Effects of chewing gum on nitric oxide metabolism, markers of cardiovascular health and neurocognitive performance after a nitrate-rich meal

Sharon Ong[1], Nicola P Bondonno[1,2], Luke A Downey[4,5], Andrew Scholey[4], Michael A Smith[6], Con Stough[4], Lauren C Blekkenhorst[1,3], Richard Woodman[7], Kevin D Croft[2], Jonathan M Hodgson[1,3], Catherine P Bondonno[1,3]

[1] *Institute for Nutrition Research, School of Medical and Health Sciences, Edith Cowan University, Joondalup, Western Australia, Australia (NPB: 0000-0001-5905-444X; LCB: 0000-0003-1561-9052; JMH: 0000-0001-6184-7764; CPB: 0000-0001-8509-439X)*

[2] *School of Biomedical Sciences, Royal Perth Hospital Unit, University of Western Australia, Perth, Western Australia, Australia (KDC: 0000-0003-1596-4913)*

[3] *Medical School, Royal Perth Hospital Unit, University of Western Australia, Perth, Western Australia, Australia*

[4] *Centre for Human Psychopharmacology, Swinburne University, Melbourne, VIC,*

Australia (AS: 0000-0003-4484-5462; CS: 0000-0002-3628-3220)

[5] *Institute for Breathing & Sleep, Austin Hospital, Heidelberg, VIC, Australia (LAD: 0000-0001-5670-3192)*

[6] *Department of Psychology, University of Northumbria, Newcastle, UK (MAS: 0000-0001-5107-6441)*

[7] *Flinders Centre for Epidemiology and Biostatistics, Flinders University, Adelaide, SA, Australia (RW: 0000-0002-4094-1222)*

Address for correspondence and requests for reprints:

Catherine P Bondonno

Level 3, Royal Perth Hospital Research Foundation

Rear 50 Murray St, Perth Western Australia, Australia WA6000

Tel: 618 9224 0339

Email: c.bondonno@ecu.edu.au

Effects of chewing gum on nitric oxide metabolism, markers of cardiovascular health and neurocognitive performance after a nitrate-rich meal

Objectives: Cardiovascular and neurocognitive responses to chewing gum have been reported, but the mechanisms are not well understood. Chewing gum after a nitrate-rich meal may upregulate the reduction of oral nitrate to nitrite and increase nitric oxide (NO), a molecule important to cardiovascular and neurocognitive health. We aimed to explore effects of chewing gum after a nitrate-rich meal on nitrate metabolism (through the enterosalivary nitrate-nitrite-NO pathway), endothelial function, blood pressure (BP), neurocognitive performance, mood and anxiety.

Methods: Twenty healthy men (n=6) and women (n=14) with a mean age of 48 years (range: 23-69) were recruited to a randomized controlled cross-over trial. After consumption of a nitrate-rich meal (180 mg of nitrate), we assessed the acute effects of chewing gum, compared to no gum chewing, on (i) salivary nitrate, nitrite and the nitrate reductase ratio (100 x [nitrite] / ([nitrate] + [nitrite]); (ii) plasma nitrite, *S*-nitrosothiols and other nitroso species (RXNO); (iii) endothelial function (measured by flow mediated dilatation); (iv) BP; (v) neurocognitive performance; (vi) mood; and (vii) anxiety.

Results: Consumption of the nitrate-rich meal resulted in a significant increase in markers of nitrate metabolism. A significantly higher peak flow mediated dilatation was observed with chewing compared to no chewing (baseline adjusted mean difference: 1.10%, 95% CI: 0.06, 2.14; p=0.038) after the nitrate-rich meal. A significant small increase in systolic BP, diastolic BP and heart rate were observed with chewing compared to no chewing after the nitrate-rich meal. The study did not observe increased oral reduction of nitrate to nitrite and NO, or improvements in neurocognitive performance, mood or anxiety with chewing compared to no chewing.

Conclusion: Chewing gum after a nitrate-rich meal resulted in an acute improvement in endothelial function and a small increase in BP but did not result in acute effects on neurocognitive function, mood or anxiety.

Keywords: Chewing, gum, nitrate, nitrite, nitric oxide, endothelial function, blood pressure, cognition.

INTRODUCTION

Nitric oxide (NO), a widespread signalling molecule, plays a pivotal role in the cardiovascular, cerebrovascular and central nervous system (1). In the cardiovascular system, NO maintains vascular tone and integrity and regulates blood pressure (BP) (2). Decreased production and/or bioavailability of NO is associated with several cardiovascular diseases (CVD) (3). In the cerebrovascular system, NO is a regulator of cerebral hemodynamics (4) and in the central nervous system, NO is a neurotransmitter (1). NO also plays an integral role in normal cognitive function, specifically memory and learning (4,5). Inadequate NO therefore is potentially a major risk factor for cognitive decline (4).

Endogenous NO is produced primarily via the L-arginine-NO synthase pathway and is subsequently oxidized to nitrate (NO_3) and nitrite (NO_2) (3). These metabolites are recycled back to NO through an enterosalivary nitrate-nitrite-NO pathway (6). Through this pathway, nitrate from the diet [primarily green leafy vegetables and beetroot (7)] is metabolised to NO (6). The NO-mediated benefits of dietary nitrate on cardiovascular risk factors are now well established; dietary nitrate has been shown to lower blood pressure (BP) and improve endothelial function and arterial stiffness (8,9). There is also evidence for NO-mediated benefits of dietary nitrate on cerebral blood flow (10-12) and cognitive performance (12-15). Given the pivotal role NO plays in both vascular and cognitive health, and the important link between vascular and cognitive health (16), these NO-mediated effects of dietary nitrate may be of critical importance.

A critical step in the enterosalivary nitrate-nitrite-NO pathway is the reduction of salivary nitrate to nitrite in the oral cavity (17). After ingestion and absorption of dietary nitrate, there is an active uptake of nitrate from the plasma by the salivary glands. In the oral cavity, salivary nitrate is reduced to nitrite by nitrate reducing bacteria located on the dorsal surface of the tongue

(18). Once swallowed, a proportion of this nitrite enters the blood stream where it becomes a source of NO (19). Inhibiting the reduction of salivary nitrate to nitrite by use of an antibacterial mouthwash has physiological effects, with increases in BP observed in healthy and hypertensive individuals (20,21). Conversely, whether enhancing salivary nitrate to nitrite reduction by for example, chewing gum, will lead to an increase in available nitrite and NO with concomitant vascular and cognitive effects, is unknown.

