Dental Research Journal

Original Article

Evaluation of salivary nitric oxide level in children with early childhood caries

AR. Senthil Eagappan[1], V. Arun Prasad Rao[2], S. Sujatha[3], D. Senthil[1], J. Sathiyajeeva[4], G. Rajaraman[5]

[1]Department of Pedodontics and Preventive Dentistry, Chettinad Dental College and Research Institute, Kelambakkam, [5]Department of Conservative Dentistry and Endodontics, Rajah Muthiah Dental College and Hospital, Chidambaram, [3]Department of Pedodontics, Saveetha Dental College and Hospital, [4]Department of Oral Pathology and Microbiology, Thai Moogambigai Dental College and Hospital, Chennai, [2]Department of Pedodontics and Preventive Dentistry, Mahatma Gandhi Post Graduate Institute of Dental Sciences, Puducherry, India

Received: November 2015
Accepted: March 2016

Address for correspondence:
Dr. AR. Senthil Eagappan, Department of Pedodontics and Preventive Dentistry, Chettinad Dental College and Research Institute, Chettinad Health City, Kelambakkam, Kancheepuram - 603 103, Tamil Nadu, India.
E-mail: dr.eaga_ars@yahoo.com

ABSTRACT

Background: Nitric oxide (NO), a highly reactive radical, participates in the nonspecific natural defense mechanism of the oral cavity. The present study was attempted to evaluate the salivary NO levels in 4–5 year-old children with early childhood caries (ECC). The objective of the present study was to assess the salivary NO concentration in children with different caries activity.

Materials and Methods: The study included 120 healthy 4–5 year-old children and they were equally divided into three groups based on decayed, missing, filled surfaces (dmfs) score; forty caries-free children (control group), forty children with dmfs 1–5 (ECC group), and forty with dmfs ≥6 (severe ECC group). Saliva collected was measured for NO concentration by Griess reaction method. The obtained data were analyzed by ANOVA and Pearson's correlation coefficient.

Results: The mean level of NO in the saliva of the control group was 51.2 ± 8.3457 and that of ECC and severe ECC were 47.1 ± 5.2614 and 33.625 ± 4.6942, respectively. The mean salivary NO concentration was significantly higher in healthy controls when compared to children with ECC and severe ECC. Moreover, a negative correlation (r = −0.6658) was observed between the salivary NO level and the mean dmfs, suggesting that as the salivary NO level decreases, the caries incidence increases.

Conclusion: The obtained results support the antimicrobial activity of salivary NO and also suggest that an increase in NO production might contribute to lower the caries occurrence in children.

Key Words: Dental caries, nitric oxide, saliva

INTRODUCTION

Dental caries is a multifactorial disease caused by the interaction of internal defense factors such as saliva, tooth surface morphology, nutritional and hormonal status, and external factors such as microbial flora, oral hygiene, diet, and fluoride availability.[1] Early childhood caries (ECC) is a virulent form of dental caries that can destroy the primary dentition of infants and preschool children.[2]

Saliva has been extensively studied in relation to dental caries because it can be easily collected, and it also allows the analysis of several local and/or systemic biological markers such as proteins, enzymes,

Access this article online

Website: www.drj.ir
www.drjjournal.net
www.ncbi.nlm.nih.gov/pmc/journals/1480

How to cite this article: Senthil Eagappan AR, Rao VA, Sujatha S, Senthil D, Sathiyajeeva J, Rajaraman G. Evaluation of salivary nitric oxide level in children with early childhood caries. Dent Res J 2016;13:338-41.

hormones, antibacterial products, host cells, and ions. Theoretically, saliva can affect the prevalence of dental caries in four general ways; first, through the cleansing effect which results in less accumulation of plaque; second, by reducing enamel solubility by means of calcium, phosphate, and fluoride; third, by the buffering effect (buffers and neutralizes the acids produced by the cariogenic microflora); and finally, by antibacterial activity.[3] The antimicrobial effect of salivary nitric oxide (NO) metabolites (nitrates and nitrites) and its effect on protection against oral diseases have been analyzed extensively in the recent years.

