Nitric Oxide 91 (2019) 15–22

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Validity and reliability of test strips for the measurement of salivary nitrite concentration with and without the use of mouthwash in healthy adults

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ARTICLE INFO

The material presented in this manuscript is original and it has not been submitted for publication elsewhere while under consideration by **Nitric Oxide**

ABSTRACT

The nitrate (NO_3^-)-nitrite (NO_2^-)-nitric oxide (NO) pathway has received considerable interest in recent years as a potential target for nutritional interventions designed to increase NO production, and elicit therapeutic effects in humans. In particular, studies have evaluated the effects of supplemental dietary NO_3^-, which serves as a 'substrate' for this pathway, on numerous different health outcomes. One challenge has been to evaluate compliance with the NO_3^- interventions. A recent advance in this field has been the development of a non-invasive, simple and rapid method to measure nitrite concentrations in saliva using small test salivary strips.

In the present study, ten healthy adults were recruited to a randomised, crossover study and received an acute dose of NO_3^--rich beetroot juice (BJ) after rinsing their mouth with either water or commercially available antibacterial mouthwash. Salivary NO_3^- and NO_2^- concentrations were measured at baseline and up to 5 h after BJ consumption using the gold-standard chemiluminescence and a colorimetric Griess assay. In addition, two salivary test strips (Berkeley Test strips, CA, USA) were used to measure NO_2^-concentrations at the same time points. Five observers read the strips and inter- and intra-observer reliability was measured. The Bland-Altman method was used to provide a visual representation of the agreement between the methods used to evaluate salivary NO_3^-/NO_2^-concentration. Sialin concentrations were measured at baseline and up to 5 h after BJ consumption.

BJ elevated salivary NO_3^- and NO_2^- concentrations when the mouth was rinsed with water (both $P < 0.01$), as assessed via both chemiluminescence and Griess methods. Rinsing the mouth with antibacterial mouthwash attenuated markedly the increase in NO_2^- ($P < 0.001$), while NO_3^-concentrations were unaffected ($P > 0.05$). The Intra-Class Coefficients of Correlation (ICC) showed a high inter- and intra-observer reliability ($r > 0.8$). A significant positive correlation was found between absolute salivary NO_2^- concentrations measured by strips and Griess and chemiluminescence methods (rho = 0.83 and 0.77, respectively) and also when expressed as changes in salivary NO_2^- concentrations (rho = 0.80 and 0.79, respectively). Bland Altman analysis indicated a poor agreement for absolute NO_2^- concentrations between salivary strips and the chemiluminescence and Griess methods. A small significant negative correlation was found between changes in salivary sialin and salivary NO_2^- concentrations ($r = -0.20$, $P = 0.04$). A non-significant positive correlation was observed between the change in salivary sialin and salivary NO_3^- concentrations ($r = 0.18$, $P = 0.06$).

This study suggests that commercially available salivary NO_2^- test strips provide a reasonable surrogate marker for monitoring changes in salivary NO_2^- concentrations in humans. However, the strips do not provide accurate estimates of absolute NO_2^- concentrations.

1. Introduction

Nitric Oxide (NO) is a reactive gas which is involved in numerous physiological processes, including blood flow regulation, immune defence and neurotransmission [2]. NO can be synthesised endogenously from L-arginine in a reaction catalysed by the NO synthase (NOS) enzymes [3]. Additionally, NO can be generated via an alternative pathway that depends on the entero-salivary circulation of NO_3^- – an inorganic anion which is present in a range of commonly consumed foods [3]. Ingested NO_3^- is absorbed rapidly from the upper

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https://doi.org/10.1016/j.niox.2019.07.002
Received 30 April 2019; Received in revised form 10 June 2019; Accepted 8 July 2019
Available online 08 July 2019

gastrointestinal tract into the blood. Approximately 25% of circulating NO_3^- is taken up by the salivary glands and concentrated, prior to being excreted into the mouth in saliva [4]. The protein sialin (SLC17A5) was recently identified as the principal NO_3^- transporter in the salivary glands and knockdown of sialin expression reduced NO_3^- transport [5]. Once in the mouth, a portion of the NO_3^- is reduced to nitrite (NO_2^-) by commensal facultative anaerobic bacteria which reside predominantly on the dorsal surface of the tongue [6]. The resulting NO_2^- is then swallowed in saliva and may be further reduced to NO via enzymatic and non-enzymatic pathways to help support or maintain NO-signaling, especially in acidic [4] and ischemic [3] conditions.

Dietary supplementation with NO_3^-, which increases NO production via the NO_3^--NO_2^--NO pathway, elicits multiple beneficial effects on physiological outcomes. Effects include a significant reduction in blood pressure (BP) [7], improved exercise performance [8,9], and enhanced cognitive function [10], although this latter finding was not confirmed in a recent meta-analysis [11]. The NO_3^- reducing bacteria which reside in the oral cavity play a fundamental role in facilitating these beneficial effects following dietary NO_3^- ingestion [6]. Indeed, several studies have shown that using antibacterial mouthwash diminishes considerably the colony size of the bacteria [1,12]. This reduction affects the production of NO_2^- in the oral cavity, concomitantly lowering the concentration of NO_2^- in saliva and plasma, and diminishes the physiological effects that may otherwise manifest following NO_3^- supplementation [1,12,13]. Thus, whilst antibacterial mouthwash could help to maintain oral health, its regular use may interfere adversely with the beneficial effects of NO_3^- on cardiovascular health. Indeed, short term (3–7 days) mouthwash use has been shown to abolish the BP lowering effects of dietary NO_3^- supplementation [1,14,15]. The effects of long term mouthwash use on oral and cardiovascular health remains to be fully elucidated, although, interestingly, a recent study found that frequent mouthwash use is associated with an increased risk of diabetes [16].