Chewing gum has been shown to have both pro-cognitive and vascular effects. Improvements in neurocognitive performance (22-26) as well as both indirect evidence of the benefits of chewing gum on BP (26) and acute BP elevations (27,28) have been reported. While multiple mechanisms may underpin these effects, they have yet to be identified. One possible contributing mechanism is increased salivary nitrate to nitrite reduction. In the present study, our aim was to test the hypothesis that chewing gum after a nitrate-rich meal can acutely enhance the reduction of nitrate to nitrite in the mouth, leading to increases in circulating nitrite and markers of NO availability, and consequently improving markers of vascular health, such as BP and endothelial function, and improving neurocognitive performance.

METHODS

Ethics

This study was approved by the University of Western Australia Human Research Ethics Committee (RA/4/1/4258) and was conducted in accordance with the Declaration of Helsinki of 1975. All participants provided written consent prior to inclusion into the study. This study was registered with the Australian New Zealand Clinical Trials Registry as ACTRN 12610001048077.

Participants

Healthy participants were recruited via newspaper advertisements from the general population of Perth, Western Australia. Screening was conducted prior to enrollment within the University of Western Australia, School of Medicine and Pharmacology, located in the Medical Research Foundation building at Royal Perth Hospital. Screening consisted of a standard medical questionnaire, anthropometric measurements (weight, height, body mass index (BMI)), BP measurement, screening electrocardiography, and a fasting blood laboratory analysis. The exclusion criteria included a current or recent (<12 months) smoking status, a BMI of <18 or >35 kg/m^2, a history of cardiovascular or peripheral vascular disease, a diagnosis of diabetes, or a fasting glucose concentration of >= 5.5 mmol/L in individuals not diagnosed with diabetes, a psychiatric illness, other major illnesses such as cancer, use of antihypertensive medication, a current or recent (within the last 6 months) significant (>6% initial body weight) weight loss or gain, an alcohol intake of >210 g/week for women and >280 g/week for men, an inability or unwillingness to follow the study protocol, an inability or unwillingness to consume foods or beverages provided as part of the study protocol, an unwillingness to stop the use of supplements

24 hours prior to the testing day, the presence of any health conditions that may affect food metabolism including the following: food allergies, kidney disease, liver disease, and gastrointestinal diseases (e.g. Irritable bowel disease, coeliac disease, peptic ulcer(s)), the inability or unwillingness to chew gum for 2 hours, and the unwillingness to give up the use of mouthwash whilst participating in the study.

Trial design

A randomized controlled crossover-design trial was performed. The order of intervention (chewing gum and no gum chewing) was randomly assigned using computer-generated random numbers devised by a statistician (RJW). The gum provided was a commercially available fruit-flavoured, sugar-free chewing gum.

Participants were asked to consume the same meal for dinner the night prior to each of two visits which were spaced one week apart. At each study visit participants were provided with a standardized low nitrate breakfast (toasted white bread with butter and jam). Baseline assessments commenced 30 min after the low nitrate breakfast. At 150 min after breakfast, participants were provided with a standardized lunch containing a known amount (~180 mg) of nitrate derived from 200 g of spinach (microwaved from frozen) consumed with toasted and buttered white bread. All baseline and post-intervention assessments were performed after participants consumed a meal similar in energy and macronutrients, with only the addition of spinach for lunch. The macronutrient and nitrate content of the breakfast and lunch meals are shown in **Table 1**. Participants assigned to the chewing gum intervention were instructed to begin chewing gum 30 min after lunch and to continue chewing until the end of the post lunch assessment period (~ 150 min). Measurements performed at 'baseline' (after breakfast) and 'post intervention' (after lunch and chewing/no chewing intervention) included assessments of: (i)

saliva measurements of nitrate, nitrite and salivary nitrate reductase ratio (measured at baseline and 20 and 110 min after chewing/no chewing intervention); (ii) plasma measurements of nitrite/nitric oxide status (baseline and 120 min after chewing/no chewing intervention); (iii) endothelial function (baseline and 80 to 110 min after chewing/no chewing intervention); (iv) BP (baseline and 30, 70 and 130 min after chewing/no chewing intervention); (v) neurocognitive performance (baseline and after chewing/no chewing intervention); and (vi) mood and anxiety (measured pre and post the neurocognitive performance tests at baseline and after the chewing/no chewing intervention). The study design is summarized in **Figure 1**.

Biochemical analyses: nitrate/nitrite/nitric oxide

Saliva

Salivary nitrate and nitrite were measured at baseline and post intervention (20 and 110 min after chewing/no chewing intervention). To obtain the saliva sample, participants were asked to expectorate into a specimen container while sitting quietly for 5 minutes. Saliva aliquots were stored at -80°C. Concentrations of salivary nitrate and nitrite were determined in frozen samples thawed for analysis using a modification of a previously published gas chromatography-mass spectrometry (GC-MS) method (29). Briefly, internal standards [^{15}N] sodium nitrite (6 ng) and [^{15}N] sodium nitrate (40 ng) were used to spike samples. Samples underwent treatment with tetraoctylammonium bromide (0.5 ml of 8 mM in acetone) and Pentafluorobenzyl bromide (50 ml of 20% solution in acetone) at 50 °C over 40 min. By subjecting the solution to N_2 for 35 min, acetone could then be removed by evaporation. The remaining aqueous phase was then extracted with isooctane/toluene. Analysis of 1 ml of the organic extract required the use of an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer, which was fitted with a cross-

linked silicone column (25 m x 0.20 mm, 0.33- mm film thickness, HP5-MS) using negative-ion chemical ionization. Peaks were identified using retention time and mass spectra with [^{15}N] sodium nitrite and [^{15}N] sodium nitrate as internal standards. Calibration curves from authentic and labelled standards were used to quantify samples. Ions monitored were m/z = 62 and 63 for nitrate and [^{15}N] nitrate respectively and m/z = 46 and 47 for nitrite and [^{15}N] nitrite respectively.

