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Clinical assessment and estimation of salivary nitric oxide levels and its relation to dental caries in cardiac compromised children- An *in vivo* study

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Abstract

Eight to ten out of every thousand live infants are affected by congenital heart disease (CHD). Studies show increased caries risk in CHD patients, with recent interest in salivary nitrites' antimicrobial effects. This research was done to evaluate the relationship among salivary nitric oxide levels and dental caries status, estimating nitric oxide levels and DMFT scores and exploring their correlation in children with and without congenital heart disease. The children werecategorized into two groups of 30 each: Study group with 30 children having Congenital Heart-Disease and Control group with 30 children without Congenital Heart-Disease. In both the groups, caries status was measured using DMFT index scores & 5ml of unstimulated saliva was collected for the estimation of salivary nitric oxide levels.An unpaired t-test was conducted using SPSS Version 25.0 to analyze the data. In the study group, mean salivary nitric oxide levels were 64.03 ± 8.86, compared to 133.59 ± 14.32 in the control group, with a considerable variation ($p < 0.005$). DMFT scores differed significantly between the groups ($p < 0.005$).Salivary nitric oxide emerges as a potential biomarker for caries risk. Dietary nitrate and probiotic therapy could offer significant protection. Boosting salivary NO levels may enhance caries protection in CHD children, pending further research validation.

Keywords: Congenital Heart Disease, Salivary Nitric Oxide, Caries.

Full length article **Corresponding Author*, e-mail:–drmittal81@gmail.com

1. Introduction

In 8–10 out of every 1000 live births, congenital heart disease (CHD) is one of the most common developmental disorders in children. These disorders, which are thought to be the most prevalent kind of birth defects, are anomalies in the cardiocirculatory system or function brought on by aberrant heart development during fetal life[1].Chronic poor oral health is one of the co-morbidities caused either by the complex heart disease or by the therapy. Dental caries and periodontal problems are one of the most important oral health concerns in these patients.This is attributed to many factors that include: Difficulty in nutrition during early stage of life that is associated with frequent vomiting that increases the need for frequent and night meals for compensation of energy requirements of the body; Sucrose containing syrups as medications; Ignorance of oral hygiene owing to a more serious illness etc.,[2].Dental caries is an infectious microbiologic disorder that begins with an acid attack on tooth enamel. This acid attack is mostly caused by bacteria like Lactobacillus species and Streptococcus mutans, which are involved in the metabolism of carbohydrates.While, Lactobacillus is present in the oral cavity even prior to the onset of tooth eruption, S. mutans colonizes the oral cavity following the first tooth eruption [3].

S. Mutans is mainly responsible for initiation of enamel carious lesion followed by involvement of lactobacilli in the advanced deep carious lesions.Previous studies highlight factors increasing susceptibility to caries in children with heart conditions which are accelerated streptococcus mutans growth in CHD patientsand structural enamel and dentin alterations observed by Yousry M. El-Hawary et al., (2014)[4-5].Nonetheless, the importance of nitrates and nitrites in preventing dental caries has drawn more attention in recent years [6].Salivary nitrates are readily converted into nitrites by some bacteria in the oral cavity under anaerobic conditions. This nitrite exerts an antimicrobial effect against the acid producing streptococci thus showing an anti-cariogenic effect.Nitrates,final oxidation products of NO, are crucial for cardiovascular and gastric mucosa protection, as well as various metabolic processes. NO is synthesized via two pathways: The Intrinsic Pathway, where oxygen-dependent Nitric Oxide synthase generates NO from L-arginine, and the Extrinsic Pathway, activated under hypoxia/ischemia, involving non-enzymatic synthesis from nitrates and nitrites via the nitrate–nitrite–NO pathway[7]. This shift in systemic nitrates toward NO production may affect nitrate concentrations in salivary glands and saliva.Thus, this study aims to assess salivary nitric oxide levels and their correlation with dental caries status in children with or without congenital heart diseases (CHD) and with objectives to

- Estimate NO levels in the saliva of children with or without CHD.
- Evaluate dental caries status in these children using DMFT Scores.Investigate the correlation between salivary NO levels and dentalcaries occurrence.