Evidence that dietary NO_3^- may improve NO bioavailability and thus enhance a range of physiological functions has attracted researchers to develop a range of simple techniques to monitor systemic NO-bioavailability, including, amongst others, NO salivary test strips. These strips allow the estimation of salivary NO_2^- concentration, which can be used as a surrogate for NO bioavailability [17]. These strips could also help to provide information on compliance with dietary NO_3^- interventions. The strips have been validated in non-supplemented [18] and supplemented subjects [19] and correlate significantly with salivary NO_2^- measured via gold-standard techniques (i.e. ozone-based chemiluminescence). However, to our knowledge, no study has investigated in a randomised study whether antiseptic mouthwash could affect the sensitivity of the salivary strips by inhibiting the conversion of NO_3^- into NO_2^- [14]. In addition, previous studies validated these strips using simple correlation analyses and adjusting analyses for mouthwash use [18], and have not applied the Bland-Altman method to evaluate the magnitude, variability and direction of the measurement bias [20].

Therefore, the purpose of this study was to test the validity of these strips against reference standard laboratory measures (i.e. ozone-based chemiluminescence) of salivary NO_2^- and NO_3^- concentrations with and without the use of mouthwash, using the Bland-Altman method. In addition, as these strips are based on a modified Griess reagent reaction, we also measured salivary NO_2^- using the Griess method for further comparison. Finally, we also took the opportunity to investigate the effect of both NO_3^- supplementation and mouthwash on salivary sialin concentrations, which functions as a NO_3^- transporter in the plasma membrane of salivary glands.

2. Methods

2.1. Subjects

Ten healthy, non-smoking, normal weight or overweight (body mass index (BMI) range: 20–29.9 kg/m^2) participants aged ≥20 years were recruited via email from Newcastle University staff and students to take part in this study. Exclusion criteria included: smoking, history of clinical conditions and medical treatments likely to interfere with the study outcome, pregnancy and breastfeeding. All participants were fasting for at least 12 h prior to participating in the experiment. All participants provided written, informed consent and the study was approved by the Faculty of Medical Sciences, Newcastle University (1459/3414/2018).

2.2. Experimental protocol

This study was a cross-over, randomised, validation study consisting of two experimental trials (without or with mouthwash) conducted on two separate visits and with a washout period of 24 h. At present, there is limited *in vivo* evidence on the minimum time taken for the oral NO_3^--reducing microbiome to recover following administration of antibacterial mouthwash [21,22]. For practical reasons, a washout period of 24 h was selected and this also allowed us to elucidate whether the oral microbiome remained compromised a day after mouthwash use. Eligible participants were invited for their first experimental visit early in the morning (~8.30–9.00 a.m.) after an ~12-h overnight fast and having avoided consumption of high NO_3^- foods for the previous 24 h. Body weight was measured, and participants were asked to collect a baseline saliva sample followed by the application of two NO Test Strips (Berkeley Test®, CA, USA), as per the manufacturer's instructions. Baseline resting BP was then measured, and participants were randomised to either rinse their mouth with 20 ml of low NO_3^- water (Buxton water) or 20 ml of antiseptic mouthwash (Corsodyl, Chlorhexidine Digluconate 0.2%, UK) for 2 min. After 15 min, participants consumed one 70 ml 'shot' of concentrated BJ (Beet-it, James White Company). This juice contains approximately 400 mg (~6.5 mmol) of NO_3^-, which is roughly equivalent to eating a large portion 200–300 g of lettuce or rocket. Participants were asked to collect saliva samples, apply the salivary strips, and measure their resting BP at 1, 2, 3, 4 and 5 h post-consumption. During this period, participants were asked not to eat any food, except for consumption of a low NO_3^- chocolate bar after collection of the third saliva sample. The consumption of low NO_3^- water was allowed *ad libitum*, but was prohibited in the 15 min prior to the collection of each saliva sample. An overview of the protocol is shown in Fig. 1. Dietary instructions and necessary materials for the collection of saliva samples were provided. Participants were asked to refrain from using mouthwash in the 24 h period before each trial, and throughout each experimental trial.

2.3. Blood pressure measurements

Baseline resting BP was measured in duplicate using automated BP monitor (Omron M3, Omron Healthcare Ltd., Kyoto, Japan). The mean of the two records was taken as the baseline BP. At 1, 2, 3, 4 and 5 h post administration of BJ, participants measured their own BP in duplicate.

2.4. Saliva samples collection

For the measurement of NO_3^-, NO_2^- and sialin concentrations, saliva samples were collected by chewing a cotton ball for 1–2 min. The cotton ball was then placed in a 20 ml syringe, which was used to squeeze the saliva into a 1.5 ml Eppendorf tube. Samples were stored at −20 °C within 30 min from collection for further analyses.

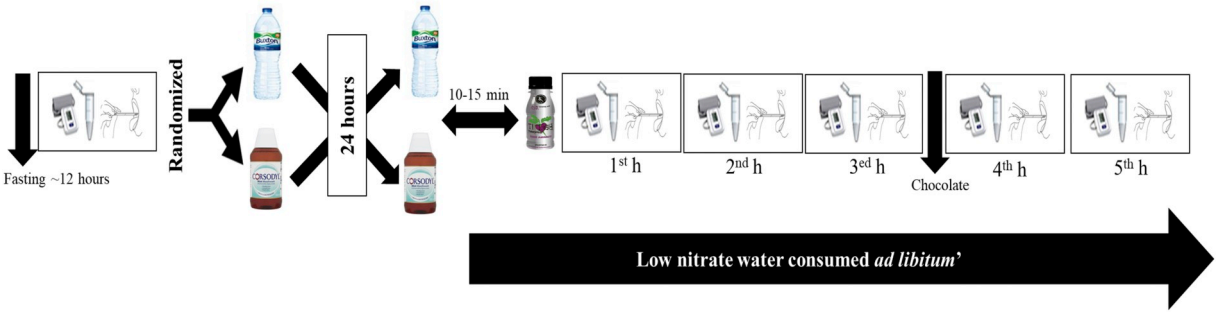

Fig. 1. Overview of the study protocol.

