

TITLE

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JOURNAL

Journal of Strength and Conditioning Research

DATE DEPOSITED

8 September 2016

This version available at

<https://research.stmarys.ac.uk/id/eprint/1187/>

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Effects of dietary nitrate, caffeine, and their combination on 20 km cycling time-trial performance.

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Running head: Dietary nitrate and caffeine on cycling

ABSTRACT

The aim of this study was to examine the acute supplementation effects of dietary nitrate, caffeine, and their combination on 20 km cycling time-trial performance. Using a randomized, counterbalanced, double-blind, Latin-square design, 14 competitive, female cyclists (age: 31 ± 7 years; height: 1.69 ± 0.07 m; body mass: 61.6 ± 6.0 kg) completed four 20 km time-trials on a racing bicycle fitted to a turbo-trainer. 2.5 hours before each trial, subjects consumed a 70 ml dose of concentrated beetroot juice containing either 0.45 g of dietary nitrate, or with the nitrate content removed (placebo). 1 hour before each trial, subjects consumed a capsule containing either $5 \text{ mg}\cdot\text{kg}^{-1}$ of caffeine or maltodextrin (placebo). There was a significant effect of supplementation on power output ($p = 0.001$), with *post hoc* tests revealing higher power outputs in caffeine (205 ± 21 W) versus nitrate (194 ± 22 W) and placebo (194 ± 25 W) trials only. Caffeine-induced improvements in power output corresponded with significantly higher measures of heart rate (caffeine: 166 ± 12 vs. placebo: $159 \pm 15 \text{ b}\cdot\text{min}^{-1}$; $p = 0.02$), blood lactate (caffeine: 6.54 ± 2.40 vs. placebo: $4.50 \pm 2.11 \text{ mmol}\cdot\text{L}^{-1}$; $p < 0.001$), and respiratory exchange ratio (caffeine: 0.95 ± 0.04 vs. placebo: 0.91 ± 0.05 ; $p = 0.03$). There were no effects ($p \geq 0.05$) of supplementation on cycling cadence, ratings of perceived exertion, $\dot{V}\text{O}_2$, or integrated electromyographic activity. The results of this study support the well-established beneficial effects of caffeine supplementation on endurance performance. In contrast, acute supplementation with dietary nitrate appears to have no effect on endurance performance and adds nothing to the benefits afforded by caffeine supplementation.

Key words: Ergogenic aids; nitric oxide; nitrite; endurance exercise.

INTRODUCTION

Nitrate supplementation, via natural (beetroot juice) and pharmacological (nitrate salts) methods, is an emerging area of research due to its potential to improve endurance performance. Many, though not all (4,5,13,36), studies have reported a reduction in the oxygen cost of exercise following nitrate supplementation (1,2,11,28,29,30,33). Correspondingly, several studies have reported improvements in time-to-exhaustion (1,2,28) and time-trial (6,11,27) protocols; with improvements in the latter of 1.2 – 2.8% in events lasting from 6 – 27 mins (11,27). Mechanisms to explain these performance enhancements, though unresolved, are suggested to result from a bioconversion cascade from nitrate, via nitrite, to nitric oxide leading to improvements in mechanical (1) and/or mitochondrial-coupling efficiency (29). As such, and despite several contradictory findings (4,5,12,13,31,33,36,43), acute nitrate supplementation in the form of concentrated beetroot juice is currently marketed to athletes as a means of enhancing endurance performance.

In contrast to the above, the effects of caffeine supplementation on endurance performance are more clearly defined (10,20); with doses of 3 – 6 mg·kg⁻¹ producing positive effects (1.2 – 4.2%) in time trial events lasting 5 – 60 mins (8,9,26,32,39,42). Although the effects of caffeine were originally purported to emanate from a glycogen-sparing mechanism of action, the absence of a corroborative change in respiratory exchange ratio (RER), combined with evidence of significant effects in events where glycogen availability would not be a limiting factor (20), led researchers to consider alternative explanations. At present, although a small peripheral effect via enhanced calcium mobilisation remains a possibility (40), the key mechanism by which caffeine is believed to enhance endurance performance is via a central mechanism involving an antagonism of adenosine receptors and leading to increases in neurotransmitter release, motor unit firing rates, and pain suppression (25); with particular support for the latter (40). Given the apparent differences in their mechanisms of action (peripheral [nitrate] versus central [caffeine]) and the fact that caffeine has been shown to have no scavenging effects on nitric oxide, at least in rodents (38), it is possible that the two supplements

combined could have a greater effect on endurance performance than either supplement alone. The aim of this study was therefore to examine the acute supplementation effects of dietary nitrate, caffeine, and their combination on subsequent 20 km time-trial performance in well-trained athletes.