2. Materials and Methods

Children between the ages of 4 and 14 participated in the current study, which was carried out at the Government Dental College and Hospital in Ahmedabad's Pedodontics and Preventive Dentistry Department. The Institutional Ethical Committee approved the study protocol. All parents/guardians of the children involved in this study gave their informed consent.Thekids were split into two equal groups: the study group and the control group. Salivary nitric oxide levels were estimated in both groups using 5ml of unstimulated saliva and DMFT index scores to determine the caries state. The thirty children in the study group had congenital heart disease and range in age from 4 to 14 years. They do not have oral lesions and have not taken any vitamins or other medications unrelated to cardiac disease in the last six months. Thirty healthy youngsters, aged four to fourteen, who were chosen at random and had not taken medicine in the preceding six months or had a history of heart disease, make up the control group. Children who were on medications and systemic diseases other than congenital heart disease, mentally and physically disabled children and those parents/guardians not willing to give their consent were excluded from the study.

2.1. Decayed Missing Filled Index

Following a rigorous, tried-and-true process (WHO standards for assessment of dental caries), each child underwent a comprehensive dental clinical examination for dental caries from experienced dental experts. Whenever necessary, teeth were cleaned. After drying every surface of the tooth that needed to be checked, a mouth mirror and probe were used to examine it. According to the DMFT index, scores were assigned.

2.2. Method of salivary sample collection

The researcher took 5 ml of unstimulated whole saliva samples by passive drooling and having the child sit in Coachman's position, which involves putting both hands on the thighs and bending forward slightly[8]. During the saliva collection procedure, the individuals were instructed not to swallow or move their lips or tongue. Saliva was allowed to build up in their mouths and drool in the receiving channel.For a minimum of two hours before to saliva collection, subjects were instructed not to eat, drink and practice good oral hygiene in order to reduce the possibility of sample contamination. To lessen the impact of sympathetic tone on saliva production, the participants were instructed to sit comfortably upright without moving or conversing. Saliva was gathered and placed in a sterile, 15 ml plastic jar devoid of lead. To avoid circadian bias, all saliva samples were done between the hours of 8 and 11 in the morning [9]. After being collected, saliva samples were brought to the lab in an icebox and kept there at 4°C until they were processed. Without knowing the oral health status of the individuals, the researcher used the Griess reaction method to identify salivary nitric oxide (NO) within 24 hours.

2.3. Estimation of salivary nitric oxide levels

Prior to measuring the NO, the saliva samples were chilled and then decoded. Saliva was tested for NO using the technique outlined by Miranda et al., (2001).

2.3.1. Principle

The reduction reaction of any nitrate to nitrite by vanadium is used in this approach to estimate the total nitrite/nitrate level. Total nitrite (intrinsic + nitrite obtained from reduction of nitrate) is then detected by Griess reagent. In the Griess reaction, sulfanilamide is diazotized by acidic nitrite, forming a pink chromophore. This chromophore is then coupled with bicyclic amines, such as N (1 naphthyl) ethylenediamine, which may be detected calorimetrically at 540 nm.

2.3.2. Procedure

0.75 ml of cold absolute ethanol was added to 0.75 ml of saliva in an Eppendorf tube (HaimenShengbangLaboratory Equipment Co., Ltd.) in order to lessen the turbidity. The mixture was refrigerated for 48 hours to achieve complete protein precipitation. After that, a cooling centrifuge was used to centrifuge it for 30 minutes at 4000 rpm and 12°C.Merely 250 microliters of the generated supernatant was transferred to a 96 well enzyme-linked immunosorbentassay plate.

2.3.3. Reagent Preparation

- 1M HCl Preparation: Mix 8.2 ml of 37% HCl with 100 ml of total distilled water (TDW).
- Vanadium (III) Chloride (VCl_3): Dissolve 400 mg VCl_3 in 50 ml 1M HCl.Filter, store in the dark at 4°C for <2 weeks.

- Griess Solution:Add 250 ml TDW to Griess Reagent bottle.Invert bottle for 5 minutes.