2.5. Salivary NO_2^- assessment using strips

Salivary NO_2^- strips (Berkeley Test strips, CA, USA) were used as per the manufacturer's guidelines. Specifically, the test strip with the 'saliva here' side was placed on the tongue and swabbed over a 10 s period covering different areas including the dorsal surface of the tongue. The two ends of the strip were folded and pressed gently for 10 s. The colour of the NO test pad was then allowed to develop over a 45 s period. The intensity of the colour was compared with a colour chart using a mobile phone based application developed by the manufacturers (Berkeley Test Application, CA, USA). The application has a long colour chart and each colour is associated with a quantitative value for NO_2^- concentration, with darker colours corresponding to higher NO_2^- concentrations. To evaluate the repeatability of this method, participants estimated their salivary NO_2^- concentration using two, separate test strips, with a 1-min interval between them. In addition, five observers read each of the strips independently to quantify inter-observer reproducibility.

2.6. Salivary nitrate and nitrite analyses

Salivary NO_3^- and NO_2^- concentrations were quantified using gas-phase chemiluminescence and a colorimetric Griess assay as described below:

Chemiluminescence: Salivary NO_3^- and NO_2^- concentrations were analysed using a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). Sodium iodide in acetic acid was used as a reductant for NO_2^- to NO, while vanadium chloride in hydrochloric acid at 95 °C was used to determine NO_3^- concentrations by the reduction of NO metabolites to NO and subsequent subtraction of NO_2^- concentration. The concentrations of NO_3^- and NO_2^- were determined by plotting signal area (mV) against a calibration plot of known concentration NO_3^- and NO_2^- standards and data were analysed using the Sievers® NOAnalysis™ Software Version 3.2.

Griess method: A commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI, US) was used to measure NO_3^- and NO_2^- concentrations using the Griess method.

2.7. Sialin (SLC17A5) analysis

Sialin (SLC17A5) concentrations in saliva were quantified using a commercial BioAssay™ ELISA Kit (Human) from Stratech Scientific Ltd, in a 96-well format.

2.8. Statistics

A two factor ANOVA for conditions (water and mouthwash) with repeated measures for sampling time was applied to determine the effects of the BJ intervention on BP, salivary NO_3^- and NO_2^-,and salivary sialin. Bland-Altman analysis was applied [20] to provide a visual representation of the agreement between methods used to analyse salivary NO_3^- and NO_2^- concentrations. Normal distribution was checked via the Shapiro-Wilk test, and data were log transformed when necessary. Spearman's correlation analysis was performed to evaluate whether changes in NO_3^- and NO_2^- concentrations were associated with changes in BP and sialin concentrations. In addition, we evaluated whether changes in salivary NO_2^- concentrations measured by salivary strips and Griess and chemiluminescence methods were significantly associated. To evaluate the effects of mouthwash/water use on recovery time of the NO_3^- reducing capacity of oral bacteria the areas under the curve (AUC) of NO_2^- concentrations for participants receiving the mouthwash on the first day (n = 5) was compared with the AUC derived from participants receiving the mouthwash on the second day (n = 5) (independent sample *t*-test). All data are presented as mean ± SEM unless otherwise indicated. Statistical significance was accepted when $P < 0.05$. The Statistical Package for Social Sciences (IBM SPSS, version 23, NY, USA) was used to perform the analysis.

3. Results

3.1. Participants' baseline characteristics

Ten healthy young participants were recruited (6 females and 4 males) with an age range of 20–45 years and a BMI range of 21.1–29.8 kg/m^2 (Table 1). Baseline systolic and diastolic blood pressure (SBP and DBP) were not different between the water and mouthwash experiments ($P = 0.91$ and $P = 0.60$ for SBP and DBP, respectively).

3.2. Salivary NO_3^- concentration

There was no significant difference in salivary NO_3^- concentration at baseline between the mouthwash and water conditions, as determined by both chemiluminescence ($P = 0.34$) and Griess methods ($P = 0.43$). Following BJ ingestion, salivary NO_3^- concentration rose rapidly to peak within 1–3 h after which concentrations declined. Time to peak appeared to be delayed after use of the mouthwash. This pattern of response in salivary NO_3^- concentrations was similar when measurements were made by the chemiluminescence and Griess methods

Table 1
Baseline characteristics of the participants (n = 10).

Characteristic	Mean	SD
Age (years)	31.2	8.7
Height (cm)	163.0	12.9
Weight (kg)	65.5	13.8
Body mass index (kg/m^2)	24.3	2.7
SBP (mmHg):		
Water experiment	115.4	8.3
Mouthwash experiment	115.0	7.9
DBP (mmHg):		
Water experiment	71.5	9.7
Mouthwash experiment	73.6	9.3

SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

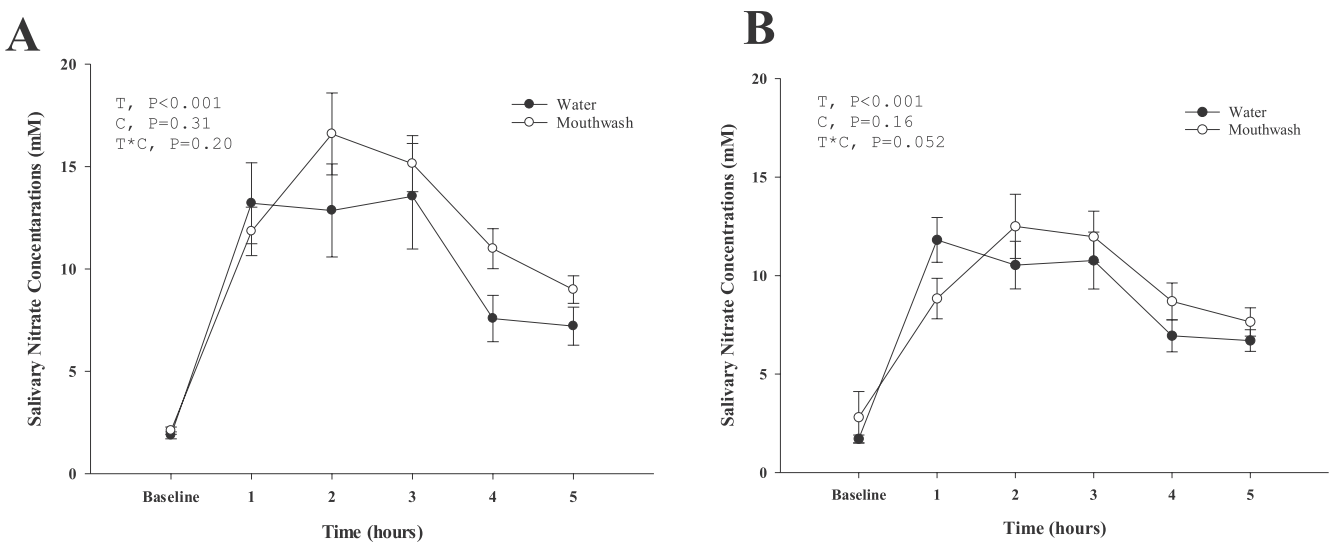

Fig. 2. Mean salivary nitrate concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before the ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

but, overall, concentrations determined by the Griess method were ~12% lower (Fig. 2). Salivary NO_3^- concentration was higher than baseline at all-time points after BJ ingestion ($P < 0.001$).

3.3. Salivary NO_2^- concentration

There was a significant main effect for time on salivary NO_2^- concentration (chemiluminescence: $P < 0.001$; Griess: $P = 0.004$). In addition, a significant effect for condition (both $P < 0.001$) and interaction between time*condition (both $P < 0.001$) was observed. Following BJ ingestion, salivary NO_2^- concentration rose significantly to peak within 2–3 h in participants drinking water, and it remained elevated until the end of the observation period. However, this increase in salivary NO_2^- concentration vanished after using anti-bacterial mouthwash (Fig. 3). This pattern of response in salivary NO_2^- concentration was similar when measurements were made by the chemiluminescence and Griess methods but, overall, measurement derived from the Griess method were ~38% and ~27% lower for the water and mouthwash conditions, respectively (Fig. 3). There was no significant difference ($P = 0.32$) in the AUC for NO_2^- concentration measured in participants receiving the mouthwash on the first day compared with participants receiving it on the second day (Figure S1 of the Online Supplementary Material).

3.4. Salivary NO_2^- strips

The effects of BJ on salivary NO_2^-, as determined by the salivary strips, are presented in Fig. 4. Overall, the salivary NO_2^- strips detected changes in salivary NO_2 concentration following to BJ supplementation similar to those measured by the chemiluminescence and Griess methods. The response was virtually abolished when participants rinsed their mouth with antibacterial mouthwash before consuming the BJ. There were significant main effects for time, conditions and for their interaction (time*condition) ($P < 0.01$). Overall, in the water experiment, the strips underestimated NO_2^- concentration by more than 50% and by ~27% compared with chemiluminescence and Griess methods, respectively.

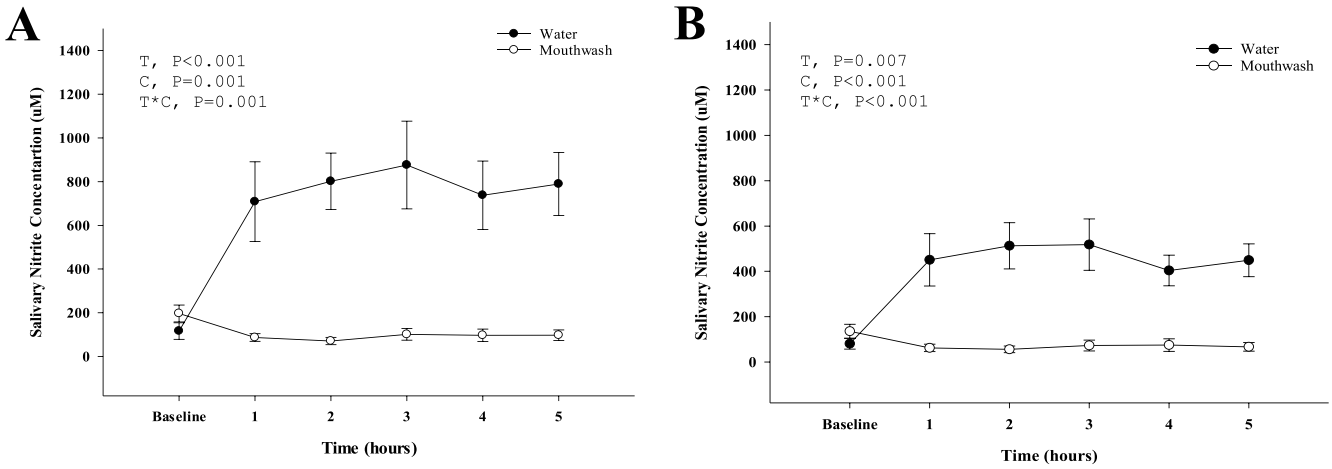

Fig. 3. Mean salivary nitrite concentrations measured by chemiluminescence (A) and Griess (B) methods after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time *condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