METHODS

Experimental Approach to the Problem

In this investigation, subjects were required to complete six trials consisting of a familiarization trial (Trial 1), a stepwise incremental trial (Trial 2), and four supplementation trials (Trials 3-6). The stepwise incremental trial was used to provide descriptive data, and to enable time-trial performance to be evaluated relative to standard physiological parameters. The supplementation trials followed a Latin-square design and were double-blind, randomized, and counterbalanced. Subjects were instructed to maintain their normal diet throughout the testing period, to follow the same diet for 24 hours prior to each trial, to avoid food and drink in the hour before each trial, and to refrain from strenuous exercise for 24 hours before each trial. Subjects were provided with lists of caffeine- and nitrate-rich foods and instructed to abstain from consumption of these for 24 hours and 48 hours, respectively, prior to each trial. A questionnaire was used to investigate the possible effect that normal caffeine consumption may have on the results of the investigation. Subjects were provided with low-nitrate bottled drinking water (Buxton Still, Nestlé Waters UK Ltd, Buxton, UK) to assist with adherence to the dietary restrictions. In addition, subjects were instructed to refrain from using anti-bacterial mouthwash for 48 hours prior to each trial in order to avoid disruption of nitrate-reducing bacteria in the entero-salivary circulation (19). 2.5 hours before each of the supplementation trials, subjects consumed a 70 ml dose of concentrated beetroot juice (Beet IT Sport Shot; James White Drinks Ltd., Ashbocking, Suffolk, UK) containing either 0.45 g (~ 7.3 mmol) of dietary nitrate, or, as supplied specifically by the manufacturer for research purposes, with the nitrate content removed (placebo: ~ 0.01 mmol nitrate). 1 hour before each trial, subjects consumed a gelatine capsule containing either 5 mg·kg⁻¹ of caffeine (Sigma-Aldrich, Steinheim, Germany) or placebo

(maltodextrin: My Protein, Manchester, UK). The supplementation trials therefore consisted of four conditions: placebo (placebo + placebo), nitrate (nitrate + placebo), caffeine (caffeine + placebo), and caffeine + nitrate.

Subjects

Fourteen well-trained, competitive, female athletes (cyclists and triathletes) volunteered for the study which was approved by St Mary's University Ethics Committee. Prior to testing, subjects received written and verbal instructions regarding the nature of the investigation and completed a training history questionnaire, which indicated that all had been actively involved in sport for approximately 13 years and that, at the time of the investigation, time spent training each week was 10.7 ± 2.2 hours. Prior to commencement, all subjects completed a health-screening questionnaire and provided written informed consent. Means \pm standard deviation for age, height, body mass, and body fat, of the subjects were: 31 ± 7 years, 1.69 ± 0.07 m, 61.6 ± 6.0 kg, and $24.9 \pm 4.3\%$, respectively.

Procedures

All trials were performed at approximately the same time of day (± 1 hr) in an air-conditioned laboratory maintained at a temperature of 19°C . In all trials, subjects exercised on a racing bicycle (Claud Butler San Remo, Claud Butler, Brigg, UK) seated on a motor-braked turbo trainer (Tacx Fortius, Wassenaar, Netherlands), which has been shown to have very good test-retest reliability (Coefficient of variation: 1.6%) for 20 km time-trial performance (37). The bicycle was fitted with clipless pedals and subjects cycled using their own cycling shoes. Rear tyre pressure was maintained at 100 psi and the trainer was calibrated before each trial in accordance with the manufacturer's instructions. Prior to every familiarization trial, the saddle height was adjusted for each subject and noted for future replication.

