



Blunted Salivary Nitrite Surge as a Predictor of Post-Glucose Microvascular and Vagal Recovery in Habitual Antiseptic-Mouthwash Users

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ABSTRACT

The enterosalivary nitrate–nitrite–nitric oxide (NO) pathway contributes to systemic NO bioavailability via commensal oral bacteria that reduce dietary nitrate to nitrite. Antiseptic mouthwashes, particularly chlorhexidine, suppress oral nitrate-reducing capacity, but the acute physiological consequences on autonomic and microvascular function following a metabolic challenge remain poorly characterised. This was a prospective, randomized, double-blind, placebo-controlled, parallel-group trial. Fifty healthy adults (17–32 years) were randomised (1:1) to receive 0.12% chlorhexidine (CHX, n=25) or placebo mouthwash (PLA, n=25) twice daily for seven days. On day 8, participants underwent a 75 g oral glucose tolerance test with measurements at 0, 30, 60, 90, 120, 180, and 240 minutes post-glucose. Chlorhexidine reduced oral nitrate-reducing capacity by 65% compared to placebo ($p < 0.001$). Salivary nitrite concentrations were significantly lower in the CHX group at all postprandial time points, with a blunted absolute rise (9.4 vs. 32.9 $\mu\text{mol/L}$; $p < 0.001$) and reduced AUC_{0-240} (13,690 vs. 25,246 $\mu\text{mol}\cdot\text{min/L}$; $p < 0.001$). A group $\times$ time interaction was highly significant for salivary nitrite in a mixed model analysis (Wald $\chi^2 = 347.9$, $p < 0.001$). CHX exhibited significantly less vagal recovery (i.e., decreased late-phase RMSSD, $g = 0.80$; increased LF/HF ratio, $g = 3.08$). The CHX group had significantly delayed microvascular reperfusion, as evidenced by increased thermal half-time ($g = 2.91$), decreased perfusion recovery AUC ($g = 3.65$), and decreased rewarming slope ($g = 3.17$, $p < 0.001$). Salivary nitrite AUC was an independent predictor of late RMSSD ($p < 0.05$) and rewarming slope ($p < 0.01$), and oral nitrate-reducing capacity was an independent predictor of perfusion recovery AUC ($p < 0.01$). The significant negative coefficients for chlorhexidine allocation remained in all models. The number of mild oral adverse events was higher in the CHX group (40% vs 8%; $p = 0.018$), but there were no serious adverse events.

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INTRODUCTION

The pleiotropic signaling molecule nitric oxide (NO) is essential for preserving vascular homeostasis, controlling microvascular perfusion, adjusting autonomic nervous system activity, and reducing inflammation. It is mostly produced endogenously thru the L-arginine–nitric oxide synthase (SON) pathway. However, NOS activity is compromised in diseases like metabolic syndrome and aging, a condition called endothelial NOS uncoupling, which results in decreased NO bioavailability and elevated oxidative stress (Janaszak-Jasiecka et al., 2023; Tain & Hsu, 2022). The pleiotropic signaling molecule nitric oxide (NO) is essential for preserving vascular homeostasis, controlling microvascular perfusion, adjusting autonomic nervous system activity, and reducing inflammation. It is mostly produced endogenously thru the L-arginine–nitric oxide synthase (SON) pathway. However, SON activity is compromised in diseases like metabolic syndrome and aging, a condition called endothelial NOS uncoupling, which results in decreased NO bioavailability and elevated oxidative stress (Donato et al., 2015; Hsu & Tain, 2026). This mechanism is crucially dependent on oral nitrate-reducing bacteria, especially taxa like *Neisseria*, *Rothia*, *Veillonella*, *Actinomyces*, and others (Chai et al., 2024; Morou-Bermúdez et al., 2025). Salivary bacterial nitrate reduction activity (DNRA) is inversely correlated with insulin resistance (HOMA-IR), fasting glucose, and 2-hour postprandial glucose levels, and it can be influenced by lifestyle factors such as physical activity, toothbrushing frequency, and smoking, according to a recent cross-sectional study (Goh et al., 2019). Oral nitrate-reducing bacteria have been proposed by some researchers as potential future probiotics for blood pressure regulation due to emerging evidence that nitrate itself fosters the growth of these beneficial bacteria in a bidirectional symbiotic connection (Chai et al., 2024; Paiva et al., 2024). On the other side, by removing nitrate-reducing bacteria, antiseptic mouthwash—especially those containing chlorhexidine—acts as a powerful disruptor of this system, lowering salivary and plasma nitrite concentrations, raising blood pressure, and decreasing vascular function (Bescos et al., 2025; Toonen et al., 2026). Its use has been connected in recent research to increased insulin resistance, systemic inflammation, oral microbial dysbiosis, and even higher mortality in some populations (Morou-Bermúdez et al., 2025; Sindhusa et al., 2025). According to a recent comprehensive analysis, using antiseptic mouthwash frequently raises serum glucose levels, systolic and diastolic blood pressure, and salivary and plasma nitrate/nitrite concentrations as well as the NO metabolic axis (Sindhusa et al., 2025). The metabolism of glucose is intimately related to this route. The ability of the oral microbiota to produce nitrite is necessary for the metabolic advantages of dietary nitrate, as shown by the ORIGINS study Oral Infections Glucose

Intolerance and Insulin Resistance Study (Goh et al., 2024). In order to control glucose uptake, storage, and hepatic glucose synthesis after oral glucose consumption, insulin causes vasodilation and vagal nerve activity) vagal tone) NO is a crucial mediator in this vascular and autonomic response (Lima et al., 2024). Salivary nitrite levels, however, do not seem to be studied as a predictor of delayed vagal reactivation and poor microvascular recovery following a glucose challenge, as are the levels of blunted salivary nitrite. This is a research gap that needs to be filled (Salama et al., 2026; Yang et al., 2025). By assessing the prognostic ability of the attenuated salivary nitrite rise among regular mouthwash users for impaired microvascular recovery and delayed vagal re-activation, this study seeks to close this gap and hypothesizes an independent relationship.

