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Comparative assessment of Salivary Nitrite-Related Measurements by Griess Reaction and Colorimetric Test Strips in Children with ECC

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Abstract **Background:** Early childhood caries (ECC) is a major public-health concern. Salivary nitric oxide (NO) may be associated with caries susceptibility through the nitrate–nitrite–NO pathway. The Griess reaction is a reference method for measuring salivary NO, whereas a simple colorimetric strip may provide a practical, non-invasive method for young children. **Aim:** To compare salivary Griess-reactive analyte measurements from a reference liquid assay and colorimetric test strips in children with early childhood caries (ECC). The analytical distinction between nitrite, nitrate after reduction, and nitric oxide must be maintained. **Method:** Thirty-four children aged 4–5 years with ECC were recruited from private dental clinics after ethical approval and parental consent. Unstimulated saliva was collected by passive drooling after a 3-hour fast. Samples were centrifuged and tested using a colorimetric strip based on the Griess reaction, with digital image histogram analysis for measurement. A solution-based Griess assay was also performed using spectrophotometry at 540 nm. Results were compared using a paired t-test. **Results:** Among 34 children with ECC, the reported mean concentrations were 39.24±8.23 µmol/L using the reference reaction and 36.18±8.17 µmol/L using the strip (unpaired descriptive means; mean paired difference 3.06 µmol/L). The originally reported paired test statistic ($t = 1.538$, $df = 33$) corresponds to a two-sided $p = 0.134$, not 0.064. The standard deviation of the paired differences and a method-agreement analysis were not available from the submitted aggregate data. **Conclusion:** A statistically non-significant paired test does not establish agreement or clinical interchangeability. Validation requires calibrated analyte definitions, paired-difference confidence intervals, limits of agreement, reproducibility and testing in caries-free as well as ECC cohorts before any chairside diagnostic or caries-risk claim can be supported.

Key Words Biomarkers, Colorimetry, Early Childhood Caries, Griess Reaction, Nitrate Reductase, Nitrite, Salivary Flow Rate

INTRODUCTION

Dental caries is a common chronic disease in children and is influenced by biological, social, economic, and environmental factors [1]. Caries development involves oral biofilm, demineralisation and remineralisation, fluoride exposure, and dietary factors [2-3]. *Streptococcus mutans* contributes to early enamel lesions, whereas lactobacilli are more often associated with lesion progression into dentine [4]. Early childhood caries (ECC) is defined as one or more decayed, missing, or filled tooth surfaces in any primary tooth in a child aged 5 years or younger [5]. It is a common childhood condition and a major public-health concern worldwide.

Salivary nitrate and nitrite have been studied in relation to individual caries susceptibility [6]. Nitric oxide is produced through two main pathways. The endogenous pathway uses nitric oxide synthase to convert L-arginine into NO [7,8]. The second pathway involves dietary nitrate and nitrite and functions under low-oxygen conditions.7,9 Human tissues do not possess nitrate reductase. Salivary nitrate is therefore reduced by facultative anaerobic bacteria present in the posterior tongue during anaerobic respiration [9].

Salivary nitrate concentration depends on dietary nitrate intake, bacterial nitrate reductase activity, salivary flow rate,

and endogenous nitrate production.7,9 When nitrate-rich saliva reaches the acidic environment around the tooth surface, nitrite can form reactive nitrogen compounds such as nitrous acid and NO [10,11]. These compounds have antimicrobial effects against several microorganisms, including enteric bacteria, dermatophytes, *Candida* species, skin pathogens, and cariogenic oral bacteria [2,6,10,11]. This process may contribute to the control of cariogenic bacteria and may partly explain the association between salivary nitrate metabolism and caries development [12].