Each stepwise incremental trial began at 100 W and increased by 20 W increments until blood lactate was $> 4 \text{ mmol}\cdot\text{L}^{-1}$. The duration of each increment was 3.5 minutes, and a 20 μl capillary blood sample was obtained in the last 30 s of each increment for the evaluation of blood lactate via an automated analyser (Biosen C-Line, EKF Diagnostic, Ebendorfer Chaussee 3, Germany). After a five minute passive rest period, subjects completed a second incremental test, again starting at 100 W and increasing by 20 W increments; however, for this phase of the trial the duration of each increment was only 1 minute. The trial was terminated when subjects reached volitional exhaustion, at which time a final blood lactate measurement was obtained. Oxygen uptake ($\dot{V}\text{O}_2$) was monitored (breath-by-breath) throughout using an on-line gas analyser (Jaeger Oxycon Pro, Hoechberg, Germany). The analyser was calibrated before each trial using oxygen and carbon dioxide gases of known concentrations (Cryoservice, Worcester, UK) and the flowmeter was calibrated using a 3-litre syringe (Viasys Healthcare GmbH, Hoechberg, Germany). During the trials subjects breathed room air through a facemask (Hans Rudolph, Kansas City, MO, USA) that was secured in place by a head-cap assembly (Hans Rudolph, Kansas City, MO, USA). $\dot{V}\text{O}_{2\text{max}}$ was determined as the highest 30 s average $\dot{V}\text{O}_2$ recorded during the trial provided that at least two of the following criteria had been met: 1) A plateau in $\dot{V}\text{O}_2$; as determined by an increase of less than $2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ over the previous stage; 2) A respiratory exchange ratio ≥ 1.15 ; 3) A heart rate within $10 \text{ b}\cdot\text{min}^{-1}$ of age predicted maximum; 4) A blood lactate concentration $\geq 8 \text{ mmol}\cdot\text{L}^{-1}$.

The familiarization and supplementation trials began with subjects completing a 5 minute warm-up at 100 W, followed by a 5 minute period of passive rest. Subjects then completed a 20 km time-trial against a resistance designed to replicate outdoor, level-gradient, cycling conditions. All measures of elapsed time were removed from the testing environment and the only data visible to the subjects throughout each time-trial was the distance completed. Verbal encouragement was provided throughout. Subjects were free to change gears throughout familiarisation; however, the gearing and

cadence typically chosen were noted and used to standardise subsequent warm-up performance and the starting intensity for subsequent time-trials. After the start of each supplementation time-trial, subjects were free to change gears if they wished. Power output, distance completed, and cadence were recorded at 1 Hz throughout each time-trial. Expired air was monitored, breath-by-breath, for the evaluation of $\dot{V}O_2$ and RER. Heart rate was monitored at 5 s intervals using a heart rate monitor (Polar s610, Polar Electro Oy, Kempele, Finland). Ratings of perceived exertion (RPE) were recorded at 5 km intervals using a 15-point scale (7).

5 minutes before the start of each supplementation trial, a venous blood sample was drawn from a branch of the basilic vein, collected in lithium-heparin tubes (Vacutainer, Becton Dickinson, New Jersey, USA), mixed, and immediately centrifuged at 3000 rpm for 10 minutes at 4°C. Subsequently decanted plasma samples were frozen at -80°C until analyzed for nitrate/nitrite and caffeine content using chemiluminescence and high-performance liquid chromatography, respectively. Analysis of plasma nitrate and nitrite content via chemiluminescence was performed using the same procedures outlined by Peacock et al. (36).

Prior to the supplementation trials, subjects lay supine on an inclined couch while pre-gelled disposable hypoallergenic 1 cm snap-electrodes (Performance Plus, Vermed, VT, USA), were located over the belly of the *vastus lateralis* of the right leg for the evaluation of muscle activity using integrated electromyography (iEMG). Positioning of the electrodes was made using SENIAM (Surface Electromyography for the Non-Invasive Assessment of Muscles) guidelines. Skin surfaces were shaved, if necessary, and swabbed with alcohol prior to electrode placement. Electrodes were placed 2.5 cm apart, parallel to the direction of muscle fibres, with a reference electrode located above the tibia. The position of the electrodes was outlined with indelible pen in order to replicate electrode placement in subsequent trials. EMG data were sampled at 1000 Hz using a data acquisition system (Biopac MP150, Biopac Systems Inc. CA, USA). Data were sampled for 30 s midway through each

warm-up and in the final minute before the end of each 5 km interval during each time-trial. The raw data was band-pass filtered (10 – 500 Hz), rectified, and integrated over each 30 s time period. iEMG data from each time-trial were normalised to the warm-up data to allow for any subtle movements in electrode placement between trials.

The influence of supplementation on tissue oxygenation was evaluated using a continuous wave near-infrared spectroscopy (NIRS) system (PortaMon, Artinis Medical Systems, Zetten, The Netherlands), which uses two wavelengths of light (842 and 762 nm) to measure concentration changes in oxyhemoglobin ([HbO₂]) and deoxyhemoglobin ([HHb]), as well as providing an index of tissue saturation (TSI). The midpoint between the light source (optode) and the receiver was located over the belly of the left *vastus lateralis* using the same procedure for electrode placement outlined above, with the device aligned parallel to the direction of the muscle fibres. Prior to location, the area beneath the device was shaved, if necessary, swabbed with alcohol, and covered with a 6 × 7 cm transparent adhesive dressing (Tegaderm, 3M, MN, USA) to limit movement and the risk of sweat interfering with the device. The device was secured in place with 50 mm lightweight elasticated adhesive bandage (Tiger Tear, Tiger Tapes, Havant, UK) and covered with a black, light-absorbing cloth to minimize the risk of extraneous light interfering with the signal. Data were sampled continuously at 10 Hz at rest, during the warm-up, and throughout each time-trial.