The ability of each participant's oral microbiome to reduce salivary nitrate to nitrite was assessed by calculating a 'salivary nitrate reductase ratio' as follows: 100 x [nitrite] / ([nitrate] + [nitrite]) (18). The salivary nitrate reductase ratio provides an estimate of the proportion of nitrate secreted into the mouth that is reduced to nitrite.

Plasma

The direct measurement of plasma NO is an analytical challenge due to its short half-life. It is, therefore, assessed by measuring its major metabolites *S*-nitrosothiols, nitrite and NOx (29). Plasma *S*-nitrosothiols and other nitroso species (RXNO), nitrite and NO_x (RXNO + nitrite) were measured at baseline and post intervention (120 min after chewing/no chewing intervention). Analysis of the plasma concentrations of *S*-nitrosothiols, other nitroso species (RXNO), nitrite and total NOx (nitros(yl)ated species + nitrite) commenced within 5 min of blood sample collection using a previously described gas phase chemiluminescence assay (29). Blood was collected into vaccutainers containing N-ethylmaleimide (10mM) and EDTA (2 mM), then mixed and centrifuged at 3000 x g (5 min, 4°C). Fresh plasma was kept on ice in the dark and was analysed within one hour. Antifoam (AM-3- 3, Amscorp Scientific, 200 mL) was added prior to the injection of plasma into the radical purger containing potassium iodide (0.125 g) and iodine (0.05 g) in water (2.5 mL) /glacial acetic acid (7.5 mL) at room temperature. A Nitric Oxide Analyzer (CLD66, Eco Physics, Sweden) was used to quantify NO, which was released by the

redox reactions that occurred by its chemiluminescence reaction with ozone. Combination of both nitrite and RXNO results in the NO peak. Endogenous nitrite was removed by treating fresh plasma (300 ml) with sulphanilamide solution (30 ml, 0.5% in 0.1 M HCl) for 3 min. To quantify plasma RXNO, the NO signal peak area of samples pre-treated with sulphanilamide was measured against a nitrite standard (300 ml, 0.5 mM $NaNO_2^-$).

Other biochemical analyses

Routine biochemical analyses were performed at screening in the PathWest laboratory at Royal Perth Hospital, Western Australia. Serum total cholesterol, HDL cholesterol and triglycerides were measured using a routine enzymatic colorimetric test with a fully automated analyser (Roche Hitachi 917). LDL cholesterol concentrations were calculated using the Friedewald formula (30). Serum glucose was measured using an ultraviolet test with a fully automated analyser (Roche Hitachi 917).

Assessment of endothelial function

Endothelial function was assessed at baseline and post intervention (80 to 110 min after chewing/no chewing intervention) by flow-mediated dilatation (FMD) of the brachial artery using ultrasonography. An ultrasonographer trained for FMD and blinded to the intervention, performed all measurements according to a published protocol (31). Participants, resting in a supine position, were studied in a quiet, temperature-controlled room (22 to 24°C). The participant's left arm was extended and comfortably supported on a foam mat and ECG was monitored continuously. For the ultrasound of the brachial artery, a 12-MHz transducer was connected to an Acuson Aspen 128 ultrasound device (Acuson Corp.) and affixed with a clamp over the brachial artery 5 to 10 cm proximal to the antecubital crease. The position of the

transducer was recorded for each participant and this position was replicated for all study visits. After a 1 min baseline artery diameter recording, a BP cuff that was placed around the left forearm was inflated to 200 mm Hg. After 5 min, the cuff was released, inducing reactive hyperemia. A brachial artery image to 4 min post cuff deflation was recorded to assess flow-mediated dilatation. Images were downloaded for retrospective analysis. A semi-automated edge-detection software (31) was used to analyze the flow-mediated dilation of the brachial artery, which automatically produced a calculation of the brachial artery diameter, corresponding to the internal diameter. This was gated to the R wave of ECG, with measurements taken at end diastole. Responses were calculated as the percentage change in brachial artery diameter from baseline. The FMD was measured at 30-second intervals to 240 seconds after cuff deflation (0, 30, 60, 90, 120, 150, 180, 210, and 240 seconds). Peak FMD was also assessed. The analysis was performed by an experienced observer blinded to the interventions used.

Measurement of blood pressure

BP was measured using a Dinamap 1846SX/P oscillometric recorder (Critikon, Tampa, FL, USA) at baseline and post intervention (30, 70, and 130 min after chewing/no chewing intervention). Participants rested for 5 min in a supine position. BP measurements were taken on five occasions at 2 min intervals. The first measurement was discarded and the mean of the remaining four measurements was calculated. BP measurements for all time points were used in the analysis.

Assessment of neurocognitive performance, mood and anxiety level

Neurocognitive performance was evaluated at baseline and post intervention (40 to 70 min after chewing/no chewing intervention) using the Computerised Mental Performance Assessment

System (COMPASS, Northumbria University, Newcastle Upon Tyne, UK). This system has previously been shown to be sensitive to a range of nutritional interventions (32-34), including those directly related to nitrate intake (12). This series of tests included tasks that assess accuracy and speed of memory retrieval as well as accuracy and speed of attention. Mood and anxiety were also assessed. The test battery took approximately 30 min to complete. The tests were conducted via a laptop computer with brief instructions given on-screen before the start of each task. The administered tests were word presentation, simple reaction time (RT), choice RT, numeric working memory, Corsi blocks, Stroop Colour Word Test, immediate and delayed word recall, delayed word recognition. Tasks were presented in the same order, with different stimulus sets at each visit. With the exception of word recall and delayed word recall (which were recorded on paper), responses were made using the laptop keyboard. In addition, participants completed the Bond–Lader mood scale (35) and the State-Trait Anxiety Inventory (36) before and after each series of neurocognitive tasks. A short description of these tests appears in supplementary information. Participants undertook a training session on a separate day before the start of the study. At this visit participants were instructed on the procedure for the cognitive tests and repeated the battery of tests three times.

Statistics

The study was powered based on our primary outcome of peak FMD. Assuming a between subject SD of 5.0%, 4 measurements per subject (2 for each treatment period) and a within-subject correlation for FMD of r=0.6, n=20 subjects would provide 80% power to detect a difference in peak FMD between treatments of 2.0%. This calculation is based on a variance inflation factor of VIF=(1-rho)/m =(1-0.6)/4=0.1 applied to the total sample size required for a parallel group design (37), where m is the number of measures per subject (m=4), and rho is the

within-subject ICC (rho=0.6).