The experiments were conducted in a warm chamber at 37°C or at ambient temperature.250 µl of vanadium trichloride and 125 µl of modified Griess reagent were added quickly using a multichannel pipettor (Nichipet R EX). The absorbance of the pink chromophore was measured at 540 nm using a double-beam spectrophotometer (BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader) during a 30-minute incubation period at room temperature. Nitrate or total NO concentrations in the sample were ascertained by linear regression of the mean absorbance values at 540 nm, adjusted for blank values.

3. Results

This cross-sectional study comprised sixty child patients, aged 4 to 14 years, split into two groups of thirty each, male and female. The technique of randomized sampling was applied to both quantitative and qualitative data.The data were analyzed using an unpaired t-test, also known as an independent t-test. With the use of The Statistical Package for Social Sciences (SPSS, IBM Corp. Version 25.0, NY, USA), all data were encoded and combined into a computer database. An independent student t-test was used to do a statistical analysis on the collected data.Table1 compares the NO levels in children with congenital heart disease (CHD) and control using an independent t test. The study group's mean salivary nitric oxide levels were 64.0307 with a standard deviation of 8.86 (64.03±8.86), while the control group's (healthy children's) levels were (133.59 ± 14.32). With a p value of 0.001, or less than 0.005, the difference between the two groups was statistically significant.The present study also focused on the dental caries status of both the study participant groups using comparison of DMFT score in both the groups. Table 2 shows the DMFT levels in both the groups. Mean DMFT score in the study population was found to be 6.27 with a standard deviation of 2.93 (6.27 ± 2.93) whereas the mean DMFT in control group was 3.70. The difference in DMFT score of both the groups was statistically considerable with p- Value 0.001 (i.e. <0.005).Finally, the study also emphasized on the association among the salivary nitric oxide levels and caries status of the individuals with or without presence of congenital heart diseases. Table 3 shows the association among the salivary nitric oxide level and DMFT score of both the groups.Figure 1 and 2 shows graphical representation of correlation of salivary nitric oxide levels with the DMFT score in study and control group respectively. Tables show a negative correlation of salivary nitric oxide levels with the DMFT score that signifies the effect of salivary nitric oxide in the incident of dental caries. The results signify that with increase in the salivary nitric oxide levels there is a significant decrease in the DMFT score of the subjects. In other words, higher prevalence of dental caries is associated with a decrease in the levels of nitric oxide in saliva. Table 4&Figure 3 show the correlation of DMFT score with salivary nitric oxide levels in the study group using a Pearson’s correlation coefficient test with a strong negative correlation among the two variables.

4. Discussion

Dental caries is a significant concern, especially for medically compromised individuals such as those with Congenital Heart-Diseases, who may face heightened risks due to various factors including neglected oral health and frequent sucrose intake. The contemporary approach to caries management highlights the importance of preventive measures over surgical intervention. Saliva plays a crucial role in prevention of dental caries due to its various components. Nitric oxide is one such component to show antimicrobial effectsagainst dental caries in recent research.Nitric Oxide (NO) undergoes rapid conversion to nitrates (NO_3^-) and nitrites (NO_2^-) in biological systems due to its highly reactive nature and short half-life. Dietary nitrates, after absorption from duodenum and upper ileum, are concentrated in salivary glands through active transport through 'Sialin' transporter that leads to significantly higher concentrations of nitrate and nitrite in saliva compared to plasma [10].NO is synthesized through two pathways: The L-Arginine-Nitric Oxide Pathway (Intrinsic Pathway) and the NO_3--NO_2—NO (alternate) pathway. In conditions like hypoxia,(common in CHD) the activity of Nitric Oxide Synthase (NOS) decreases due to reduced oxygen supply, leading to the dominance of the alternate pathway in which nitrate reduction to nitrite occurs mainly in the oral cavity by commensal bacteria. This nitrite is further metabolized toNO and other biologically active nitrogen oxides [11]. This shift towards NO production may lead to a decrease in salivary nitrite concentration. Children with CHD often experience these situations of hypoxia and thus might have reduced levels of salivary nitrites.Therefore, the colorimetric detection (Griess reaction) method was used in this investigation to determine the content of NO as totalnitratesandnitrites(NO_3+NO_2).The findings indicate a statistically significant variation in salivary NO levels between congenital heart disease patients and the control group. These findings concur with a study carried out by SA Garg et al., (2015) [12]. Additionally, the study demonstrated a strong negative connection (Figure 3) between salivary NO levels and dental caries, as reported by StecksénBlicks et al.in the Swedishpopulation, (2004) [2].Oral flora, including over 500 species like Veillonellaatypicaand Nocardia Speciesetc., reduce nitrate into nitrite in low-oxygen areas like deep tongue clefts[11]. These nitrites exhibit bacteriostatic and bactericidal effects against acidogenic bacteria like Streptococcus Mutans. Nitrite acidification yields a blend of nitrogen oxides and nitrous acid. Nitrous acid decomposes spontaneously, forming NO and nitrogen dioxide. Interestingly, only nearby bacteria experience the acidified nitrite's selective antibacterial activity. Nitric oxide's antimicrobial effects involve DNA and respiratory complex modifications, as well as interactions with reactive species. NO may inhibit bacterial growth and enhance macrophage- mediated cytotoxicity in saliva.It permeates cell membranes easily, damaging microorganisms via various mechanisms, including mitochondrial enzyme inhibition, DNA damage, and peroxynitrite formation [13]. Similarly, salivary NO levels were lower in caries active individualsas compared to caries free persons, according to a study by HegdeMitra et al., (2012) [14].