Study Design, Participants, and Sample Size

All experimental procedures were conducted at the Human Physiology and Clinical Biochemistry Laboratories, Al-Hakim Hospital, Maysan Governorate, Iraq, during 2026. This was a prospective, randomized, double-blind, placebo-controlled, parallel-group trial. Fifty healthy, non-smoking adults aged 17–32 years with healthy periodontium (plaque and gingival indices < 1) were randomly allocated in a 1:1 ratio to receive either 0.12% chlorhexidine gluconate (CHX) or a visually identical placebo mouthwash for seven days. Randomization was performed using a computer-generated block sequence, and allocation was concealed in sealed envelopes. Sample size was estimated a priori using G*Power version 3.1.9.7 (two-sided test, allocation ratio 1:1, $\alpha = 0.05$, power = 80%, and effect size = 0.80). The calculation yielded 25 participants per group; therefore, a total of 50 participants were enrolled. Exclusion criteria included cardiovascular disease, diabetes, regular medication use, and allergy to chlorhexidine. The study protocol was reviewed and approved by the Institutional Administration/Ethics Committee of Al-Hakim Hospital. In addition, official approval for sample collection was obtained from the Iraqi Ministry of Health under approval letter No. 1077, dated April 21, 2025, prior to the commencement of sample collection. All participants volunteered to participate in the study and provided verbal and written informed consent before data and sample collection, including blood and saliva sampling and blood pressure measurement. Full confidentiality of participants' information was ensured throughout the study.

Intervention and Post-Glucose Protocol

Participants rinsed with 15 mL of their assigned mouthwash for 30 seconds twice daily (morning and evening) for seven consecutive days. On the experimental day, following a 10–12 h overnight fast, participants consumed a 75 g anhydrous glucose dissolved in 250–300

mL water within 5 minutes under supervision. Measurements were performed at baseline (0, 30, 60, 90, 120, 180, and 240) minutes post-glucose. No food was provided during the 240 minutes observation period; water intake was standardized.

Saliva Collection and Assays

Saliva Sampling: Unstimulated whole saliva was collected by passive drool for 5 minutes into pre-weighed tubes. Participants refrained from swallowing, talking, or coughing during collection. Volume was determined gravimetrically (density = 1 g/mL) and flow rate expressed as mL/min. Samples were immediately placed on wet ice, centrifuged (3000×g, 15 min, 4°C), aliquoted, and stored at -80°C.

Nitrite and Nitrate (Primary Outcome): Salivary nitrite and nitrate ($\mu\text{mol/L}$) were measured using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay Kit (Item No. 780001). The assay uses the Griess reaction with enzymatic nitrate reduction; absorbance was read at 540 nm. Samples were run in duplicate; coefficient of variation (CV) was < 10%.

Oral Nitrate-Reducing Capacity ($\mu\text{mol/L/min}$): Assessed ex vivo by incubating a fresh saliva aliquot with a standard sodium nitrate solution at 37°C, and calculating the linear rate of nitrite generation.

Capillary Glucose: Measured immediately via finger-prick using the Accu-Chek Inform II system with compatible test strips (Roche Diagnostics).

Serum Markers (Oxidative Stress & Inflammation): Venous blood was collected into serum-separating tubes, clotted for 30 min, centrifuged (1500–2000×g, 15 min, 4°C) and stored at -80°C. MDA was assayed using the Cayman TBARS Kit (Item No. 10009055); Total Antioxidant Capacity (TAC) via the Cayman Antioxidant Assay Kit (Item No. 709001); and hs-CRP via the R&D Systems Quantikine ELISA Kit (Catalog No. DCRP00). All assays were performed in duplicate (CV < 10% accepted).

Haemodynamic, Autonomic, and Arterial Stiffness Assessments

Peripheral Blood Pressure: Brachial systolic (SBP), diastolic (DBP), and heart rate (HR) were measured using the OMRON HEM-907XL Professional IntelliSense Monitor. Three readings were taken after a 5-min rest; the mean of the last two was used. MAP was calculated as $\text{DBP} + \frac{1}{3}(\text{SBP} - \text{DBP})$.

Central Haemodynamics and Arterial Stiffness: Central systolic pressure, central pulse pressure, carotid-femoral pulse wave velocity (cfPWV, m/s), and augmentation index corrected for HR 75 bpm (Alx@75) were assessed using the SphygmoCor XCEL system (AtCor Medical) with software version 1.3.

Heart Rate Variability (HRV): Continuous ECG was recorded during stable resting periods using the BIOPAC

MP160 system with ECG100C amplifier (BIOPAC Systems Inc.). HRV analysis (RMSSD, SDNN, HF power, LF/HF ratio) was performed using Kubios HRV Scientific version 4.3 (Kubios Oy).

Microvascular and Thermal Assessments

Digital Thermal Re-warming: Performed using a FLIR T540 infrared thermal camera (Teledyne FLIR; 464×348 pixels, 30 Hz). After baseline stabilisation, the hand was immersed in a 15°C water bath for 60 seconds. Rewarming was monitored for ≥ 10 minutes. Regions of interest (ROIs) were fixed across all measurements. Key derived parameters included rewarming slope and thermal half-time.

Laser Doppler Perfusion: Cutaneous microvascular perfusion (perfusion units, PU) was measured using the moorLAB-LDF laser Doppler flowmeter (Moor Instruments Ltd.). Baseline perfusion, peak reperfusion, and perfusion recovery area under the curve (AUC) were derived.

Outcome Definitions

The primary outcome was salivary nitrite AUC_{0-240} ($\mu\text{mol}\cdot\text{min/L}$), calculated by the linear trapezoidal rule. Secondary outcomes included: salivary nitrate AUC, nitrite peak, T_{max} , absolute/percentage rise, late $\text{AUC}_{120-240}$; capillary glucose AUC; HRV metrics (RMSSD, SDNN, HF, LF/HF); thermal recovery parameters (rewarming slope, half-time); laser-Doppler parameters (peak reperfusion, recovery AUC); brachial and central haemodynamics; cfPWV; Alx@75; oral nitrate-reducing capacity; and serum MDA, TAC, and hs-CRP.