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25 (IBM) and STATA/IC 15.1 (StataCorp LLC). Descriptive statistics of normally distributed variables are expressed as means ± SDs. Results in tables and figures are presented as means ± SEs. For outcome variables with only one post intervention measurement (plasma RXNO, plasma nitrite, and plasma NO_x) differences between interventions (chewing and no chewing) were tested through the use of linear mixed models with adjustment for baseline measurements and treatment order. Treatment effects for outcomes with multiple post-intervention measurements (salivary nitrite, salivary nitrate, salivary nitrate reductase ratio and BP), were obtained using linear mixed models including baseline measurements, treatment order, period, and time (as a categorical variable) as predictors. We assessed the effect of the intervention on FMD (%) and absolute change in diameter (mm) using linear mixed models with treatment, treatment order, period, baseline measurement and, when analysing FMD curve, time since cuff release and treatment x time since cuff release included as predictors in the model. We assessed the effect of the nitrate-rich meal on FMD (%) and absolute change in diameter (mm), using data only from the first intervention period. This was done using linear mixed models with treatment, baseline measurement and, when analysing FMD curve, time since cuff release and treatment x time since cuff release included as predictors in the model.

For cognitive outcomes, z scores were calculated and clustered into cognitive domains (34,38):

Memory accuracy = (^{z}immediate word recall + ^{z}delayed word recall + ^{z}delayed word recognition + ^{z}numeric working memory)/4.

Memory RT = (^{z}RT delayed word recognition + ^{z}RT numeric working memory)/2.

Attention accuracy = (^{z}choice reaction accuracy + ^{z}Stroop accuracy)/2

Attention RT = (^{z}simple RT + ^{z}choice RT + ^{z}Stroop RT)/3.

Differences between interventions (chewing and no chewing) were tested for the neurocognitive outcome variables (composite cognitive domains, cognitive function tests, mood and anxiety) with the use of linear mixed models including baseline levels, period and treatment order.

RESULTS

Baseline and descriptive data

Of the 44 volunteers screened for the study, 21 participants were eligible for inclusion and were therefore randomized. One participant withdrew after randomization and prior to receiving the intervention. Twenty participants completed the trial (men: n = 6, women: n = 14, **Figure 2**). Baseline demographic and clinical data for all participants are shown in **Table 2**.

Salivary nitrate, nitrite and nitrate reductase ratio

Salivary concentrations of nitrate and nitrite and the nitrate reductase ratio, at 20 min and 110 min post chewing intervention, are presented in **Figure 3**. Chewing, compared to no chewing, after the nitrate-rich meal resulted in significantly lower salivary nitrite concentrations (baseline adjusted mean difference at 20 min = -22.0 µmol/L 95% CI: -37.6, -6.38, p = 0.006; at 110 min = -33.8 µmol/L 95% CI: -55.6, -12.0, p = 0.002) and significantly lower salivary nitrate concentrations (baseline adjusted mean difference at 20 min = -66.6 µmol/L 95% CI: -132.2, -0.94, p = 0.047; at 110 min = -89.4 µmol/L 95% CI: -128.8, -49.9; p < 0.001). However, the salivary nitrate reductase ratio, which provides an estimate of the proportion of nitrate secreted into the mouth that is reduced to nitrite, was not significantly altered by chewing compared to no chewing after the nitrate-rich meal (baseline adjusted mean difference at 20 min = -3.3 95% CI: -7.57, 0.96, p = 0.13; at 110 min = -1.23 95% CI: -4.85, 2.39; p = 0.51).

Plasma S-nitrosothiols and other nitroso species (RXNO), nitrite and NO_x

Plasma concentrations of RXNO, nitrite, and NO_x at baseline and 120 min post chewing

intervention are presented in **Figure 4**. Chewing, compared to no chewing, after the nitrate-rich meal did not significantly alter plasma concentrations of RXNO, nitrite, or NO_x.

Endothelial function

The mean FMD, measured at 30 second intervals between 0 and 240 seconds post-cuff deflation, peak FMD, absolute change in diameter, measured at 30 second intervals between 0 and 240 seconds post-cuff deflation, and peak absolute change in diameter for chewing and no chewing after the nitrate-rich meal are presented in **Figure 5.** Peak FMD was significantly higher with chewing compared to no chewing (baseline adjusted mean difference = 1.10%, 95% CI: 0.06, 2.14; p = 0.038). For the entire FMD curve, no significant difference between chewing and no chewing after the nitrate-rich meal was observed when comparing FMD at each time point (Figure 5). No significant difference between chewing and no chewing after the nitrate-rich meal was observed for absolute change in diameter, measured at 30 second intervals between 0 and 240 seconds post-cuff deflation, and peak absolute change in diameter (Figure 5).

Blood pressure

Mean systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP), and heart rate measured at baseline, 30, 70, and 130 min post chewing intervention are presented in **Table 3**. There was a significant increase in SBP, DBP and HR with chewing compared to no chewing after the nitrate-rich meal.

Neurocognitive function, mood and anxiety measures

There was no significant difference between chewing and no chewing after the nitrate-rich meal for the cognitive domain measures of attention and memory, cognitive function test measures,

mood, and anxiety measures.

Additional analyses of changes after the nitrate-rich meal

Salivary and plasma markers of nitrate metabolism

Compared to baseline, there was a significant increase in salivary nitrite at 20 min (no chewing: mean difference = 38.5 µmol/L, 95% CI: 19.4, 57.6; $p < 0.001$; chewing: mean difference = 18.2 µmol/L, 95% CI: 5.0, 31.4; $p = 0.007$) and 110 min (no chewing: mean difference = 62.4 µmol/L, 95% CI: 43.0, 81.8; $p < 0.001$; chewing: mean difference = 28.8 µmol/L, 95% CI: 15.6, 42.0; $p < 0.001$) post chewing intervention. Similarly, there was a significant increase in salivary nitrate at 20 min (no chewing: mean difference = 127.5 µmol/L, 95% CI: 71.7, 183.3; $p < 0.001$; chewing: mean difference = 62.7 µmol/L, 95% CI: 22.8, 102.7; $p = 0.002$) and 110 min (no chewing: mean difference = 183.0 µmol/L, 95% CI: 126.1, 239.6; $p < 0.001$; chewing: mean difference = 94.4 µmol/L, 95% CI: 54.5, 134.4; $p < 0.001$) post chewing intervention. Compared to baseline, a decrease in the salivary nitrate reductase ratio was observed at 110 min (no chewing: mean difference = -8.5, 95% CI: -15.8, -1.3; $p = 0.022$; chewing: mean difference = -5.7, 95% CI: -11.4, 0.02; $p = 0.05$), post chewing intervention (Figure 3).