Table 1: Comparison of NO levels in study group and Control group using independent t test.

Group	Mean NO Levels	Standard. Deviation	Mean Difference	Standard. Error Difference	95% Confidence Interval of the variation		P value
					Lower	Upper	
Study group	64.0307	8.86226	-69.56433	3.07609	-75.72181	-63.40686	<0.001*
Control group	133.5950	14.32938					

Table 2: Comparison of DMFT in Study group and Control group using independent t test.

Group	Mean DMFT Score	Standard. Deviation	Mean Difference	Standard. Error Difference	95% Confidence Interval of the variation		P value
					Lower	Upper	
Study group	6.27	2.935	2.567	0.651	1.265	3.869	<0.001*
Control group	3.70	3.70					

*Statistically significant.

Table 3: Correlation of NO levels with respect to DMFT in both the groups.

Group		Mean	Std. Deviation	N	Correlation	P value
Study group	NO Level	64.0307	8.86226	30	-0.928	<0.001*
	DMFT	6.27	2.935	30		
Control group	NO Level	133.5950	14.32938	30	-0.714	<0.001*
	DMFT	3.70	2.020	30		

*Correlation is significant at the 0.01 level.

Table 4: Correlation of DMFT with Nitric oxide levels in study participants.

		DMFT
NO Level	Pearson Correlation	-.648*
	P value	.000
	N	30

*Correlation is significant at the 0.01 level.