Statistical Analysis:

Analyses were performed using IBM SPSS Statistics version 32.0. Normality was assessed with the Shapiro-Wilk test. Normally distributed variables are reported as mean $\pm$ SD; non-normally distributed as median (IQR); categorical as n (%). Between-group comparisons used Welch's t-test (normal) or Mann-Whitney U (non-normal) for continuous variables, and Fisher's exact for categorical variables. Effect sizes are reported as Hedges' g (bias-corrected). Repeated-measures data (salivary nitrite, nitrate, glucose, haemodynamics, HRV) were analysed using linear mixed-effects models with REML estimation. Fixed effects were group (CHX vs. placebo), time (0–240 min), and their interaction; participant was included as a random effect. Wald χ^2 and exact p-values are reported. Multiple linear regression identified independent predictors of late RMSSD, rewarming slope, perfusion recovery AUC, and nitrite AUC. Predictors included nitrite AUC, oral nitrate-reducing capacity, chlorhexidine allocation, age, BMI, and glucose AUC. Results are presented as standardised β -coefficients with 95% CI. All tests were two-tailed; significance was set at $p < 0.05$.

Table 1: Baseline Demographic, Anthropometric, Clinical, Oral-Health, and Physiological Characteristics of the Chlorhexidine and Placebo Groups

Variable	Chlorhexidine (n=25)	Placebo (n=25)	Effect estimate, 95% CI	Test statistic	p value	Effect size
Age, years	24.0 ± 3.3	25.1 ± 3.5	-1.1 (-3.0 to 0.9)	t=-1.127; df=47.8	0.265	Hedges g=-0.314
Height, cm	173.9 ± 7.0	176.1 ± 5.3	-2.2 (-5.7 to 1.3)	t=-1.248; df=44.4	0.218	Hedges g=-0.348
Weight, kg	73.0 ± 8.5	73.7 ± 7.7	-0.7 (-5.3 to 3.9)	t=-0.303; df=47.6	0.763	Hedges g=-0.084
Body mass index, kg/m ²	24.12 ± 2.13	23.76 ± 2.15	0.36 (-0.86 to 1.57)	t=0.587; df=48.0	0.560	Hedges g=0.163
Waist circumference, cm	80.3 ± 6.4	80.9 ± 5.2	-0.6 (-3.9 to 2.7)	t=-0.383; df=46.2	0.704	Hedges g=-0.107
Habitual physical activity, MET- min/week	2101 [1760– 2393]	2250 [1778– 2805]	—	U=295.0	0.742	Rank-biserial r=-0.056
Fasting capillary glucose, mmol/L	4.94 ± 0.36	4.86 ± 0.27	0.08 (-0.10 to 0.26)	t=0.875; df=44.5	0.386	Hedges g=0.244
Resting heart rate, beats/min	67.9 ± 6.3	68.2 ± 5.9	-0.4 (-3.8 to 3.1)	t=-0.205; df=47.8	0.839	Hedges g=-0.057
Resting systolic blood pressure, mmHg	115.9 ± 7.0	117.0 ± 6.1	-1.0 (-4.7 to 2.7)	t=-0.546; df=47.2	0.588	Hedges g=-0.152
Resting diastolic blood pressure, mmHg	73.8 ± 6.8	71.8 ± 3.6	2.0 (-1.2 to 5.1)	t=1.276; df=36.6	0.210	Hedges g=0.355
Mean arterial pressure, mmHg	87.8 ± 5.3	86.8 ± 3.6	1.0 (-1.6 to 3.6)	t=0.770; df=42.1	0.446	Hedges g=0.214
Plaque index	0.46 ± 0.14	0.39 ± 0.14	0.07 (-0.01 to 0.15)	t=1.835; df=48.0	0.073	Hedges g=0.511
Gingival index	0.22 ± 0.10	0.24 ± 0.09	-0.01 (-0.07 to 0.04)	t=-0.534; df=47.1	0.596	Hedges g=-0.149
Unstimulated salivary flow, mL/min	0.52 ± 0.10	0.51 ± 0.11	0.01 (-0.05 to 0.06)	t=0.225; df=47.5	0.823	Hedges g=0.063
Non-smoker, n (%)	25 (100.0%)	25 (100.0%)	0.0 percentage points	Fisher exact	1.000	OR=1.00 (0.02–52.37)
Healthy oral screening examination, n (%)	25 (100.0%)	25 (100.0%)	0.0 percentage points	Fisher exact	1.000	OR=1.00 (0.02–52.37)

RESULTS AND DISCUSSION

Participant Baseline Characteristics

Fifty subjects were enrolled and randomly allocated (25 per group) and all followed the study protocol. Table 1 Shows that no significant difference in the baseline variables was seen between the groups (all P>0.05). The mean age was 24 years in the CHX group and 25 years in

the placebo group. Similarly, body mass index (24.12 vs. 23.76 kg/m²; p = 0.560), resting systolic blood pressure (115.9 vs. 117.0 mmHg; p = 0.588), and fasting capillary glucose (4.94 vs. 4.86 mmol/L; p = 0.386) were comparable between groups. All participants (100%) in both groups were non-smokers and had a healthy oral screening examination at baseline.

Table 2: Intervention Adherence, Protocol Compliance, and Adverse Events in the Chlorhexidine and Placebo Groups

Variable	Chlorhexidine (n=25)	Placebo (n=25)	Effect estimate, 95% CI	Test statistic	p value	Effect size
Completed rinse doses, median [IQR]	7 [7–7]	7 [7–7]	—	U=312.5	1.000	Rank-biserial r=0.000
Adherence, %	98.9 ± 4.0	98.9 ± 4.0	0.0 (-2.2 to 2.2)	t=0.000; df=48.0	1.000	Hedges g=0.000
Returned rinse volume, mL	6.5 ± 5.1	6.8 ± 5.1	-0.3 (-3.2 to 2.6)	t=-0.210; df=48.0	0.835	Hedges g=-0.058
Final pre-visit dose completed, n (%)	24 (96.0%)	24 (96.0%)	0.0 percentage points	Fisher exact	1.000	OR=1.00 (0.06–16.93)
Laboratory attendance, n (%)	25 (100.0%)	25 (100.0%)	0.0 percentage points	Fisher exact	1.000	OR=1.00 (0.02–52.37)