Statistical Analyses

All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL). Measures of centrality and spread are presented as means ± standard deviation. $\dot{V}O_2$ data from each time-trial were filtered to eliminate values that were outside four standard deviations of the midpoint of a rolling 20 breath mean (attributed to ‘noise’), integrated, and averaged to provide mean responses for each trial and each 5 km split. The onset of

blood lactate accumulation (OBLA), from the incremental trials, was identified using software developed for the purpose (34). The effects of supplementation on pre-test measures of plasma nitrate, nitrite, and caffeine were evaluated using one-way ANOVAs. Two-way (supplement \times 5 km split) ANOVAs were used to determine the effects of supplementation and 5 km splits on 20 km time-trial performance measures (power output, time, and cadence) and physiological responses (heart rate, blood lactate, $\dot{V}O_2$, RPE, and RER). The possibility that any effects of caffeine supplementation on performance were influenced by habitual caffeine consumption was investigated by deriving correlations between estimated daily caffeine consumption and caffeine-induced changes (relative to placebo) in 20 km time-trial performance. The effects of supplementation on iEMG activity during the warm-up and during each time-trial were evaluated using one- and two-way (supplement \times 5 km split) ANOVAs, respectively. Finally, the effects of supplementation on NIRS data at rest, during warm-up, and during each time-trial, were determined using one-way ANOVAs. α was set at 0.05 for all analyses. Violations to assumptions of sphericity were adjusted using the Greenhouse-Geisser correction factor. Significant interactions were followed-up using *post hoc* tests with Bonferroni adjustments for multiple comparisons. The above analyses provided 95% confidence limits for all estimates.

RESULTS

Plasma Analyses

The results of the plasma nitrate, nitrite, and caffeine analyses are presented in Table 1. There were significant effects of supplementation on pre-trial plasma concentrations of nitrate ($F_{(1.8,22.9)} = 21.11$; $p < 0.001$), nitrite ($F_{(2.0,26.4)} = 12.88$; $p < 0.001$), and caffeine ($F_{(1.5,19.6)} = 29.21$; $p < 0.001$), confirming subject compliance with supplementation guidelines and dietary restrictions. *Post hoc* tests revealed significantly elevated caffeine concentrations in the caffeine-supplemented conditions and significantly elevated nitrate and nitrite concentrations in the nitrate-supplemented conditions.

The only exception to the above was the absence of a significant difference in plasma nitrite concentration between the caffeine + nitrate and the caffeine trials ($p = 0.09$).

Performance Responses

$\dot{V}O_{2\max(\text{cycling})}$ of the subjects was $3.21 \pm 0.35 \text{ L}\cdot\text{min}^{-1}$ ($52.3 \pm 4.9 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and OBLA occurred at a power output of $205.6 \pm 24.1 \text{ W}$. The effects of supplementation on power output during the time-trials are presented in Figure 1A, with effects on time and cadence presented in Table 2. There was a significant effect of supplementation on power output ($F_{(3,39)} = 6.91$; $p = 0.001$) and time ($F_{(3,39)} = 6.81$; $p = 0.001$) during the time-trials, but no effect on cadence ($F_{(3,39)} = 0.88$; $p = 0.46$). *Post hoc* analyses revealed that significant effects of supplementation on power output and time were only observed between caffeine and nitrate, and caffeine and placebo trials. In effect, caffeine supplementation resulted in significantly higher power outputs than nitrate (mean difference: 10.4 W; 95% likely range: 2.3 – 18.4 W) and placebo (mean difference: 10.3 W; 95% likely range: 0.8 – 19.8 W), with effects translating to corresponding reductions in 20 km completion time (mean difference [caffeine vs nitrate]: 42.4 s; 95% likely range: 5.7 – 79.1 s; [caffeine vs placebo]: 45.1 s; 95% likely range: 3.8 – 86.5 s). Moreover, although the effects of caffeine + nitrate on power output and time were not significantly different from those of caffeine ($p = 1.00$), they were not significantly different from nitrate ($p = 0.21$) and placebo ($p = 0.12$) conditions. The correlation between the improvement in power output, relative to placebo, following caffeine supplementation and estimated typical daily caffeine consumption ($249 \pm 131 \text{ mg}\cdot\text{d}^{-1}$) was -0.20 (95% likely range: $-0.66 - 0.37$).