NO is a short-lived gas and it acts as a free radical since it contains an extra electron that enables high chemical reactivity.[4] Being a strong reactive radical, NO participates in the nonspecific defensive mechanisms of the oral cavity.[5] It expresses its antibacterial effect in two ways – by inhibition of bacterial growth and/or by increase of macrophages-mediated cytotoxicity from saliva.[6]

NO appears in oral cavity either by the physiological reduction of dietary nitrates or from L-arginine undergoing the reaction catalyzed by inducible nitric oxide synthase,[7] the enzyme expressed in salivary glands. Some authors have proved the efficiency of salivary nitrite to have an inhibitory effect on growth and survival of cariogenic bacteria in acid through microbiological tests.[8,9] Clinically, very few studies have assessed the relationship between salivary NO and severity of dental caries in young children. Moreover, these studies have yielded mixed results indicating the need for further studies.

Hence, the present study was taken up with a view to finding the relationship between caries severity and salivary NO levels by measuring the levels of salivary NO in children with ECC, severe ECC, and healthy controls. Establishing a correlation between severity of ECC and salivary NO levels could in turn have implications for prevention of ECC in young children.

MATERIALS AND METHODS

Sample selection

The study protocol was approved by the Ethical Committee of Rajah Muthiah Dental College, Annamalai University, and informed consent was obtained from the parents of the children. About 250 healthy children in the age group of 4–5 years were selected from the schools of Chidambaram, Tamil Nadu, India. Exclusion criteria included children with systemic diseases, long-term medications, and children under antibiotic treatment for any immediate past or present illness.

Dental examination was carried out by an experienced dentist using mouth mirror under room light. The caries status was determined by dental caries index: Decayed, missing, filled surfaces [dmfs] according to the WHO criteria. Based on the dmfs score, children were divided into three groups:

- Group I (control): dmfs score - nil
- Group II (ECC): dmfs score of 1–5
- Group III (severe ECC): dmfs score ≥6.

Inclusion criteria for Group II and III were based on the NIH case definition.[10] Following the dental examination, based on the above-described criteria, 131 children fell in the caries-free group; 76 in the ECC group; and 43 in the severe ECC group. From the three groups, forty children were randomly selected from each group. The final sample consisted of 120 children with forty children in each group.

Sample collection

The subjects were instructed to refrain from eating and drinking for a minimum of 3 h before saliva collection. Approximately 2–2.5 ml of unstimulated saliva was collected by asking the patient to passively drool into a funnel inserted into a vial. The collected saliva was centrifuged, and the obtained supernatant was stored in the refrigerator at 4°C temperature. NO evaluation was done within 24 h by Griess reaction method.[11]

Sample analysis

NO is a highly reactive free radical gas that is a ready oxidizer and remains stored in tissues as nitrates (NO_3^-) or nitrites (NO_2^-).[12] Hence, in the present study, NO concentration was measured as total nitrates and nitrites (NO_3 + NO_2) by the Griess reaction method.

First, NO_3^- is reduced to NO_2^- in a salivary sample by reduced nicotinamide adenine dinucleotide phosphate in the presence of enzyme nitrate reductase. This is followed by the calorimetric Griess reaction to measure NO_2^- levels. Salivary samples (50 µl) were transferred to a 96-well enzyme-linked immunosorbent assay plate. Using a multichannel pipettor, 50 µl of the sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) followed by 50 µl of the naphthyl ethylene diamine solution (0.1% N-naphthyl ethylene diamine) was dispensed to all experimental samples.

The samples were incubated at room temperature for 5–10 min. A red/purple color was observed and its optical density was measured using a plate reader with 540 nm filter.

Statistical analysis

Salivary NO concentrations among the three groups were compared for statistical significance by ANOVA. Results are presented as a mean ± standard deviation. Pearson correlation coefficient (r) was determined to evaluate the possible relation between NO level and caries severity.

RESULTS

Analysis of NO levels revealed that the mean concentration of NO in the control group (51.2 μM) was higher than in the ECC (47.1 μM) and severe ECC group (33.625 μM) [Table 1]. The significance was confirmed with ANOVA, which revealed a statistically significant difference between the three groups [Table 2]. The *post hoc* Tukey's honestly significant difference test revealed the NO levels in the control group were significantly higher than in the severe ECC group ($P < 0.01$) and ECC group ($P < 0.05$).

