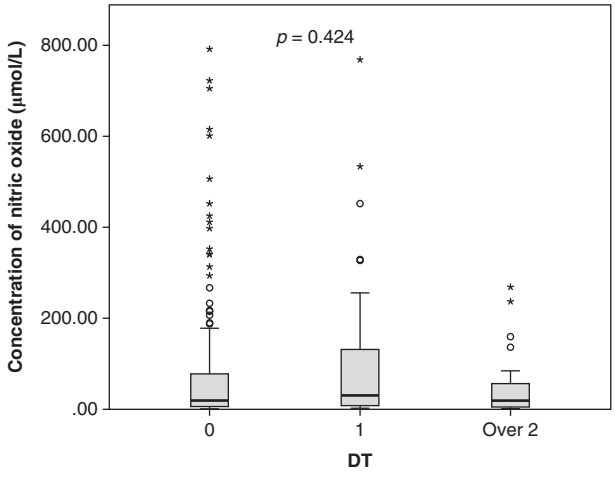

Figure 4. Distribution of NO levels according to DT ($n = 257$). In the box-plots, the lower and upper margins of the boxes indicate the 25th and 75th percentiles, respectively, and the middle lines are medians. The circles are mild outliers and the asterisks are extreme outliers. *p*-values were obtained from analysis of covariance (ANCOVA) adjusted for age, gender, salivary flow rate and salivary buffering capacity.

antimicrobial oxides of nitrogen. Accordingly, individuals with a high nitrate intake and an oral flora with the ability to reduce large amounts of nitrate to nitrite in the oral cavity may be protected against cariogenic acidogenic bacteria and, thus, dental caries.

To test the hypothesis that a high nitrite level in saliva could be protective against dental caries, we examined the levels of salivary nitrite, *S. mutans* and *Lactobacillus* spp. counts in saliva and the caries status of children recruited from CCCs across Korea. We found that those children with high salivary nitrite levels had significantly lower salivary LB levels, lending support to the hypothesis that salivary NO levels may play an important role in the innate immune response in the oral cavity. Further circumstantial evidence that nitrate reduction could be important as a defense mechanism against dental caries was provided by Doel et al. [7]. However, we did not find a significant association between salivary NO levels and dental caries. Additional studies are required to test our hypothesis and to determine the biological mechanisms underlying the link between NO and dental caries.

Although we found an association between salivary NO levels and salivary LB levels, these results could be due to several factors. First, measurement errors in salivary NO levels could have distorted the actual amount of salivary NO. Second, physiologic variables may influence the level of salivary NO levels: salivation itself may have a direct effect on NO levels and environmental factors such as diet, hormone levels and cells may indirectly influence NO levels. Third, it is now accepted that, in nature, oral bacteria exist for the most part attached to a tooth surface as a biofilm, often as a member of a polymicrobial community (or consortium) of interacting species. In health, the microbial composition of dental plaque is diverse and remains relatively stable over time (microbial homeostasis). Under certain circumstances, this microbial homeostasis can break down and diseases such as caries can occur. In dental caries, there is a shift toward increased proportions of acid-producing and acid-tolerating species, such as *mutans streptococci* and *Lactobacilli*, although other species with relevant traits can participate in demineralization [31]. However, we examined the activity of nitric oxide against individual bacteria. An appreciation of dynamic ecological dental plaque would have been critical to fully understand the relationship between the oral microflora and the host in oral health or dental caries. Application of novel imaging (confocal or epifluorescence microscopy, fluorescence *in situ* hybridization, live/dead stains, etc.) and molecular techniques (16S rRNA gene amplification and sequence comparison, proteomics, transcriptomics, reporter gene technology, etc.) would be needed in further study. Finally, there may have been selection bias when we chose subjects. Although our subjects were not a representative sample, the bias could be non-differential and thereby affect the association we found.

Further comprehensive and detailed prospective studies are required to clarify the causality between salivary NO levels and dental caries. Studies that evaluate representative samples and additional modifying factors may provide more insight into the association between salivary NO levels and dental caries.

Conclusions

Our results indicate that a high level of salivary NO could be a protective factor against *Lactobacillus* spp. in saliva. The concentration of nitric oxide in the saliva could potentially be used as a biomarker for evaluating dental caries status in children.

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