Clinical studies in children have reported lower salivary NO levels in those with rampant caries and ECC than in caries-free children [13]. Salivary NO levels decreased progressively from caries-free children to those with ECC and severe ECC, with the lowest values observed in severe ECC [14]. Another study also found lower levels in children with ECC than in caries-free children.15 Reduced salivary NO has also been observed in children with congenital heart disease, who have a higher risk of dental caries [4,16]. Salivary NO concentrations have increased after operative treatment of active caries and after restoration of carious teeth with resin composite [17-19]. Systemic and gastrointestinal conditions such as dyspepsia can also affect the relationship between salivary NO and caries, indicating that NO may represent a general mucosal defence mechanism rather than a caries-specific marker [20]. Colorimetric test strips based on the Griess reaction provide a rapid and low-cost method for measuring salivary nitrite and can be used without laboratory equipment. Previous validation has compared these strips with reference solutions and digital image histogram analysis [21-23]. Salivary NO has antimicrobial activity against oral microorganisms, including cariogenic bacteria [5,10,11,24,25]. A simple chairside method may be useful in children with ECC because saliva collection is non-invasive and does not require venepuncture. Therefore, this study aims to compare a colorimetric NO test strip with the solution-based Griess reaction assay for salivary NO estimation in children with ECC. The study also aims to determine whether the test strip provides results comparable to the reference assay and can be used as a practical chairside method for NO assessment.

METHOD

Study Participants and Saliva Collection

The study protocol was approved by the Ethical Committee of Saveetha Dental College. Written informed consent was obtained from the parents of all participating children. Thirty-four children aged 4–5 years with early childhood caries (ECC) were recruited from private dental clinics in Tirunelveli, Tamil Nadu, India. Children with systemic diseases, long-term medication use, or current or recent antibiotic treatment were excluded. The children were instructed to avoid food and drinks for at least 3 h before saliva collection. Approximately 5 mL of unstimulated saliva was collected by passive drooling into a vial through a funnel. The samples were centrifuged, and the supernatant was used for analysis.

Preparation and Analysis of the Colorimetric Test Strip

Citric acid, N-(1-naphthyl)-ethylenediamine, and sulfanilamide were obtained from Sigma-Aldrich. Whatman chromatography paper (Grade 3MM CHR) was obtained from Fisher Scientific. A 20-mil-thick vinyl roll was obtained from Hi-Tech Products, and acid-free double-sided tape was obtained from Scotch 3M. The chromatography paper was treated with a solution containing Citric acid, N-(1-naphthyl)-ethylenediamine, and sulfanilamide. The paper was then manually cut into individual strips and fixed to the vinyl backing using the double-sided tape.

The strips were based on the Griess reaction for NO_2^- measurement. When saliva containing NO_2^- contacts the treated paper, citric acid converts NO_2^- to nitrous acid. Nitrous acid reacts with sulfanilamide and N-(1-naphthyl)-ethylenediamine to form a red-violet compound. The colour intensity corresponds to the NO_2^- concentration. Each strip was immersed in a saliva sample and Colour intensity was measured. The measured intensity was converted to NO_2^- concentration using a pre-caliberated colour guide (Figure 1) prepared with solutions of known concentrations.

Reference Griess Assay and Statistical Analysis

For reference measurement, the saliva samples were stored at 4°C and analysed within 24 h using a quantitative Griess assay. Total nitrate and nitrite concentration was measured. Nitrate was first reduced to nitrite using reduced nicotinamide adenine dinucleotide phosphate in the presence of nitrate reductase. For the assay, 50 µL of each sample was transferred to a 96-well plate, followed by 50 µL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) and 50 µL of N-naphthyl-ethylenediamine solution (0.1%). The samples were incubated at room temperature for 5–10 min. Optical density was measured at 540 nm using a plate reader. The two methods were compared using a paired *t*-test. Results were expressed as mean±standard deviation (SD). Pearson's correlation coefficient (*r*) was calculated to assess the relationship between the concentrations obtained using the test strip and the reference Griess assay.

RESULTS

All 34 children with ECC provided saliva samples that were analysed by both methods. Therefore, the sample size was 34 for each method, as shown in Table 1. The mean salivary NO concentration was 39.24±8.23 µmol/L with the Griess reaction assay, with a median of 37 µmol/L and a range of 30–58 µmol/L. The mean concentration obtained with the colorimetric test strip was 36.18±8.17 µmol/L, with a median of 30 µmol/L and a range of 30–60 µmol/L. The standard deviations were similar for the two methods (8.23 and 8.17 µmol/L). The concentration ranges also showed substantial overlap, with the test strip showing a slightly higher maximum value.