Variable	Chlorhexidine (n=25)	Placebo (n=25)	Effect estimate, 95% CI		Test statistic	p value	Effect size
Fasting requirements fulfilled, n (%)	24 (96.0%)	24 (96.0%)	0.0 percentage points		Fisher exact	1.000	OR=1.00 (0.06–16.93)
Completed 240-minute protocol, n (%)	24 (96.0%)	25 (100.0%)	-4.0	percentage points	Fisher exact	1.000	OR=0.32 (0.01–8.25)
Any mild oral adverse event, n (%)	10 (40.0%)	2 (8.0%)	32.0	percentage points	Fisher exact	0.018	OR=7.67 (1.47–39.99)
Altered or bitter taste, n (%)	8 (32.0%)	1 (4.0%)	28.0	percentage points	Fisher exact	0.023	OR=11.29 (1.29–98.89)
Temporary tongue numbness, n (%)	5 (20.0%)	0 (0.0%)	20.0	percentage points	Fisher exact	0.050	OR=13.68 (0.71–262.19)
Oral burning, n (%)	4 (16.0%)	1 (4.0%)	12.0	percentage points	Fisher exact	0.349	OR=4.57 (0.47–44.17)
Mucosal irritation, n (%)	3 (12.0%)	1 (4.0%)	8.0	percentage points	Fisher exact	0.609	OR=3.27 (0.32–33.84)
Dry mouth, n (%)	3 (12.0%)	1 (4.0%)	8.0	percentage points	Fisher exact	0.609	OR=3.27 (0.32–33.84)
Minor oral discoloration, n (%)	2 (8.0%)	0 (0.0%)	8.0	percentage points	Fisher exact	0.490	OR=5.43 (0.25–118.96)
Serious adverse event, n (%)	0 (0.0%)	0 (0.0%)	0.0	percentage points	Fisher exact	1.000	OR=1.00 (0.02–52.37)
Treatment discontinuation, n (%)	0 (0.0%)	0 (0.0%)	0.0	percentage points	Fisher exact	1.000	OR=1.00 (0.02–52.37)

Adherence, Tolerability, and Safety

The compliance with the mouthwash protocol was very good and similar for both groups (Table 2). There was no significant difference in the number of completed doses of rinses (mean: 98.9 %) between the two groups ($p = 1.000$). There was no significant difference in objective compliance (returned mouthwash volume) between CHX (6.5 mL) and PLA (6.8 mL; $p = 0.835$). Twenty-four participants completed the entire 240 minutes protocol in the CHX group (96%) and 25 participants completed the entire protocol in the placebo group (100%).

The CHX group had more mild oral adverse events. The incidence of any mild oral adverse event was significantly higher in the CHX group compared with the placebo group (40% vs. 8%; $p = 0.018$). Altered or bitter taste was the most commonly reported event (32% vs. 4%; $p = 0.023$), followed by temporary tongue numbness (20% vs. 0%; $p = 0.050$). There were no serious adverse events reported and no participant withdrew from the study because of adverse events.

Table 3: Salivary Nitrate and Nitrite Kinetics, Oral Nitrate-Reducing Capacity, and Salivary Nitrite Area under the Curve

Variable	Chlorhexidine (n=25)	Placebo (n=25)	Effect estimate, 95% CI		Test statistic	p value	Effect size
Oral nitrate-reducing capacity, $\mu\text{mol/L/min}$	0.59 $\pm$ 0.16	1.69 $\pm$ 0.20	-1.10 (-1.20 to -0.99)		$t = -21.410$; $df = 45.6$	<0.001	Hedges $g = -5.961$
Baseline salivary nitrite, $\mu\text{mol/L}$	53.8 $\pm$ 12.0	89.6 $\pm$ 9.8	-35.8 (-42.0 to -29.5)		$t = -11.545$; $df = 46.1$	<0.001	Hedges $g = -3.214$
Peak salivary nitrite, $\mu\text{mol/L}$	63.1 $\pm$ 10.7	122.4 $\pm$ 9.6	-59.3 (-65.1 to -53.5)		$t = -20.601$; $df = 47.5$	<0.001	Hedges $g = -5.735$
Time to peak salivary nitrite, min	90 [60–90]	60 [60–60]	—		$U = 396.0$	0.069	Rank-biserial $r = 0.267$
Absolute nitrite rise, $\mu\text{mol/L}$	9.4 $\pm$ 3.7	32.9 $\pm$ 6.2	-23.5 (-26.4 to -20.5)		$t = -16.144$; $df = 39.2$	<0.001	Hedges $g = -4.494$
Percentage nitrite rise, %	19.3 $\pm$ 11.7	37.4 $\pm$ 9.3	-18.0 (-24.0 to -12.0)		$t = -6.043$; $df = 45.7$	<0.001	Hedges $g = -1.682$
Nitrite AUC_{0-240} , $\mu\text{mol} \cdot \text{min/L}$	13,690 $\pm$ 2,578	25,246 $\pm$ 2,433	-11,556 (-12,982 to -10,131)		$t = -16.300$; $df = 47.8$	<0.001	Hedges $g = -4.538$
Incremental nitrite AUC_{0-240} , $\mu\text{mol} \cdot \text{min/L}$	782 $\pm$ 842	3,750 $\pm$ 1,364	-2,968 (-3,616 to -2,320)		$t = -9.256$; $df = 40.0$	<0.001	Hedges $g = -2.577$
Late nitrite $\text{AUC}_{120-240}$, $\mu\text{mol} \cdot \text{min/L}$	6,635 $\pm$ 1,275	11,918 $\pm$ 1,276	-5,282 (-6,008 to -4,557)		$t = -14.644$; $df = 48.0$	<0.001	Hedges $g = -4.077$
Baseline salivary nitrate, $\mu\text{mol/L}$	320.4 $\pm$ 29.9	220.8 $\pm$ 22.4	99.5 (84.4 to 114.6)		$t = 13.298$; $df = 44.5$	<0.001	Hedges $g = 3.702$
Nitrate AUC_{0-240} , $\mu\text{mol} \cdot \text{min/L}$	78,622 $\pm$ 6,746	53,873 $\pm$ 5,470	24,749 (21,253 to 28,245)		$t = 14.248$; $df = 46.0$	<0.001	Hedges $g = 3.967$

Salivary Nitrite and Nitrate Kinetics

The effects of the chlorhexidine intervention were highly inhibitory of the enterosalivary nitrate–nitrite pathway (Table 3). Oral nitrate-reducing capacity was markedly lower in the CHX group compared to the placebo group (0.59 vs. 1.69 $\mu\text{mol/L/min}$; $p < 0.001$). Salivary nitrite levels were significantly lower in the CHX group than the placebo group at baseline (53.8 vs. 89.6 $\mu\text{mol/L}$, $p < 0.001$). The highest level of salivary nitrite reached after 240 minutes after eating was significantly reduced in the CHX group (63.1 $\mu\text{mol/L}$ vs. 122.4 $\mu\text{mol/L}$; $p < 0.001$).