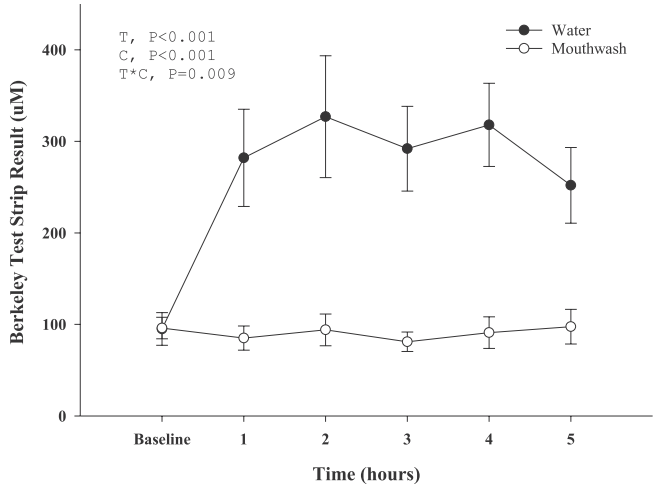

Fig. 4. Mean salivary nitrite concentrations measured by salivary NO_2^- strips after acute ingestion of BJ (70 ml). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time *condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

3.5. Agreement analysis (Bland & Altman method)

Concentrations of salivary NO_2^- estimated using the salivary strips were significantly and strongly correlated with measurements obtained using the chemiluminescence (rho = 0.77, P < 0.001) and Griess (rho = 0.83, P < 0.001) methods. Similarly, changes in salivary NO_2^- measured by the strips were significantly correlated with changes in concentration measured by the chemiluminescence (rho = 0.79, P < 0.001) and Griess (rho = 0.80, P < 0.001) methods. As expected there was a significant, strong correlation between salivary NO_3^- concentration measured by the chemiluminescence and Griess methods (P < 0.001, r = 0. 86). However, despite these statistically significant correlations, the limits of agreement between methods illustrated in the Bland Altman analysis (Fig. 5) were wide, indicating a lack of accuracy of the Griess method. In addition, the Griess and salivary strips methods showed a significant differential bias as magnitude of the differences became larger with increasing concentrations. For salivary NO_2^- measured by Griess and chemiluminescence, the estimated bias was −150 μM (95% CI −193 to −107, P = 0.0001) and the 95% limits of agreement were fairly wide (−618, 318 μM). For salivary NO_2^- measured by the salivary strips and chemiluminescence the estimated bias was −201 μM (95% CI −266 to −136, p = 0.0001) and the 95% limits of agreement were also wide (−909, 506 μM). For salivary NO_2^- measured by the salivary strips and Griess the estimated bias was −64 μM (95% CI -97 to −32, p = 0.0001) and the 95% limits of agreement ranged from −418 to 289 μM. The differences between measurements increased with higher NO_2^- concentrations. For salivary NO_3^- measured by Griess and chemiluminescence, the estimated bias was −2 mM (95% CI− 2 to −1, p = 0.0001) and 95% limits of agreement were between −8 and 5 mM).

3.6. Reliability of salivary NO_2^- strips

3.6.1. Reproducibility

Table 2 shows the mean concentrations of salivary NO_2^- estimated using the salivary strips for all 10 participants at each time-point obtained from five different observers. The intra-class correlation coefficient (ICC) showed a high reproducibility between the five observers.

3.6.2. Repeatability

Table 3 shows the results of NO_2^- measurements by salivary strips performed by five observers on two different occasions. The high ICCs indicated a high repeatability of the salivary NO_2^- strips.

3.6.3. Salivary sialin

There was no significant difference between salivary sialin concentrations measured at baseline between the water and mouthwash conditions (P = 18). There were no significant effects of condition (water v. mouthwash; P = 0.54) or time (P = 0.49), or time*condition (P = 0.41) interaction. We found a trend for an increase in sialin in the mouthwash experiment compared to water experiment but this increment was not significant at any of the time points (P > 0.05) (Fig. 6). A weak but significant correlation was found between the change in salivary sialin and the change in salivary NO_2^- concentration (r = −0.20, P = 0.04). Conversely, there was a weak positive correlation between the change in salivary sialin and the change in salivary NO_3^- concentration (r = 0.18, P = 0.06) (data not shown).

3.6.4. Blood pressure

There was no significant difference in SBP and DBP at baseline between the mouthwash and water conditions (P = 0.91 and P = 0.60, respectively). Over the 5 h following ingestion of BJ, there were no significant changes in SBP and DBP (P > 0.05) with, or without, use of the mouthwash (Fig. 7).

4. Discussion

This study investigated for the first time the validity of salivary NO_2^- strips against the gold standard chemiluminescence technique and Griess methods after acute consumption of NO_3^- rich BJ consumption with and without the use of mouthwash. Furthermore, this study evaluated for the first time whether acute NO_3^- supplementation, with and without mouthwash, altered sialin concentrations in saliva. Overall, salivary strips provide a simple and user-friendly method to detect changes in salivary NO_2^- concentrations after the consumption of high-NO_3^- foods, which could be useful for the monitoring of compliance in longer-term high-NO_3^- nutritional interventions. The strips are also characterised by a high repeatability and reproducibility, but they underestimated NO_2^- concentration compared with other laboratory methods (Griess and chemiluminescence) especially at higher salivary NO_2^- concentrations. In addition, when study participants used the mouthwash, salivary sialin concentration tended to increase following the ingestion of BJ and salivary sialin concentrations correlated with salivary NO_2^- concentration.