Since children from control group did not have caries, the investigation of the correlation between dmfs and salivary NO concentration was conducted only in the ECC and severe ECC groups. The two groups were clustered together, and Pearson's correlation coefficient was determined. A negative correlation ($r = -0.6658$) was observed between the salivary NO level and the mean dmfs, suggesting that as the salivary NO level decreases, the caries incidence increases.

Table 1: Descriptive statistics of salivary nitric oxide concentration in control, early childhood caries, and severe early childhood caries groups

Groups	*N*	Mean (NO) μM±SD	Minimum	Maximum
Group I - Control	40	51.2±8.3457	40	71
Group II - ECC	40	47.1±5.2614	37	58
Group III - Severe ECC	40	33.625±4.6942	25	43

ECC: Early childhood caries; NO: Nitric oxide; SD: Standard deviation

Table 2: Analysis of variance - salivary nitric oxide concentration

Source	Sum of squares	df	Mean square	*F*	*P*
Between groups	6763.550	2	3381.775	84.992	0.0001 (significant)
Within groups	4655.374	117	39.790		
Total	11,418.924	119			

DISCUSSION

In the oral cavity, salivary nitrate comes in contact with bacteria that are capable of rapidly reducing nitrate to nitrite as part of their respiration.[13] Several nitrite producing organisms have been identified in saliva include *Veillonella* species, *Staphylococcus aureus* and *S. epidermis*, *Nocardia* species, and *Corynebacterium pseudodiptheriticum*.[14] These bacteria are facultative anaerobes which, under hypoxic conditions, use nitrate instead of oxygen as an electron acceptor to oxidize carbon components to manufacture adenosine triphosphate and derive energy. In doing so, they reduce the nitrate to nitrite. For the bacteria, nitrite is a waste product but for the mammalian host, it is an antimicrobial agent.[15]

This nitrite, when it encounters the acid environment around the teeth, leads to the formation of the complex mixture of nitrous oxide and nitrous acid. Acid surrounding is obtained by the cariogenic bacteria (*Lactobacillus*, *Streptococcus mutans*, and *Actinomyces*). This nitrous acid is unstable and will spontaneously decompose to produce nitric oxide and nitric dioxide (NO_2).[6] NO has a strong antibacterial effect. It can easily penetrate the cell membrane and cause damage to the microorganism by inhibiting iron-containing DNA synthases, reacting with an iron-sulfur center of mitochondrial respiratory chain enzymes or by combining with superoxide to highly reactive hydroxyl radical.[16] The antimicrobial effects of NO result in autoinhibition of cariogenic bacteria through the mechanism described above.

The antibacterial effect of NO is also reported by Han *et al*.[17] They found a significant inverse correlation between salivary NO level and salivary lactobacilli count among 257 Korean children suggesting that a high level of salivary NO could be a protective factor against *Lactobacillus* spp.

The results of the present study showed that the concentration of NO and its metabolites in saliva of children with healthy natural teeth (control group) is significantly higher compared to ECC and severe ECC group, suggesting the defense role of NO in relation to caries. In line with our results, the previous studies have found an inverse association between the salivary NO and dental caries in children[13,18] and adults[19] whereas some studies have reported

contradicted results.[20,21] These latter studies that have reported contradicted results are in disagreement with the vast majority of literature that describes the lower concentration of NO in children with dental caries.

The result obtained in our study is also supported by findings of Li *et al*.,[22] who suggested that nitrate is an electron acceptor that represses acid fermentation and thereby buffers salivary acidity which in turn helps in protecting the tooth against dental caries.

In the present study, the salivary NO levels were correlated with dental caries severity. This yielded a higher negative correlation value which implies, a decrease in salivary NO concentration was associated with an increase in caries severity.

CONCLUSION

Prevalence of dental caries is on the rise, especially in the developing countries. In order to prevent this complex disease, an in-depth understanding of the risk factors is needed. The results of the current study bring to light the level of salivary NO as a biomarker for dental caries.

Financial support and sponsorship
Nil.

Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

REFERENCES

1. Lenander-Lumikari M, Loimaranta V. Saliva and dental caries. Adv Dent Res 2000;14:40-7.
2. Msefer S. Importance of Early Diagnosis of Early Childhood Caries. JODQ 2006;Suppl April:6-8.
3. Llena-Puy C. The role of saliva in maintaining oral health and as an aid to diagnosis. Med Oral Patol Oral Cir Bucal 2006;11:E449-55.
4. Moncada S, Palmer RM, Higgs EA. Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-42.
5. Pašić E, Dedić A, Huskić J, Hadžić S. Concentration of nitric oxide in patients' saliva from various metal restorative activities in the oral cavity. Acta Med Acad 2011;40:159-65.
6. Surdilovic D, Stojanovic I, Apostolovic M, Igic M, Kostadinovic L. The role of nitric oxide in saliva in reduction of caries. Acta Fac Med Naissensis 2008;25:93-5.
7. Olin AC, Aldenbratt A, Ekman A, Ljungkvist G, Jungersten L, Alving K, *et al.* Increased nitric oxide in exhaled air after intake of a nitrate-rich meal. Respir Med 2001;95:153-8.
8. Silva Mendez LS, Allaker RP, Hardie JM, Benjamin N. Antimicrobial effect of acidified nitrite on cariogenic bacteria. Oral Microbiol Immunol 1999;14:391-2.
9. Xia DS, Liu Y, Zhang CM, Yang SH, Wang SL. Antimicrobial effect of acidified nitrate and nitrite on six common oral pathogens *in vitro*. Chin Med J (Engl) 2006;119:1904-9.
10. Drury TF, Horowitz AM, Ismail AI, Maertens MP, Rozier RG, Selwitz RH. Diagnosing and reporting early childhood caries for research purposes. A report of a workshop sponsored by the National Institute of Dental and Craniofacial Research, the Health Resources and Services Administration, and the Health Care Financing Administration. J Public Health Dent 1999;59:192-7.
11. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors 2003;3:276-84.
12. Menaka KB, Ramesh A, Thomas B, Kumari NS. Estimation of nitric oxide as an inflammatory marker in periodontitis. J Indian Soc Periodontol 2009;13:75-8.
13. Doel JJ, Hector MP, Amirtham CV, Al-Anzan LA, Benjamin N, Allaker RP. Protective effect of salivary nitrate and microbial nitrate reductase activity against caries. Eur J Oral Sci 2004;112:424-8.
14. Li H, Duncan C, Townend J, Killham K, Smith LM, Johnston P, *et al.* Nitrate-reducing bacteria on rat tongues. Appl Environ Microbiol 1997;63:924-30.
15. Benjamin N. Nitrates in the human diet- good or bad? Ann Zootechnie 2000;49:207-16.
16. Hegde MN, Kumari S, Hegde N, Shetty S, Nireeksha. Evaluation of the status of salivary nitric oxide in patients with dental caries. Nitte Univ J Health Sci 2012;2:6-9.
17. Han DH, Kim MJ, Jun EJ, Kim JB. Nitric oxide modulates levels of salivary lactobacilli. Acta Odontol Scand 2013;71:1156-61.
18. Hegde AM, Neekhra V, Shetty S. Evaluation of levels of nitric oxide in saliva of children with rampant caries and early childhood caries: A comparative study. J Clin Pediatr Dent 2008;32:283-6.
19. Hegde MN, Hegde ND, Ashok A, Shetty S. Salivary nitric oxide (NO_2 + NO_3) as biomarker of dental caries in adults: An *in vivo* study. Int Res J Pharm 2012;3:100-2.
20. Javadinejad SH, Talebi M, Aslani G. Nitric oxide concentrations in saliva in relation to caries; experience in 6-12 years old children. J Isfahan Dent Sch 2007;3:71-5.
21. Ghasempour M, Qujeq D, Rabiee M, Hamzeh M. Measurement of nitrite and nitrate in saliva of children with different caries activity. J Contemp Dent Pract 2014;15:623-5.
22. Li H, Thompson I, Carter P, Whiteley A, Bailey M, Leifert C, *et al.* Salivary nitrate – An ecological factor in reducing oral acidity. Oral Microbiol Immunol 2007;22:67-71.