The absolute nitrite rise was reduced by approximately 70% in the CHX group compared to placebo (9.4 vs. 32.9 $\mu\text{mol/L}$; $p < 0.001$), and the percentage rise was similarly blunted (19.3 vs. 37.4; $p < 0.001$). The total postprandial nitrite exposure, quantified as nitrite AUC_{0-240} , was significantly lower in the CHX group (13,690 vs. 25,246 $\mu\text{mol}\cdot\text{min/L}$; $p < 0.001$). Conversely, both the salivary nitrate concentration and the nitrate AUC_{0-240} were significantly greater in the CHX group (both $p < 0.001$), which indicates less bacterial reduction of nitrate to nitrite.

Table 4: Repeated-Measures Mixed-Model Results

Outcome	Effect	Wald χ^2	df	Exact p value
Salivary nitrite	Group	135.311	1	2.82×10^{-31}
Salivary nitrite	Time	1292.973	6	3.60×10^{-276}
Salivary nitrite	Group $\times$ time	347.852	6	4.46×10^{-72}
Salivary nitrate	Group	177.467	1	1.73×10^{-40}
Salivary nitrate	Time	101.098	6	1.48×10^{-19}
Salivary nitrate	Group $\times$ time	9.052	6	0.171

For the purposes of a formal analysis of the temporal trajectories, a repeated measures mixed model analysis was conducted (Table 4). The difference in response to glucose ingestion for salivary nitrite was significant across all groups (Wald $\chi^2 = 135.3$, $p < 0.001$), significant across time (Wald $\chi^2 = 1293.0$, $p < 0.001$) and highly significant for the interaction of group $\times$ time (Wald $\chi^2 = 347.9$, $p < 0.001$), indicating that the response to glucose ingestion differed

fundamentally between groups. Significant main effects for group and time were found for salivary nitrate, but the group $\times$ time interaction was not (Wald $\chi^2 = 9.05$, $p = 0.171$); this means the temporal trend for change was the same for each group, but there were consistently higher levels of nitrate in the CHX group.

Table 5: Post-Glucose Capillary Glucose, Brachial Blood Pressure, Heart Rate, Central Haemodynamic, and Arterial-Stiffness Responses

Variable	Chlorhexidine (n=25)	Placebo (n=25)	Effect estimate, 95% CI	Test statistic	p value	Effect size
Fasting capillary glucose, mmol/L	4.98 $\pm$ 0.40	4.86 $\pm$ 0.35	0.12 (−0.10 to 0.33)	t=1.092; df=47.3	0.280	Hedges g=0.304
Peak capillary glucose, mmol/L	8.01 $\pm$ 0.42	7.88 $\pm$ 0.31	0.13 (−0.08 to 0.34)	t=1.281; df=44.4	0.207	Hedges g=0.357
Time to peak capillary glucose, min	30 [30–30]	30 [30–30]	—	U=300.0	0.337	Rank-biserial r=−0.040
Capillary glucose at 240 min, mmol/L	5.01 $\pm$ 0.42	4.97 $\pm$ 0.29	0.04 (−0.17 to 0.25)	t=0.393; df=42.7	0.697	Hedges g=0.109
Glucose AUC_{0-240} , mmol·min/L	1,473 $\pm$ 87	1,460 $\pm$ 68	13 (−31 to 58)	t=0.611; df=45.4	0.545	Hedges g=0.170
Mean post-glucose systolic BP, mmHg	117.1 $\pm$ 7.0	118.0 $\pm$ 5.8	−0.9 (−4.5 to 2.8)	t=−0.475; df=46.3	0.637	Hedges g=−0.132
Mean post-glucose mean arterial pressure, mmHg	88.6 $\pm$ 5.3	87.5 $\pm$ 3.5	1.1 (−1.5 to 3.7)	t=0.876; df=41.8	0.386	Hedges g=0.244
Mean post-glucose heart rate, beats/min	72.2 $\pm$ 6.3	72.8 $\pm$ 6.0	−0.6 (−4.1 to 2.9)	t=−0.351; df=47.9	0.727	Hedges g=−0.098
Mean post-glucose central systolic pressure, mmHg	104.4 $\pm$ 6.9	104.3 $\pm$ 6.3	0.1 (−3.7 to 3.9)	t=0.045; df=47.6	0.964	Hedges g=0.013
Mean post-glucose central pulse pressure, mmHg	34.1 $\pm$ 4.2	35.6 $\pm$ 4.6	−1.5 (−4.0 to 1.0)	t=−1.218; df=47.6	0.229	Hedges g=−0.339

Mean post-glucose carotid-femoral PWV, m/s	6.07 ± 0.53	5.88 ± 0.42	0.18 (-0.09 to 0.46)	t=1.349; df=45.8	0.184	Hedges g=0.375
Mean post-glucose augmentation index@75, %	3.5 ± 4.2	3.9 ± 4.0	-0.4 (-2.7 to 1.9)	t=-0.328; df=47.9	0.744	Hedges g=-0.091

Glycaemic and Haemodynamic Responses

The glycaemic and postprandial haemodynamic responses are summarised in Table 5. Fasting capillary glucose (4.98 vs. 4.86 mmol/L; $p = 0.280$), peak glucose (8.01 vs. 7.88 mmol/L; $p = 0.207$), and glucose AUC_{0–240} (1,473 vs. 1,460 mmol•min/L; $p = 0.545$) did not differ

between groups. Additionally, mean brachial and central haemodynamic indices after a meal (systolic blood pressure, mean arterial pressure, heart rate, carotid-femoral pulse wave velocity, and augmentation index at 75 beats per minute) were similar between groups (all $p > 0.05$).