The reported paired t-statistic (t = 1.538, df = 33) implies a two-sided p = 0.134. The strip average was 36.18±8.17 µmol/L versus 39.24±8.23 µmol/L for the Griess-reactive assay, yielding an observed mean paired difference of 3.06 µmol/L. If the reported t-statistic is correct, its implied 95% confidence

Table 1: Descriptive Statistics of Salivary NO Concentration by Method (N = 34)

Group / Method	N	Mean (µmol/L)	SD	Minimum (µmol/L)	Median (µmol/L)	Maximum (µmol/L)
ECC - Griess Reaction	34	39.24	8.23	30	37	58
ECC - Colorimetric Strip	34	36.18	8.17	30	30	60

Table 2: Paired Comparison of Griess Reaction Versus Colorimetric Strip (N = 34)

Comparison	Test	*t*-value	*p*-value	Significance
ECC: Griess reaction vs colorimetric strip	Paired *t*-test	1.538	0.134 (two-sided; df = 33)	Not statistically significant; agreement not established

interval for the paired mean difference is approximately –0.99 to 7.11 µmol/L; this calculation cannot substitute for verification from the original 34 matched pairs. Neither an insignificant difference test nor simple correlation establishes method agreement, and the strip values cluster at the reported measurement floor of 30 µmol/L.

DISCUSSION

Analytical interpretation: The Griess reaction responds to nitrite; nitrate contributes only when reduced beforehand, and neither measurement is a direct assay of fleeting free nitric oxide. The reported paired t statistic implies a two-sided p value of 0.134 for 34 paired observations. Agreement between methods cannot be inferred from the p value alone, and an appropriate Bland–Altman analysis would require individual paired measurements.

Salivary NO assessment may provide useful information in children with ECC. A simple method for measuring salivary NO could make its assessment easier in routine paediatric dental practice. The main finding of this study was that the colorimetric test strip produced salivary NO concentrations similar to those obtained with the reference solution-based Griess reaction. The paired analysis indicates that the test strip may provide a practical alternative to laboratory-based spectrophotometric measurement in children with ECC. Venous blood collection and prolonged laboratory procedures can be difficult in children aged 4–5 years. A simple, non-invasive test strip may therefore facilitate salivary NO assessment for screening and follow-up in paediatric dental practice [13-15].

The role of salivary NO metabolites in dental caries involves the entero-salivary nitrate–nitrite–NO pathway. Dietary nitrate enters saliva and is reduced to nitrite by nitrate-reducing bacteria on the posterior tongue [7,9]. Human cells do not carry out this reaction. Nitrite can then form nitric oxide and other reactive nitrogen compounds under acidic conditions produced by acidogenic bacteria. These compounds can inhibit bacterial growth and, at higher concentrations, can kill bacteria involved in enamel demineralisation [10,11]. Acidified nitrite has antimicrobial activity against enteric bacteria, dermatophytes, *Candida* species, and cariogenic oral bacteria [2,6,10,11]. NO is also produced through the nitric-oxide-synthase pathway.7,8 Salivary NO levels can therefore depend on both endogenous production and bacterial nitrate reduction.9 Changes in dietary nitrate intake, salivary flow, or nitrate-reducing oral bacteria may alter this process and may contribute to changes in caries susceptibility [12].

The present results were obtained in children with ECC and can be compared with studies that have assessed salivary NO in children with different caries conditions. Indian studies have reported lower salivary NO concentrations in children with rampant caries or ECC than in caries-free children [13,15]. Salivary NO concentrations increased after treatment of active carious lesions, both after conventional restorative treatment [17] and after composite resin restoration [19]. These findings suggest that salivary NO may change with caries activity. Children with congenital heart disease have also shown altered salivary NO concentrations and an increased risk of caries [4,16]. Gastrointestinal conditions such as dyspepsia can also affect the relationship between salivary NO and caries [20]. Therefore, salivary NO may be affected by oral disease and systemic factors.

The salivary NO concentrations obtained in the present study are similar to values reported in other paediatric cohorts. Senthil Eagappan *et al.* [14] reported mean salivary NO concentrations of 51.2±8.35 µmol/L in caries-free children, 47.1±5.26 µmol/L in children with ECC, and 33.625±4.69 µmol/L in children with severe ECC. Salivary NO also showed a negative correlation with dmfs (r = –0.6658). Hegde *et al.* reported mean concentrations of 75.04±1.13 µmol/L in caries-free children and 34.99±1.22 µmol/L in children with rampant caries/ECC, with a negative correlation between NO and caries status (r = –0.566) [13].