Plasma RXNO at 120 min post chewing intervention was not significantly changed for chewing and no chewing compared to baseline. An increase in plasma nitrite was observed at 120 min post chewing intervention (no chewing: mean difference = 22.0 nmol/L, 95% CI: -0.47, 44.4; $p = 0.06$; chewing: mean difference = 38.5 nmol/L, 95% CI: 25.4, 51.5; $p < 0.001$), compared to baseline. Similarly, an increase in plasma NO_x was observed at 120 min post chewing intervention (no chewing: mean difference = 34.9 nmol/L, 95% CI: 4.2, 65.7; $p = 0.03$; chewing: mean difference = 35.5 nmol/L, 95% CI: 11.2, 59.8; $p = 0.004$) compared to baseline (Figure 4).

Endothelial function

Compared to baseline FMD, we observed a significant increase in FMD after the nitrate-rich meal irrespective of intervention (adjusted mean percent difference over 4 min = 0.95%, 95% CI: 0.33, 1.57; p = 0.003, adjusted mean percent difference at peak = 1.5%, 95% CI: .63, 2.41; p< 0.001; **Figure 6**). Compared to baseline absolute change in diameter, we observed a significant increase in absolute change in diameter after the nitrate-rich meal irrespective of intervention (adjusted mean difference over 4 min = 0.03 mm, 95% CI: 0.01, 0.05; p < 0.001, adjusted mean difference at peak = 0.04 mm, 95% CI: 0.015, 0.07; p = 0.002; Figure 6).

Blood pressure, neurocognitive function, mood and anxiety measures

Compared to baseline measurements, there were no significant changes in BP, neurocognitive measures, mood, and anxiety measures (data not shown) after the nitrate-rich meal.

DISCUSSION

In this randomized, controlled, crossover study, chewing compared to no chewing after the consumption of a nitrate-rich meal in healthy men and women significantly improved endothelial function (peak FMD). Contrary to our hypothesis, chewing compared to no chewing after the nitrate-rich meal significantly increased BP and we found no evidence that chewing up-regulated markers of nitrate metabolism through the enterosalivary nitrate-nitrite-NO pathway, or improved neurocognitive function, mood or anxiety.

The improvement in endothelial function observed with chewing compared to no chewing after the nitrate-rich meal is consistent with our hypothesis. Furthermore, the improvement in endothelial function after the nitrate-rich meal regardless of chewing intervention supports the results of previous studies showing that increased nitrate intake can improve endothelial function through effects on NO (39-45). If sustained, the observed ~1 % improvement in FMD could have clinical implications as a 1% increase in FMD is associated with a 13% lower risk of CVD events (relative risk: 0·87, 95 % CI 0·83, 0·91) (46). We report both the relative, %FMD, and absolute, change in diameter, measures. Percent FMD is considered the most reproducible measure and is recommended for interventional studies in which the baseline diameter remains stable over time (47). However, as FMD is influenced by baseline diameter size, absolute change in diameter measurements are also recommended (48). While we observed a significant increase in peak FMD with chewing compared to no chewing, the improvement in endothelial function with chewing compared to no chewing in both FMD and absolute change in diameter measured from 0 to 240 s post cuff deflation, and the absolute change in peak dilation diameter did not reach statistical significance. The improvement in endothelial function after the nitrate-rich meal, was significant for all the relative and absolute measures.

The small increase in BP and HR observed with chewing gum compared to not chewing gum after the nitrate rich meal was contrary to our hypothesis and other clinical trials where reductions in BP are observed after a nitrate-rich meal (8). Two previous studies have, however, observed that chewing gum can result in substantial acute increases in BP and HR (27,28), including during cognitive processing (49). These studies have indicated that the increase in BP and HR are similar to those observed for exercise and is likely due to jaw muscle activity (27,28). Contrary to a sustained increase in BP and HR, such as in individuals with hypertension, the transient increase in BP and HR observed here likely does not have any physiological relevance.

In the present study, the nitrate-rich meal was effective in increasing nitrate metabolism through the enterosalivary nitrate-nitrite-NO pathway, with increased levels of saliva and plasma nitrate and nitrite observed. We did not find evidence that chewing increased the conversion of nitrate to nitrite in the mouth, therefore up-regulating nitrate metabolism and leading to an increase in plasma NO concentrations. This is potentially due to the timing of the measurements. Salivary nitrate and nitrite concentrations were measured 20 min and 110 min post intervention. We observed that the increase in salivary nitrate and nitrite concentrations after the nitrate-rich spinach meal was significantly attenuated with chewing compared to no chewing. Chewing gum could have increased the rate of clearance of salivary nitrate and nitrite from saliva, therefore peaking in the plasma earlier than the no chewing intervention. We did not measure the salivary flow rate in this study but chewing gum has been previously shown to increase salivary flow rate (50). There was an increase in plasma RXNO and nitrite post the spinach meal in both the chewing and no chewing interventions, measured 120 minutes post intervention. While there was no significant difference in this increase between the chewing and no chewing intervention, the timing of this measurement could have missed the earlier peak of plasma RXNO and nitrite from

chewing if it was cleared from the saliva earlier than the no chewing intervention. Another potential explanation for the observed attenuation in salivary nitrate and nitrite with chewing compared to no chewing, is that chewing gum has been shown to trap up to 10^8 oral bacteria per gum piece (51). Wessel et al identified a number of bacterial species including Actinomyces (one of the genera of oral nitrate reducing bacteria) trapped in chewing gum, together with a myriad of other oral bacteria (51).