Table 6: Repeated-Measures Mixed-Model Results

Outcome	Group χ^2 (df), p	Time χ^2 (df), p	Group × time χ^2 (df), p
Capillary glucose	1.289 (1), $p=0.256$	6646.299 (6), $p<0.001$	5.395 (6), $p=0.494$
Brachial systolic BP	0.151 (1), $p=0.697$	24.037 (6), $p<0.001$	3.117 (6), $p=0.794$
Brachial diastolic BP	0.786 (1), $p=0.375$	7.062 (6), $p=0.315$	6.499 (6), $p=0.370$
Mean arterial pressure	0.285 (1), $p=0.594$	24.280 (6), $p<0.001$	5.328 (6), $p=0.502$
Heart rate	<0.001 (1), $p=0.996$	572.764 (6), $p<0.001$	7.141 (6), $p=0.308$
Central systolic pressure	0.018 (1), $p=0.893$	56.392 (6), $p<0.001$	6.344 (6), $p=0.386$
Central pulse pressure	1.324 (1), $p=0.250$	89.395 (6), $p<0.001$	17.132 (6), $p=0.009$
Carotid-femoral PWV	1.859 (1), $p=0.173$	83.728 (6), $p<0.001$	1.412 (6), $p=0.965$
Augmentation index@75	2.402 (1), $p=0.121$	87.699 (6), $p<0.001$	61.148 (6), $p=2.63 \times 10^{-11}$

Table 6 shows the results of the repeated measures mixed model analysis of glucose and haemodynamic parameters. The main effects of time were significant for most variables ($p < 0.001$), as would be expected due to the physiology of the ingestion of glucose. No significant main effects were observed for group for any variable (all $p > 0.05$), however. Interestingly, there were significant group × time interactions for central PP (Wald $\chi^2 = 17.13$, $p = 0.009$) and augmentation index at 75 (Wald $\chi^2 = 61.15$, $p < 0.001$), meaning the temporal trends of these indices of central haemodynamics were different between the two groups despite similar means. There were no significant group × time interactions for the capillary glucose (all $p > 0.05$) or any other haemodynamic variable.

Autonomic and Microvascular Recovery

The magnitude of group differences in autonomic and microvascular recovery parameters was depicted in the temporal effect-size map (Figure 1), in early (30–90 min) and late (120–240 min) postprandial phases. In the early phase, the chlorhexidine group had moderately larger effect sizes for the LF/HF ratio ($g = 2.05$, $p < 0.001$), thermal half-time ($g = 2.34$, $p < 0.001$) and rewarming slope ($g = 2.27$, $p < 0.001$). In the late period, the effect size for the LF/HF ratio, thermal half-time, rewarming slope, perfusion recovery AUC and time to peak reperfusion were very large and highly significant ($g = 3.08$, $p < 0.001$; $g = 2.91$, $p < 0.001$; $g = 3.17$, $p < 0.001$; $g = 3.65$, $p < 0.001$; and $g = 1.81$, $p < 0.001$, respectively). This suggests that recovery of these parameters was delayed and impaired in the chlorhexidine group and became worse over time.

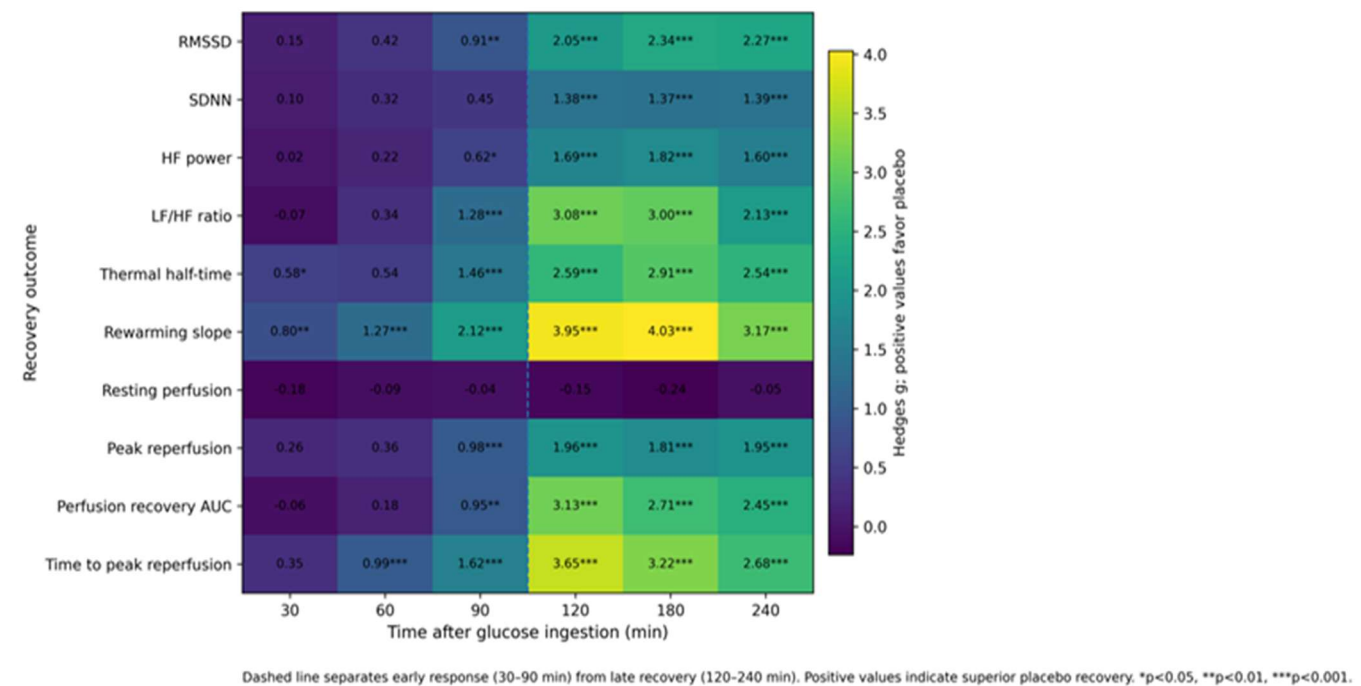


Figure 1: Temporal Effect-Size Map of Post-Glucose Recovery

These quantitative results were confirmed visually by a representative thermogram (Figure 2) obtained with FLIR T540 infrared camera. During the late rewarming period, the warm colours (red/yellow) in the proximal finger were greater in the Placebo group, suggesting greater thermal

recovery in Placebo. Conversely, the distal (cool-purple) part of the cool area was larger in chlorhexidine participants, and the temperature drop between the distal and proximal parts was greater, suggesting impaired microvascular reperfusion.