Clodfelter et al., tested different brands of NO saliva test strips and found that they reacted with solution containing sodium nitrite ($NaNO_2^-$) but not with sodium NO_3^-, indicating that these strips can be utilised for the selective detection of NO_2^- in biological fluids [23]. The study found that the colour intensity of the strips increased with greater concentrations of $NaNO_2^-$ and that the lowest limit of detection was 10 μM. However, the Clodfelter et al., study was performed *ex-vivo*. When the strips were applied on the tongue, we observed that colour intensity increased after the consumption of high NO_3^- BJ. However, there was no increase in colour on the strips after the use of mouthwash which confirmed the lack change in salivary NO_2^- concentration when measured by standard laboratory methods (chemilunimesence and Griess). This finding clearly indicates the sensitivity of the strips in detecting the effect of the antibacterial mouthwash, which is known to block the activity of the oral bacterial NO_3^- reductase and inhibit the conversion of NO_3^- into NO_2^- [1]. This study also demonstrated a high level of repeatability and reproducibility of the strips. In addition, our study revealed the capacity of the strips to detect changes in salivary NO_2^- concentrations following an acute oral dose of NO_3^- rich BJ (400 mg). This is in agreement with McDonagh and colleagues who found that the strips measured changes in salivary NO_2^- concentrations following the ingestion of a range of doses of NO_3^- (~5.76 and ~1.40 mmol of NO_3^-) [19].

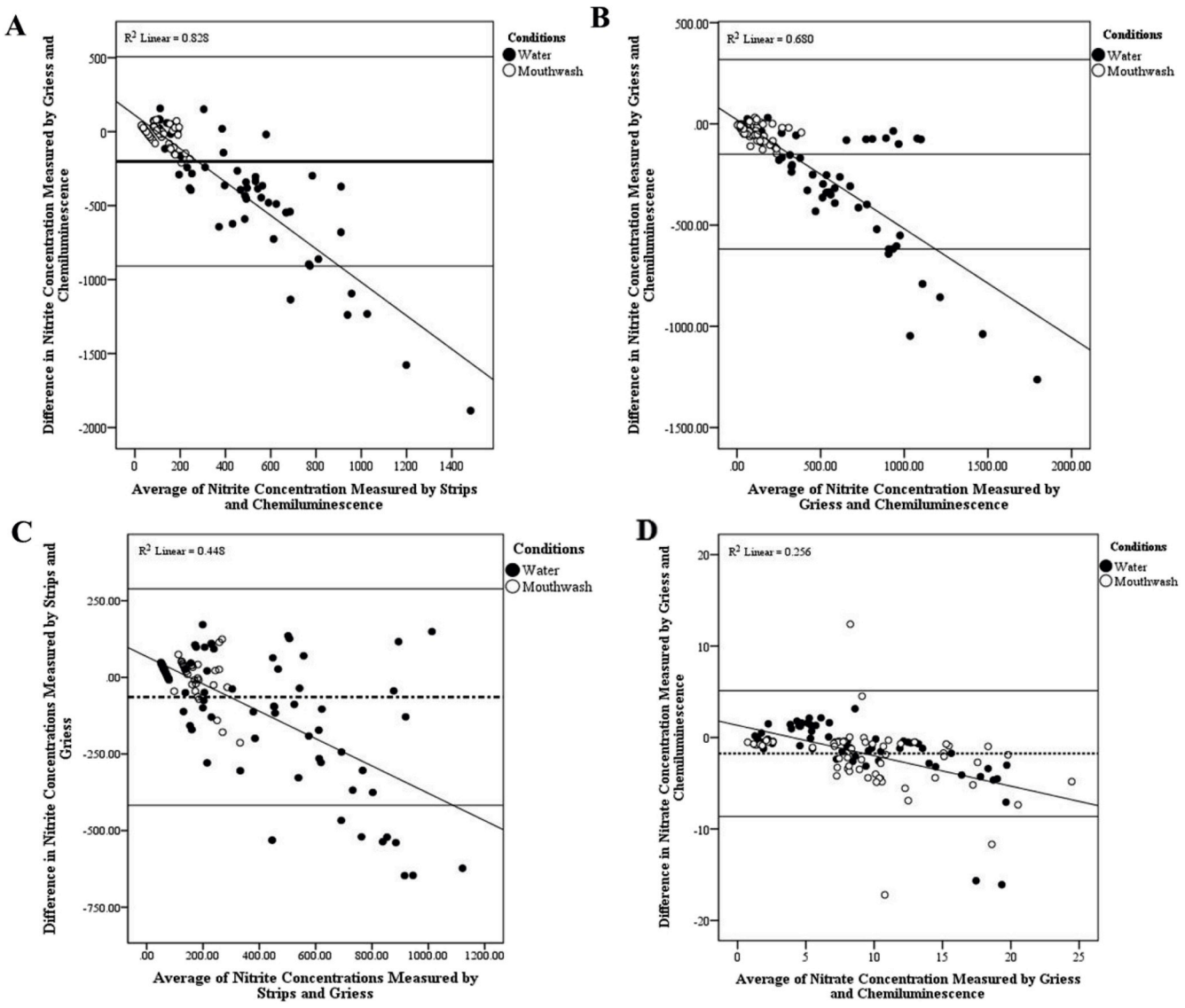

Fig. 5. Comparison of mean salivary nitrite (A, B and C) and nitrate (D) concentrations measured by two different methods. Black dash horizontal line shows the mean difference and the ± 2S.D. range (fine, black line). A regression line was fitted to the points to evaluate differential bias.