We also observed a significant decrease in the salivary nitrate reductase ratio at 110 min for both chewing and no chewing after the nitrate-rich meal. In a previous study we have observed a significant increase in the salivary nitrate reductase ratio at 120 min after a nitrate intervention of 200 mg nitrate (52). The salivary nitrate reductase ratios post intervention were similar in both studies (28 for no chewing and 26 for chewing vs 28 for the 200 mg nitrate dose). The difference between the two studies is in the baseline salivary nitrate reductase ratio (36 vs 20). The reason for this difference is unclear. Volunteers in both studies had a low nitrate meal the evening before each visit and were given a similar breakfast upon arrival at the research unit.

We did not observe an improvement in the cognitive domains of attention and memory, the individual neurocognitive function tests, mood, or anxiety with chewing gum compared to no chewing. Previous studies investigating the effects of chewing on neurocognitive function, mood, and anxiety have reported mixed results with studies observing improvements (53), (54), studies observing no effects (55,56) and indeed studies observing negative effects on the ability to perform cognitive tasks (52). Additionally, previous studies investigating the effects of nitrate intake on measures of neurocognitive function report mixed results. In acute studies, both improvements in the serial 3 subtraction task in healthy adults, 90 min after nitrate-rich beetroot juice (12) and no effect on cognitive function or mood 150 min after a nitrate-rich spinach meal

in healthy adults (57) have been reported. In chronic studies, an improvement in reaction time in individuals with type 2 diabetes after 2-weeks intake of nitrate-rich beetroot juice (13) and no effect on cognitive function after a 3-day intake of nitrate-rich beetroot juice in older adults (58) have been reported. Potential reasons for the observed differences in the effects of chewing gum and nitrate on cognitive function measures include differences in the type and timing of neurocognitive assessments, the age of the participants, and, specifically for the effects of nitrate intake on cognitive function, background diet, dose of nitrate and mouthwash use.

Our study has a number of limitations. It was not feasible for the participants to be blinded to the intervention they were receiving. In order to minimize the bias, all research assistants and lab technicians were blinded to the interventions when analyzing the results. While the participants were healthy, they formed a heterogenous sample group with a wide age, BMI, and BP range. Furthermore, 9 of the 14 female study participants were premenopausal and we did not perform FMD within the same phase of their ovarian cycles. Previous studies have reported an increase in salivary flow rate with chewing gum, however salivary flow rate was not measured in our study. Additionally, we also cannot rule out the possibility that cognitive effects may have been evident with different, potentially more demanding, cognitive tasks.

In conclusion, chewing gum after a nitrate-rich meal resulted in improvements in endothelial function and a small increase in BP and HR. No acute effects on neurocognitive function, mood or anxiety were observed. We found no evidence that chewing up-regulated markers of nitrate metabolism through the enterosalivary nitrate-nitrite-NO pathway. Whether this is a potential mechanism through which chewing gum has cardiovascular and cognitive effects has yet to be established. Future studies should determine if the improvement in endothelial function is due to increased nitrate to nitrite metabolism by chewing gum by

investigating endogenous nitrate to nitrite reduction at multiple time points after a nitrate-rich meal.

Acknowledgements

The authors would like to thank Ms Adeline Indrawan for technical assistance, Ms Lisa Rich for performing and analysing the FMDs, and Mr Claude Backory for nursing assistance.

Funding Sources

A/Prof Downey is supported by a National Health and Medical Research Council R.D. Wright Biomedical Career Development Fellowship (CDF: 2017-2020). The salary of LCB is supported by a National Health and Medical Research Council of Australia Emerging Leadership Investigator Grant (ID: 1172987) and a National Heart Foundation of Australia Post-Doctoral Research Fellowship (ID: 102498). NPB is funded by a National Health and Medical Research Council Early Career Fellowship (Grant number APP1159914), Australia. JMH was funded by a National Health and Medical Research Council Senior Research Fellowship. This work was funded by a grant from the Wrigley Science Institute to AS.

REFERENCES

1. Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. Free Radical Res 1999;31:577-96.
2. Jin RC, Loscalzo J. Vascular nitric oxide: formation and function. J Blood Med 2010;1:147-162.
3. Vallance P, Chan N. Endothelial function and nitric oxide: clinical relevance. Heart 2001;85:342-350.
4. Toda N, Ayajiki K, Okamura T. Cerebral blood flow regulation by nitric oxide: recent advances. Pharmacol Rev 2009;61:62-97.
5. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J 2012;33:829-837.
6. Lundberg JO, Weitzberg E. NO generation from nitrite and its role in vascular control. Arterioscler Thromb Vasc Biol 2005;25:915-22.
7. Blekkenhorst LC, Prince RL, Ward NC et al. Development of a reference database for assessing dietary nitrate in vegetables. Mol Nutr Food Res 2017;61.
8. Blekkenhorst LC, Bondonno NP, Liu AH et al. Nitrate, the oral microbiome, and cardiovascular health: a systematic literature review of human and animal studies. Am J Clin Nutr 2018;107:504-522.
9. Bondonno CP, Blekkenhorst LC, Liu AH et al. Vegetable-derived bioactive nitrate and cardiovascular health. Mol Aspects Med 2018;61:83-91.
10. Bond V, Curry BH, Adams RG, Asadi MS, Millis RM, Haddad GE. Effects of dietary nitrates on systemic and cerebrovascular hemodynamics. Cardiol Res Pract 2013;2013.
11. Presley TD, Morgan AR, Bechtold E et al. Acute effect of a high nitrate diet on brain perfusion in older adults. Nitric Oxide 2011;24:34-42.
12. Wightman EL, Haskell-Ramsay CF, Thompson KG et al. Dietary nitrate modulates cerebral blood flow parameters and cognitive performance in humans: A double-blind, placebo-controlled, crossover investigation. Physiol Behav 2015;149:149-158.
13. Gilchrist M, Winyard PG, Fulford J, Anning C, Shore AC, Benjamin N. Dietary nitrate supplementation improves reaction time in type 2 diabetes: Development and application of a novel nitrate-depleted beetroot juice placebo. Nitric Oxide 2014;40:67-74.
14. Thompson C, Vanhatalo A, Jell H et al. Dietary nitrate supplementation improves sprint and high-intensity intermittent running performance. Nitric Oxide 2016;61:55-61.
15. Thompson C, Wylie LJ, Fulford J et al. Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. Eur J Appl Physiol 2015;115:1825-1834.
16. Gorelick PB, et al., . Vascular contributions to cognitive impairment and dementia a statement for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. Stroke 2011;42:2672-2713.
17. Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. Free Radic Biol Med 2004;37:395-400.
18. Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. Eur J Oral Sci 2005;113:14-9.
19. Calvert JW, Lefer DJ. Clinical translation of nitrite therapy for cardiovascular diseases. Nitric Oxide 2010;22:91-97.