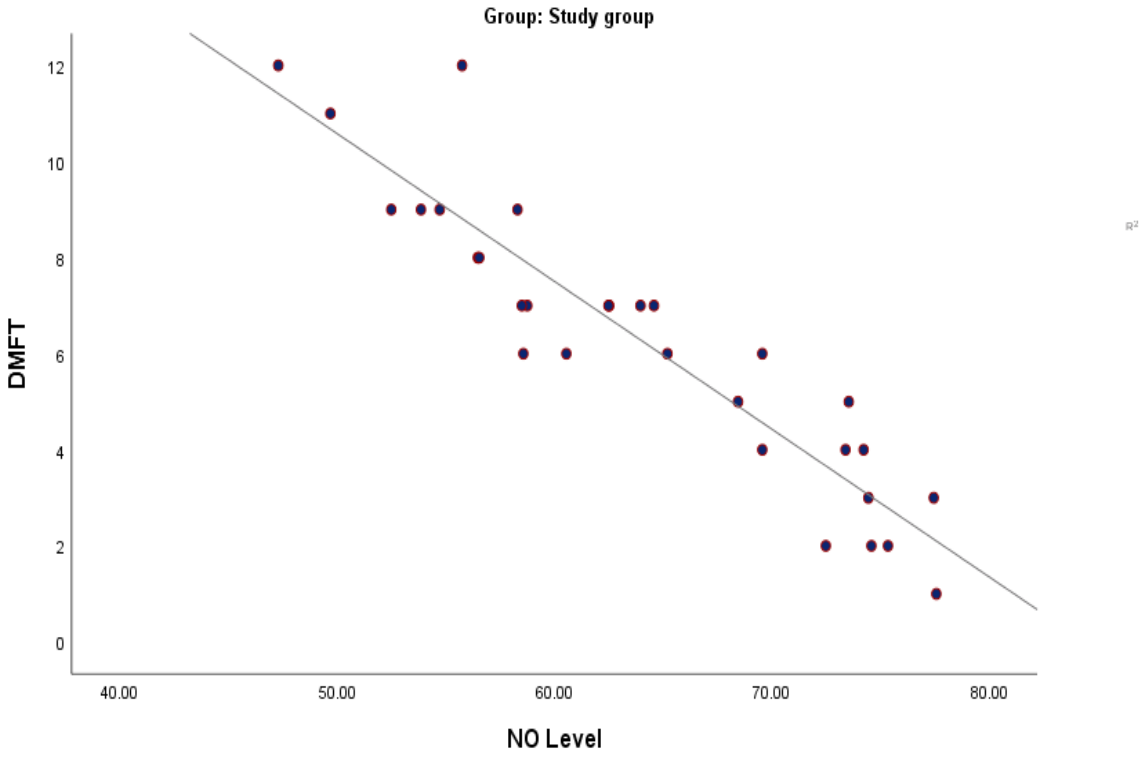

Figure 1: Graphical Correlation of DMFT with nitric oxide levels in Study group.

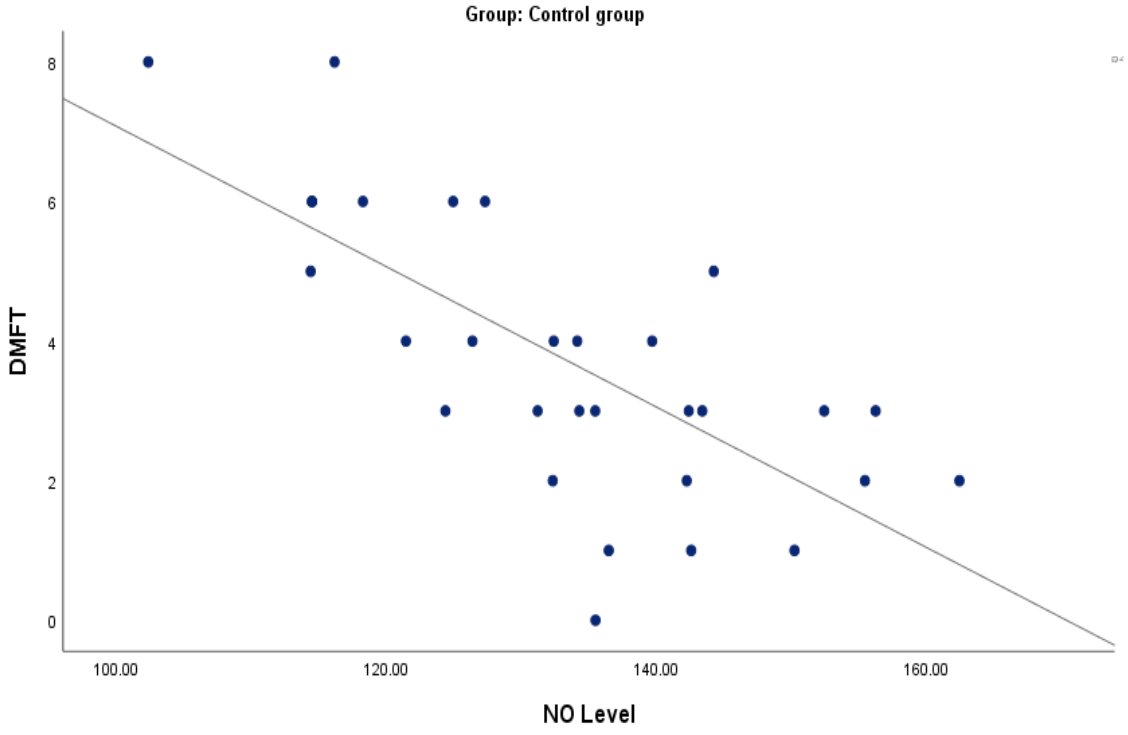

Figure 2: Graphical Correlation of DMFT with nitric oxide levels in Control group.