In the present study, the mean salivary NO concentration was 39.24±8.23 µmol/L with the Griess assay and 36.18±8.17 µmol/L with the colorimetric strip. These values are close to the concentrations reported in children with ECC and severe ECC [13,14]. The similar concentrations obtained by the two methods also indicate that the test strip produced values within the range reported for children with ECC.

The present study included only children with ECC and did not include a caries-free or severe-ECC group. Therefore, the findings cannot establish a relationship between salivary NO concentration and caries severity within the study sample. The comparison with other studies is based on their reported values and should be interpreted accordingly.

Variation in absolute NO concentrations between studies is expected because measurement methods and participant characteristics can differ. Saliva collection procedures, assay calibration, colour reference curves, digital image analysis, microplate measurement, age, and dietary nitrate intake can affect the measured concentration. Therefore, comparison of the present findings with other

studies should focus on the reported concentration ranges rather than exact numerical values [13,14]. The paired design of the present study provides a direct comparison because each child was assessed using both the colorimetric strip and the reference Griess assay.

Previous studies have evaluated strip-based colorimetric methods for nitrite measurement against Griess assays using calibrated solutions and digital image analysis [21-23]. The similar results obtained with the two methods in the present study indicate that the strip can provide NO estimates comparable to the reference assay. Both methods measure nitrate and nitrite, which are stable oxidation products of NO and are therefore suitable for NO assessment [24-26]. A simple chairside strip may be useful for salivary NO assessment in young children and could be considered for caries-risk assessment in dental clinics, community programmes, and school-based screening where laboratory facilities are limited [27-29].

Salivary NO may have a role in caries-risk assessment in young children when measured by either the reference assay or the colorimetric strip. Its interpretation should account for factors such as dietary nitrate intake, oral bacterial activity, and systemic conditions that can alter nitrate reduction and NO production [7,9].

A colorimetric strip that produces results similar to a laboratory assay may be useful in dental clinics, community programmes, and school-based screening. These settings may have limited access to microplate readers and laboratory facilities. The strip could be used with existing caries-risk assessment methods to identify children who may require dietary advice, fluoride treatment, or dental referral without venous sampling or laboratory processing [5,15,27]. Salivary NO is not specific to caries and can be affected by dietary nitrate intake, oral hygiene, tongue and plaque bacteria, congenital heart disease, and gastrointestinal conditions [4,20]. A low NO concentration should therefore be considered as one factor in caries-risk assessment rather than as a diagnostic value. Future longitudinal studies should assess salivary NO together with dietary nitrate intake, oral microbiota, and caries progression to determine suitable NO thresholds for caries-risk assessment.

Strengths and Limitations

Each child provided saliva for both methods, allowing a direct paired comparison. The same saliva samples were used for the colorimetric strip and the reference Griess assay. Standardised fasting and passive drooling provided uniform sample collection. The reference assay also allowed direct comparison with the strip method. The sample size of 34 children limits statistical power, and the recalculated two-sided p value of 0.134 does not exclude a small difference between the methods. The single-centre design and narrow age range limit generalisability to other paediatric populations. Dietary nitrate intake, salivary flow rate, and oral microbiota were not assessed. Diagnostic accuracy, useful cut-off values, and changes in salivary NO overtime were also not evaluated.

CONCLUSION

The colorimetric strip provides a simple and non-invasive method for salivary NO assessment in young children. The low cost and easy application may allow its use in paediatric dental clinics, community programmes, and school-based screening. Future studies should include larger multi-centre samples, wider age groups, and caries-free controls. The effects of dietary nitrate intake, salivary flow rate, oral microbiota, and caries progression should also be assessed. Standardised calibration and clinically relevant NO cut-off values are required to assess diagnostic accuracy. Longitudinal studies can determine whether repeated strip measurements detect changes in caries activity. Further studies should compare the strip with established caries-risk assessment methods to define its role in clinical practice.

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