The current study revealed significant strong correlations between the strips and other laboratory methods for the measurement of absolute salivary NO_2^- concentrations and also for the measurement of changes in salivary NO_2^- concentrations. Overall, the strength of the correlations found in this study (rho = 0.80 and 0.79 for the Griess and chemiluminescence methods, respectively) was greater than the correlation (r = 0.57) reported by McDonagh et al., [16]. The different strength of the associations reported in the two studies may be explained by the use in our study of a mobile phone based application that provides a more detailed colour chart providing an assigned, quantitative value of NO_2^- concentration to each colour. McDonagh et al. used a simple colour chart which simply classified NO_2^- concentrations as depleted, low, threshold, target and high. McDonagh et al. [16]

Table 3
Intra-observer repeatability of the two strips used at each time point.

Observers	First strip	Second strip	ICC	P-value
Observer 1	173 ± 145	179 ± 153	0.938	< 0.001
Observer 2	200 ± 147	200 ± 146	0.813	< 0.001
Observer 3	185 ± 127	174 ± 103	0.833	< 0.001
Observer 4	158 ± 76	156 ± 72	0.918	< 0.001
Observer 5	76 ± 141	73 ± 137	0.720	< 0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. P-values indicate that the readings of the two strips used at each time point are significantly correlated between observers.

Table 2
Inter-observer reproducibility of strips.

Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	ICC	P-value
176 ± 147	200 ± 140	193 ± 117	157 ± 72	75 ± 129	0.91	< 0.001

ICC, intraclass correlation coefficient (absolute agreement). Salivary nitrite values are presented as mean ± SD. The average of two strips' readings of salivary nitrite strips was reported. P-value indicate that the strips reading are significantly correlated between observers.

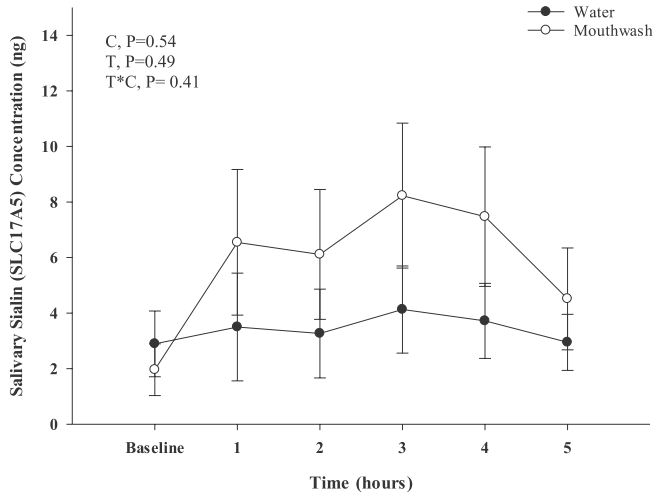

Fig. 6. Mean salivary sialin concentrations. Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*-condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

concluded that salivary strips are a practical method to estimate salivary NO_2^- concentrations after the consumption of dietary NO_3^-. However, the poor level of agreement and the significant bias between the methods may limit the application of the strips for absolute measurement of salivary nitrite concentrations [24]. We used the Bland Altman method to assess the agreement between the strips and Griess and chemiluminescence methods and found that the limits of agreement between salivary strips and other, more precise, laboratory methods are wide, suggesting that salivary strips may not provide accurate estimates of salivary NO_2^- concentrations. However, the strips detected changes to acute ingestion of high doses of NO_3^- and therefore they may be useful for monitoring the compliance in nutritional interventions testing the effects of inorganic NO_3^-. This could be a simple cost-effective method of monitoring for NO_3^- intake as the results can be seen almost instantaneously and this method can also provide an indication of NO bioavailability, without requiring access to expensive laboratory equipment. Further, this method could represent a convenient and effective solution to research studies conducted in situations where the collection and storage of saliva samples for later analysis may be problematic (e.g. studies conducted in rural areas and/or developing countries).

In pigs, Qin and co-authors identified sialin as the primary NO_3^-transporter in salivary glands and observed inhibition of NO_3^-transport after sialin expression was knocked down [5]. To our knowledge, this is the first study to examine the association between salivary sialin concentrations and changes in salivary NO_2^- and NO_3^- concentrations in humans. The acute dose of NO_3^- rich BJ did not affect sialin concentrations in saliva as concentrations remained similar to baseline levels during the experimental period. Sialin concentrations tended to increase, however, after blocking the NO_3^- conversion into NO_2^- via mouthwash and a weak but significant correlation between salivary sialin and salivary NO_2^- concentrations was observed. Tentatively, these results may suggest the existence of a feedback mechanism linking NO_3^-transport and conversion to sialin expression, but mechanistic studies with larger sample size are needed to confirm this.

Previous studies have showed that SBP can be reduced after 3 h of BJ consumption [7,25]. In addition, in a recent study, Woessner and co-workers found a significant difference in SBP changes between water and mouthwash over a 4-h period post BJ consumption [12]. In our study, we found no effect of BJ on SBP or on DBP and no effect of use of mouthwash. A possible explanation for this difference is the higher SBP baseline values in the study by Woessner et al. compared with our study (124 vs 115 mmgH), which may make individuals more responsive to the BP lowering effect of NO_3^- [26].

In this study we administered mouthwash on one occasion, which contrasts with several previous investigations where mouthwash has been administered two or more times daily over several days [14,15,27]. We found that acute mouthwash use abolished the increase in salivary NO_2^- concentration consequent to beetroot juice ingestion, suggesting that one-time use of mouthwash is sufficient to blunt, at least temporarily, the NO_3^--reducing capacity of the oral microbiome. However, in contrast with some (e.g. Ref. [14]) but not all (e.g. Ref. [27]) prolonged mouthwash studies, we did not observe a mouthwash-induced increase in BP. Future studies are warranted to determine potential differential effects of acute versus chronic mouthwash use on markers of NO bioavailability and physiological responses.