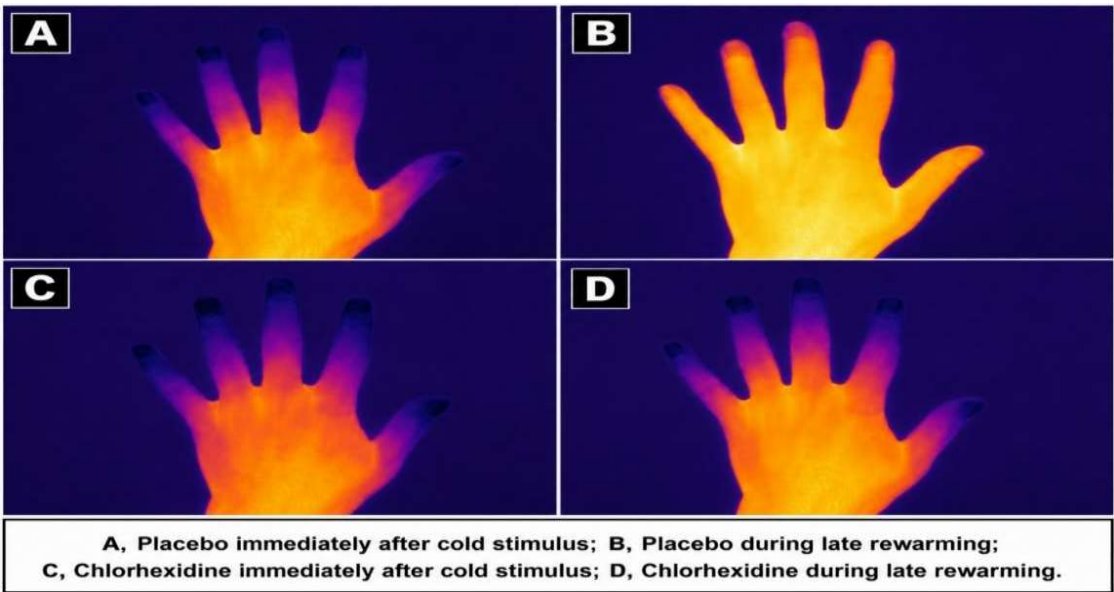


Figure 2: Direct FLIR T540 Radiometric Thermograms Demonstrating Delayed Digital Rewarming After Chlorhexidine Exposure

Independent Predictors of Autonomic and Microvascular Recovery

To identify independent predictors of salivary nitrite availability, vagal recovery, thermal rewarming, and microvascular reperfusion, multiple linear regression

models were created (Figure 3). The higher exposure to salivary nitrite (AUC_{0-240}) independently predicted higher late RMSSD and faster rewarming slope (both $p < 0.05$). Larger AUC of perfusion recovery and greater AUC for salivary nitrite (both $p < 0.01$) were independently

predicted by oral nitrate reducing capacity. The negative coefficients of chlorhexidine allocation in all models suggest a separate negative effect on each outcome. The relationship between chlorhexidine allocation and late RMSSD was weakened after accounting for salivary nitrite ($p > 0.05$), but the other chlorhexidine effects remained

significant (all $p < 0.05$), suggesting both nitrate-mediated and residual effect pathways of treatment. Covariates included were not significant in any model: age, BMI and glucose AUC (all $p > 0.05$).

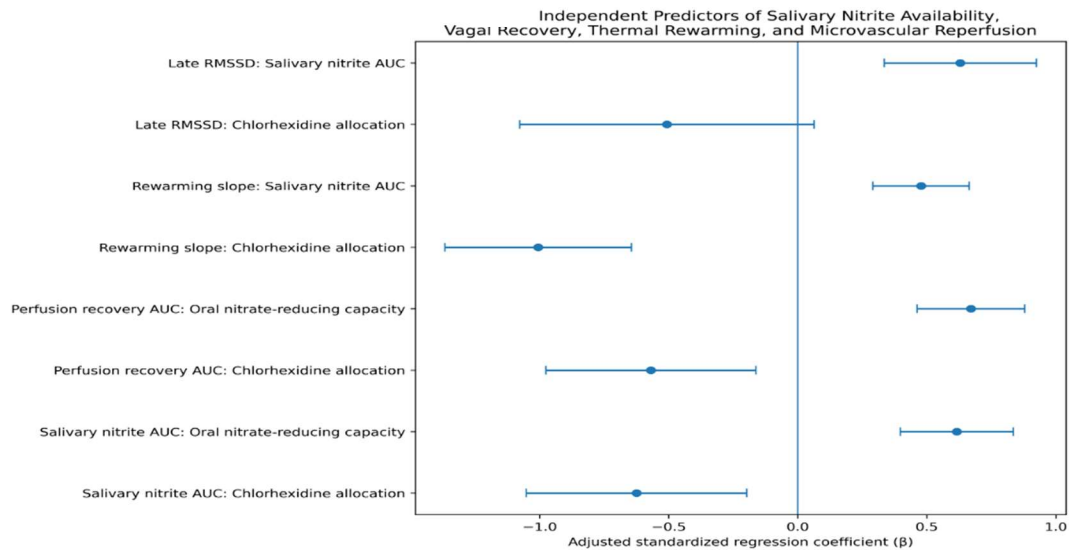


Figure 3: Presents coefficients from adjusted multivariable models

Discussion

The present study provides evidence that a 0.12% chlorhexidine mouthwash disrupts the enterosalivary nitrate–nitrite–nitric oxide (NO) axis, causing a reduction of the increase in salivary nitrite after a meal and a corresponding reduction in the vagal response and delay in microvascular reperfusion after an oral glucose challenge in healthy young adults. The results indicate that an antiseptic mouthwash that inhibits the reduction of nitrate in the oral cavity may alter autonomic and microvascular function, and have implications for cardiovascular health. Similar to previous studies, treatment with chlorhexidine mouthwash resulted in a reduction of oral nitrate-reducing capacity by about 65%. At all postprandial time points, this was associated with significantly lower concentrations of salivary nitrite, and a significantly dampened nitrite surge ($p < 0.001$). CHX eliminates nitrate reducing bacteria which are necessary for NO production, thus disrupting the nitrate–nitrite–NO pathway, and CHX mouthrinses potentially have an impact on blood pressure (Lima et al., 2024). found The mixed-model analysis yielded a highly significant group $\times$ time interaction for salivary nitrite (Wald $\chi^2 = 347.9$, $p < 0.001$) reflecting the fact that the temporal pattern of nitrite response to glucose ingestion was quite different between groups (placebo group, the expected postprandial nitrite surge, and chlorhexidine group, a flat, less intense response throughout the 240-minute observation period). This persistent suppression is