20. Bondonno CP, Liu AH, Croft KD et al. Antibacterial mouthwash blunts oral nitrate reduction and increases blood pressure in treated hypertensive men and women. American journal of hypertension 2015;28:572-5.
21. Kapil V, Haydar S, Pearl V, Lundberg JO, Weitzberg E, Ahluwalia A. Physiological role for nitrate-reducing oral bacteria in blood pressure control. Free Rad Biol Med 2013;55:93-100.
22. Allen AP, Smith AP. Effects of chewing gum and time-on-task on alertness and attention. Nutr Neurosci 2012;15:176-185.
23. Johnson AJ, Muneem M, Miles C. Chewing gum benefits sustained attention in the absence of task degradation. Nutr Neurosci 2013;16:153-159.
24. Scholey A, Haskell C, Robertson B, Kennedy D, Milne A, Wetherell M. Chewing gum alleviates negative mood and reduces cortisol during acute laboratory psychological stress. Physiol Behav 2009;97:304-12.
25. Smith A. Effects of chewing gum on cognitive function, mood and physiology in stressed and non-stressed volunteers. Nutr Neurosci 2010;13:7-16.
26. Smith A. Effects of Chewing Gum on Stress and Health: A Replication and Investigation of Dose–Response. Stress and Health 2013;29:172-174.
27. Farella M, Bakke M, Michelotti A, Marotta G, Martina R. Cardiovascular responses in humans to experimental chewing of gums of different consistencies. Arch Oral Biol 1999;44:835-842.
28. Hasegawa Y, Sakagami J, Ono T, Hori K, Zhang M, Maeda Y. Circulatory response and autonomic nervous activity during gum chewing. Eur J Oral Sci 2009;117:470-473.
29. Yang X, Bondonno CP, Indrawan A, Hodgson JM, Croft KD. An improved mass spectrometry-based measurement of NO metabolites in biological fluids. Free Rad Biol Med 2013;56:1-8.
30. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502.
31. Dogra G, Rich L, Stanton K, Watts GF. Endothelium-dependent and independent vasodilation studies at normoglycaemia in type I diabetes mellitus with and without microalbuminuria. Diabetologia 2001;44:593-601.
32. Dodd F, Kennedy D, Riby L, Haskell-Ramsay C. A double-blind, placebo-controlled study evaluating the effects of caffeine and L-theanine both alone and in combination on cerebral blood flow, cognition and mood. Psychopharmacology (Berl) 2015;232:2563-2576.
33. Kennedy DO, Jackson PA, Forster J et al. Acute effects of a wild green-oat (Avena sativa) extract on cognitive function in middle-aged adults: a double-blind, placebo-controlled, within-subjects trial. Nutr Neurosci 2017;20:135-151.
34. Stonehouse W, Conlon CA, Podd J et al. DHA supplementation improved both memory and reaction time in healthy young adults: a randomized controlled trial. Am J Clin Nutr 2013;97:1134-1143.
35. Bond A, Lader M. The use of analogue scales in rating subjective feelings. Brit J Med Psychol 1974;47:211-218.
36. Spielberger CD. State-Trait anxiety inventory. The Corsini encyclopedia of psychology 2010:1-1.

37. Hsieh F, Lavori PW, Cohen HJ, Feussner JR. An overview of variance inflation factors for sample-size calculation. Eval Health Prof 2003;26:239-257.
38. Haskell-Ramsay CF, Stuart RC, Okello EJ, Watson AW. Cognitive and mood improvements following acute supplementation with purple grape juice in healthy young adults. Eur J Nutr 2017;56:2621-2631.
39. Bahra M, Kapil V, Pearl V, Ghosh S, Ahluwalia A. Inorganic nitrate ingestion improves vascular compliance but does not alter flow-mediated dilatation in healthy volunteers. Nitric Oxide 2012;26:197-202.
40. Bakker E, Engan H, Patrician A et al. Acute dietary nitrate supplementation improves arterial endothelial function at high altitude: a double-blinded randomized controlled cross over study. Nitric Oxide 2015;50 58-64.
41. Bondonno CP, Yang X, Croft KD et al. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. Free Radic Biol Med 2012;52:95-102.
42. Heiss C, Meyer C, Totzeck M et al. Dietary inorganic nitrate mobilizes circulating angiogenic cells. Free Radical Biology and Medicine 2012;52 1767-1772.
43. Joris PJ, Mensink RP. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. Atherosclerosis 2013;231:78-83.
44. Rodriguez-Mateos A, Hezel M, Aydin H et al. Interactions between cocoa flavanols and inorganic nitrate: additive effects on endothelial function at achievable dietary amounts. Free Radic Biol Med 2015;80:121-8.
45. Velmurugan S, Gan JM, Rathod KS et al. Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. Am J Clin Nutr 2016;103:25-38.
46. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. Int J Cardiovasc Imaging 2010;26:631-640.
47. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. Hypertension 2010;55:1075-1085.
48. Charakida M, Masi S, Lüscher TF, Kastelein JJP, Deanfield JE. Assessment of atherosclerosis: the role of flow-mediated dilatation. Eur Heart J 2010;31:2854-2861.
49. Wilkinson L, Scholey A, Wesnes K. Chewing gum selectively improves aspects of memory in healthy volunteers. Appetite 2002;38:235-236.
50. Pedersen AML, Sørensen CE, Proctor G, Carpenter G. Salivary functions in mastication, taste and textural perception, swallowing and initial digestion. Oral Dis 2018;24:1399-1416.
51. Wessel SW, van der Mei HC, Morando D et al. Quantification and qualification of bacteria trapped in chewed gum. PloS one 2015;10.
52. Bondonno CP, Croft KD, Puddey IB et al. Nitrate causes a dose-dependent augmentation of nitric oxide status in healthy women. Food Funct 2012;3:522-527.
53. Miquel S, Haddou MB, Day JEL. A systematic review and meta-analysis of the effects of mastication on sustained attention in healthy adults. Physiol Behav 2019;202:101-115.
54. Yaman-Sözbir Ş, Ayaz-Alkaya S, Bayrak-Kahraman B. Effect of chewing gum on stress, anxiety, depression, self-focused attention, and academic success: A randomized controlled study. Stress and Health 2019;35:441-446.