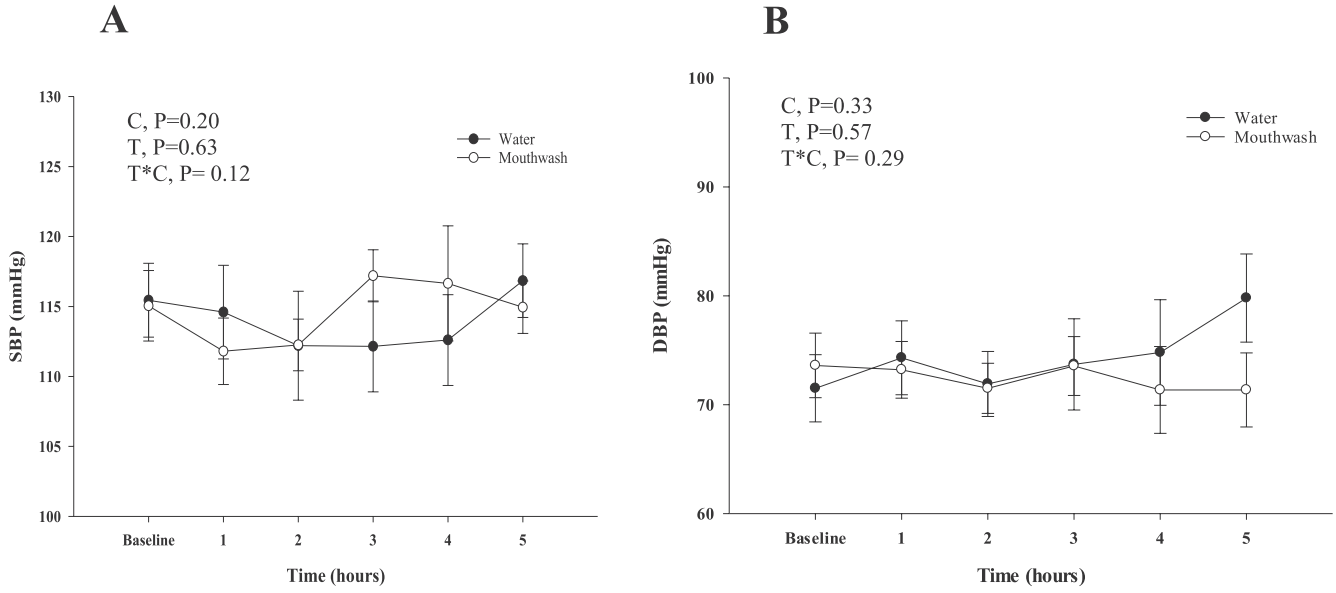

Fig. 7. Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP). Filled circles represent times when individuals rinsed their mouth with water 15 min before beetroot ingestion. Open circles represent times when individuals rinsed their mouth with mouthwash. Data were analysed with a 2-factor repeated measures (time*condition) ANOVA. Data are expressed as mean ± SEM, n = 10.

A limitation of the current study is the small sample size, which reduced the power to detect significant changes in BP and sialin concentrations after BJ ingestion. However, the primary purpose of the study was to test the validity of the salivary strips for which the study size was deemed as adequate based on the sample size of previous studies with a similar study design [13]. The short washout period between the two experiments (mouthwash and water) could be considered a potential limitation if the oral microbiota had not recovered from the acute use of mouthwash. However, there was no significant difference in the AUC for salivary NO_2^- ($P = 0.34$) between the participants who used mouthwash on the first experimental day and those who used mouthwash on the second day. This indicates that the NO_3^--reducing capacity of the oral bacteria may recover within 24 h following the use of mouthwash. It is challenging to reconcile this finding with the currently limited evidence on the effects of antiseptic mouthwash on oral bacteria. *Ex vivo* studies have shown that, after exposure to chlorhexidine digluconate (0.2%) for 3 min, the proportion of viable bacteria is reduced by approximately 30% within a few hours and that full recovery of the bacteria requires up to 5 weeks [21]. The vitality of plaque bacteria after treatment with chlorhexidine digluconate (0.2%) was investigated in 6 volunteers studied over four days [22]. At 24 h after the last exposure to the mouthwash, plaque bacteria vitality was 60%. Our observation of no increase in NO_2^- concentrations over the 5 h following use of mouthwash indicates immediate, and total, suppression of the NO_3^--reducing capacity of the bacteria. However, 24 h after mouthwash use, the capacity of oral NO_3^--reducing bacteria has been re-established as we observed no difference in the AUCs of salivary NO_2^- concentrations measured during the two water experiments. These findings suggest differential kinetics after mouthwash use for total plaque bacteria viability and for the specific bacteria responsible for NO_3^--reduction. The effects of frequent mouthwash treatment on NO_3^--reducing oral bacteria and its impact on the recovery the bacterial flora after stopping the treatment are very relevant research questions that should be explored in future studies.

In conclusion, the commercially available salivary NO_2^- strips applied in this study showed a high level of reproducibility and repeatability in detection of changes in salivary NO_2^- concentrations following acute ingestion of inorganic NO_3^-. However, Bland Altman plots indicated that there is a poor agreement between salivary strips, chemiluminescence and Griessmethods, which means that these strips are not sufficiently accurate for the measurement of absolute concentrations of NO_2^- in saliva. Salivary strips may be a cost effective and simple method for monitoring changes in salivary NO_2^- concentrations and for monitoring compliance in intervention studies focussed on increasing dietary NO_3^- intake. The preliminary findings suggesting an association between salivary NO_2^- concentrations with the salivary NO_3^- transporter (sialin) are intriguing and should be explored in future mechanistic studies.

Conflicts of interest

The authors have no conflict of interest to declare.

Funding

This study was funded by Umm-Al Qura University, Saudi Arabia, Makkah, and Newcastle core budget.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.07.002.

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