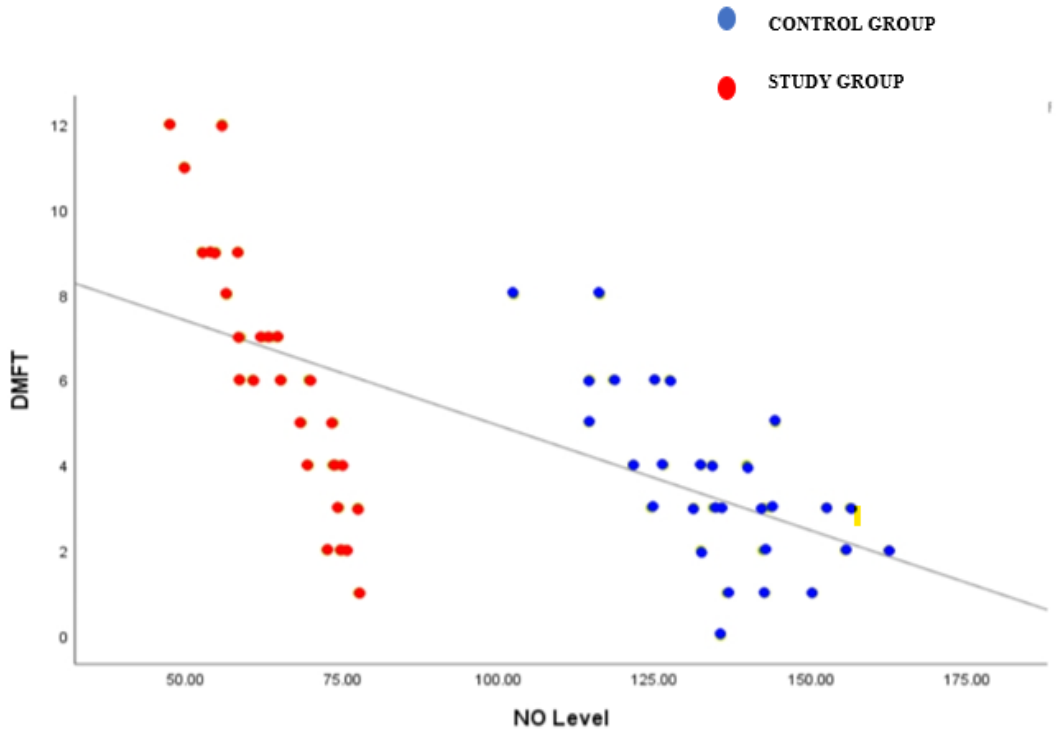

Figure 3: Graphical Correlation of DMFT with Nitric oxide levels in study participants.

It should be noted, nevertheless, that saliva can influence all of the salivary antibacterial components in a variety of ways, making it exceedingly challenging to assess any one antibacterial element in isolation. Furthermore, according to Kirstila et al., (1998), there is no specific salivary antibacterial component that is particularly significant in determining caries risk in vivo.Therefore, more research, both clinical and experimental, is required to evaluate the effect of nitrite in preventing dental caries.

5. Conclusions

Salivary nitric oxide emerged as a potential biomarker for caries risk in such children, with dietary nitrate intake being a crucial determinant. Recommendations for increased consumption of nitrate-rich foods and probiotic therapy to promote colonization of nitrate-reducing bacteria could be beneficial in caries prevention. The findings emphasize the importance of raising salivary NO levels for better protection against dental caries in children with CHD. Within the limitations of the study, it can be concluded that increasing nitrate intake in children especially children with congenital heart diseases or any other cardiac defects that lead to alteration of nitrate equilibrium inside body may be especially significant in restraining the growth of acid producing cariogenic bacteria and thereby protecting teeth against dental caries.However, further controlled research is necessary to validate the link between salivary NO levels,CHD, and dental caries over the long term.

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