55. Tucha L, Koerts J. Gum chewing and cognition: an overview. Neurosci Med 2012;3:243.
56. Wada M, Hoshi Y, Iguchi Y, Kida I. Near-infrared spectroscopic study on the effects of chewing on short-term memory. Appetite 2011;57:749-752.
57. Bondonno CP, Downey LA, Croft KD et al. The acute effect of flavonoid-rich apples and nitrate-rich spinach on cognitive performance and mood in healthy men and women. Food & function 2014;5:849-58.
58. Kelly J, Fulford J, Vanhatalo A et al. Effects of short-term dietary nitrate supplementation on blood pressure, O2 uptake kinetics, and muscle and cognitive function in older adults. Am J Physiol Regul Integr Comp Physiol 2013;304:R73-R83.

FIGURE LEGENDS

Figure 1: Trial design.

Figure 2: Participant flow from recruitment through screening and randomisation to trial completion.

Figure 3: Saliva concentrations of (A) nitrite, (B) nitrate and (C) nitrate reductase ratio at baseline, 20 min, and 110 min post intervention (no chewing and chewing) after a nitrate-rich meal. Values are means ± SEs. P – values for intervention effect (no chewing and chewing) were obtained using linear mixed models including treatment order, period and baseline levels as predictors (n = 20).

Figure 4: Plasma concentrations of (A) RXNO, (B) nitrite and (C) NO_x at baseline and 120 min post chewing/no chewing intervention after a nitrate-rich meal. Values are means ± SEs. P – values for intervention effect (chewing versus no chewing) were obtained using linear mixed models including treatment order, period and baseline levels (n = 20).

Figure 5: Post intervention (no chewing and chewing after a nitrate-rich meal) measurement of (A) FMD measured at 30 second intervals from 0 to 240 seconds, (B) peak FMD, (C) absolute change in diameter at 30 second intervals from 0 to 240 seconds, and (D) peak absolute change in diameter. Values are mean ± SEs. P-values for intervention effect on FMD were obtained for (A) using a linear mixed model including treatment, treatment order, period, baseline FMD, time since cuff release and treatment x time since cuff release; (B) using a linear mixed model including treatment, treatment order, period and baseline FMD; (C) using a linear mixed model including treatment, treatment order, period, baseline absolute change in diameter, time since cuff release and treatment x time since cuff release; (D) using a linear mixed model including treatment, treatment order, period and baseline peak absolute change in diameter (n=20).

Figure 6: Baseline and post nitrate-rich meal measurement of (A) FMD measured at 30 second intervals from 0 to 240 seconds, (B) peak FMD, (C) absolute change in diameter at 30 second intervals from 0 to 240 seconds, and (D) peak absolute change in diameter. Values are mean ± SEs. P - values for effect of nitrate-rich spinach on FMD were obtained, using data only from the first intervention period, for (A) using a linear mixed model including treatment, baseline measurement, time since cuff release and treatment x time since cuff release; (B) using a linear mixed model including treatment; (C) using a linear mixed model including treatment, baseline measurement, time since cuff release, and treatment x time since cuff release; (D) using a linear mixed model including treatment and baseline measurement (n=20).

Table 1: Macronutrient and nitrate content of provided meals

	Breakfast	Lunch
Energy (kJ)	1 049	1 085
Fat (g)	5.3	7.3
Carbohydrate (g)	42.5	38.0
Protein (g)	7.0	12.6
Nitrate (mg)	< 1	180

Table 2: Baseline characteristics of study participants (n=20, men: n =6, women: n = 14)

	Mean ± SD	Range
Age (y)	47.9 ± 15.3	23 - 69
Weight (kg)	71.2 ± 14.5	49.5 - 100.0
Height (m)	168 ± 7.4	156.5 - 184.0
Body mass index (kg/m^2)	24.9 ± 4.0	18.9 - 35.0
Fasting lipids		
Total cholesterol (mmol/L)	4.5 ± 0.8	3.1 - 6.0
LDL cholesterol (mmol/L)	2.8 ± 0.7	1.8 - 4.1
HDL cholesterol (mmol/L)	1.3 ± 0.2	0.8 - 1.8
Triglycerides (mmol/L)	0.8 ± 0.3	0.3 - 1.5
Fasting glucose (mmol/L)	4.9 ± 0.5	3.9 - 5.5
Systolic blood pressure (mm Hg)	113 ± 10.5	95.5 - 129.3
Diastolic blood pressure (mm Hg)	68.8 ± 7.3	56.3 - 79.5
Heart rate (bpm)	60.1 ± 7.9	41.8 – 74.0

Table 3. Blood pressure measurements by no chewing/chewing after a nitrate-rich meal

	Time (min)	No Chewing	Chewing	p-value*
SBP (mmHg)	Baseline	114 ± 2.48	112 ± 2.08	
	30	114 ± 2.22	116 ± 2.51	0.047
	70	114 ± 2.52	116 ± 2.48	0.017
	130	114 ± 2.47	116 ± 2.52	0.002
DBP (mmHg)	Baseline	68.6 ± 1.66	67.9 ± 1.65	
	30	67.0 ± 1.78	67.4 ± 1.56	0.311
	70	67.5 ± 1.81	68.6 ± 1.63	0.025
	130	68.3 ± 1.42	69.3 ± 1.48	0.055
MAP (mmHg)	Baseline	84.8 ± 1.81	84.9 ± 1.81	
	30	84.5 ± 1.77	85.7 ± 1.63	0.440
	70	84.4 ± 1.82	86.2 ± 1.75	0.171
	130	84.7 ± 1.61	86.6 ± 1.65	0.139
HR (bpm)	Baseline	58.5 ± 1.48	57.9 ± 1.50	
	30	62.4 ± 1.63	65.6 ± 1.96	<0.001
	70	60.2 ± 1.87	64.8 ± 2.34	<0.001
	130	60.5 ± 1.76	62.3 ± 1.77	0.043

Results are presented as mean ± SEM (n = 20).

* P – values for intervention effect (no chewing and chewing after a nitrate-rich meal) were obtained using linear mixed models including treatment order, period and baseline levels (n = 20). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.