consistent with the findings that recovery of nitrate-reducing bacteria does not occur until after use of chlorhexidine has stopped (Toonen et al., 2026). One of the main and novel findings of this study is that the blunted salivary nitrite exposure was linked to the impaired recovery of the vagus nerve function, evidenced by the reduced RMSSD and the increased LF/HF ratio in the chlorhexidine group. There was no significant difference in effect size between the groups in early phase ($p > 0.05$), but a significant decrease in effect size in the late phase ($p < 0.01$); the LF/HF ratio progressively increased in effect size from early ($g = 2.05$) to late ($g = 3.08$) phases. NO has been shown to modulate cardiac autonomic function, with effects that include potentiation of muscarinic receptor activation on heart rate regulation and facilitating vagal neurotransmission by enhancing acetylcholine release from parasympathetic nerve terminal (Pang et al., 2025).The nitrate–nitrite–NO pathway offers an endothelial NOS-independent supply of NO which could be especially significant in the postprandial state when endothelial NOS activity may be reduced (Salama et al., 2026). Vagal dysfunction, reflected by a lower RMSSD and a higher LF/HF ratio, has been linked to higher cardiovascular risk, insulin resistance, and lower metabolic flexibility after meals (Sindhusa et al., 2025). The association of the reduction of salivary nitrate, glucose metabolism, and lifestyle factors indicates that the oral microbiome is likely to be involved in glucose metabolism and insulin sensitivity (Guo et al., 2025). Our observations

are clinically relevant, given that higher salivary nitrite was linked to better glycemic control and insulin sensitivity in those with type 2 diabetes (Bahadoran et al., 2026).

The most interesting results obtained are related to microvascular function. In the late postprandial phase, the thermal recovery half-time, rewarming slope and perfusion recovery AUC were significantly reduced in the chlorhexidine-treated participants. The magnitude of these effects is very large and suggests a deep defect in the microvascular reperfusion capacity which worsens with time. NO is a potent vasodilator, that controls microvascular tone, increases capillary recruitment and promotes post ischaemic reperfusion (Blot, 2021).

The alternative source of NO is the nitrate-nitrite-NO pathway that is more relevant in the postprandial state of low oxygen and low pH which occurs in metabolically active tissues. This selectivity in inhibiting the nitrite reservoir, and the hypothesis that disruption of the oral microbial nitrate metabolism is related to decreased NO bioavailability, and that decreased NO bioavailability is related to the occurrence or worsening of pathologies like atherosclerosis, diabetes, and sepsis, was fulfilled by the findings of chlorhexidine (Bagabir et al., 2024). This was confirmed by the regression analysis, which showed that the perfusion recovery AUC was independently predicted by the oral nitrate-reducing capacity, while the salivary nitrite AUC was independently predicted by the rewarming slope (both $p < 0.01$). The nitrate-nitrite-NO pathway is important for regulating adequate endothelial function, and it is counterbalanced by the microbiome and dietary nitrate consumption. But reactive oxygen species (ROS) and the uncoupling of eNOS can cause loss of bioavailability and exacerbate ROS production that establishes a vicious circle (Li et al., 2025).

While mean brachial and central haemodynamic indices did not significantly differ between groups, significant group $\times$ time interactions for central PP ($p = 0.009$) and augmentation index@75 ($p < 0.001$) were observed.

These results suggest that there was no difference between the groups in terms of average values but there was a difference in terms of temporal patterns of central haemodynamic responses. It has been previously reported that CHX mouthwash causes an increase in SBP in normotensive humans, and literature suggests that CHX mouthrinses may decrease the capacity of nitrate-reducing bacteria, which could result in elevated blood pressure (Kapil et al., 2013).

Agents that disrupt this pathway, such as antiseptic mouthwash, proton pump inhibitors and others, are mechanisms that impair blood pressure responses to nitrite and nitrate (Batista et al., 2021; Sanchez-Orozco et al., 2025). Antiseptic mouth wash disrupts the enterosalivary circuit of nitrate, but oral nitrite bypasses this disruption to promote blood pressure lowering and

nitrosation effects (Kapil et al., 2013) thus suggesting therapeutic strategies to avoid its disruption.

Our results were in line with, and extended, the newly proposed model of the oral microbiome – nitrate – nitrite – NO pathway and its association with heart health (Sanchez-Orozco et al., 2025; Yang et al., 2025). A recent study published by (Bescos et al., 2025) showed that a bioactive oral rinse containing inorganic nitrate and antioxidants, which positively alters the oral microbial environment to favor the enterosalivary pathway, lowers BP over 1 week and downregulates the oral microbiome compared to an oral rinse containing chlorhexidine, which disrupts the enterosalivary pathway. Additionally, it was found that regular use of routine antibacterial mouthwash could have harmful effects on the bioavailability of NO, blood pressure and diabetes risk, even after adjusting for relevant confounders, and may also lead to further poor glycemic control via shared pathways of action mediated by NO (Batista et al., 2021; Sindhusha et al., 2025).

The therapeutic potential of the nitrate-nitrite-NO pathway was further supported by the beneficial effects of inorganic nitrate on metabolic parameters, when compared with metformin in a mouse model of cardiometabolic disease (Cordero-Herrera et al., 2020; Pang et al., 2025).

CONCLUSION

Seven days of chlorhexidine reduces oral nitrate-reducing capacity, flattening the postprandial surge in saliva nitrite, and slows down the recovery of the vagal function and microvascular reperfusion in healthy adults. These effects are time dependent (maximal between 120 and 240 min) and are partly mediated by the nitrate-nitrite-NO pathway. The use of chlorhexidine is still appropriate for indications but not for routine use for the time being due to safety concerns in the cardiovascular system, which require further research for long-term use of enterosalivary NO axis disruption.

RECOMMENDATIONS

Chlorhexidine should be used for specific indications, not used for at least 24 to 48 hours before metabolic and vascular evaluations, and should be avoided in persons with hypertension, diabetes and cardiovascular disease. Routine use of antiseptic mouthwash is associated with an increased risk of cardiovascular disease, and public health interventions should encourage people to consume dietary nitrate and also inform them of the cardiovascular risks of using mouthwash regularly. Long-term effects, at-risk populations, nitrate supplementation, alternative formulations and microbiome-targeted strategies to maintain nitrate reducing bacteria should be explored in future research.

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