Irrespective of the form of supplementation, there was a significant main effect of 5 km split on power output ($F_{(1,3,17.5)} = 13.79$; $p < 0.001$) and time ($F_{(1,4,17.6)} = 15.39$; $p < 0.001$) across the time-trials; though once again, there was no effect on cadence ($F_{(1,2,16.1)} = 0.84$; $p = 0.40$). *Post hoc* analyses revealed that subjects produced a significantly greater power output and a correspondingly faster time

in the final 5 km of each time-trial in comparison with each of the other 5 km splits. No significant differences were observed in power output or time for any of the remaining 5 km split comparisons; moreover, there were no significant supplement \times 5 km split interactions for measures of power output ($F_{(5.2,67.0)} = 1.69$; $p = 0.15$), time ($F_{(5.0,64.6)} = 2.12$; $p = 0.075$), or cadence ($F_{(4.6,59.9)} = 0.57$; $p = 0.71$).

Physiological Responses

Heart rate

Heart rate increased progressively throughout the 20 km time-trials ($F_{(1.7,21.9)} = 104.04$; $p < 0.001$), with significantly greater values at each 5 km split (see Figure 1B). There was also a significant effect of supplementation on heart rate ($F_{(3,39)} = 11.95$; $p < 0.001$); *post hoc* analyses revealing that values were significantly higher in caffeine versus placebo (mean difference: 6.5 b·min⁻¹; 95% likely range: 0.9 – 12.1 b·min⁻¹), caffeine + nitrate versus placebo (mean difference: 8.0 b·min⁻¹; 95% likely range: 3.2 – 12.8 b·min⁻¹), and caffeine + nitrate versus nitrate (mean difference: 5.2 b·min⁻¹; 95% likely range: 1.3 – 9.2 b·min⁻¹) trials. There was no supplement \times split interaction for heart rate ($F_{(3.9,50.5)} = 1.88$; $p = 0.13$).

$\dot{V}O_2$

There was a significant effect of 5 km split on $\dot{V}O_2$ ($F_{(1.5,19.2)} = 13.53$; $p < 0.001$), with values increasing between the first two 5 km splits (mean difference: 0.16 L·min⁻¹; 95% likely range: 0.08 – 0.24 L·min⁻¹), but with no significant differences between the 5-10, 10-15, and 15-20 km splits (see Table 3). There was, however, no effect of supplementation on $\dot{V}O_2$ ($F_{(3,39)} = 1.39$; $p = 0.26$), and no supplement \times split interaction ($F_{(3.9,50.3)} = 1.18$; $p = 0.32$).

Respiratory exchange ratio

The pattern of the RER response to the time trials is presented in Figure 1C. There was a significant effect of 5 km split ($F_{(1,2,15,9)} = 23.51$; $p < 0.001$) on RER, with significant differences between all comparisons apart from that between the 5-10 and 15-20 km splits. There was also a significant effect of supplementation ($F_{(3,39)} = 6.61$; $p = 0.001$), with *post hoc* tests revealing significantly higher RER values for caffeine versus placebo (mean difference: 0.033; 95% likely range: 0.002 – 0.063) and caffeine versus nitrate (mean difference: 0.034; 95% likely range: 0.001 – 0.064) trials only. There was no significant supplement \times split interaction ($F_{(4,0,52,0)} = 1.08$; $p = 0.38$) for RER.

Ratings of perceived exertion

There was a significant effect of 5 km split on RPE during the time trials ($F_{(1,6,21,3)} = 144.75$; $p < 0.001$). *Post hoc* comparisons revealed a progressive increase in RPE throughout the time-trials with significant differences between all contrasts (see Table 3). There was, however, no significant effect of supplementation ($F_{(3,39)} = 1.62$; $p = 0.20$) and no supplement \times split interaction ($F_{(9,117)} = 0.82$; $p = 0.60$).

Blood lactate

Blood lactate responses to the time-trials are presented in Figure 1D. There was a significant effect of 5 km split ($F_{(1,6,20,7)} = 23.94$; $p < 0.001$), with mean values increasing throughout the time-trials, and with *post hoc* analyses revealing significant differences between all contrasts apart from those between 0-5 and 5-10 km, and 5-10 and 10-15 km splits. There was also a significant effect of supplementation ($F_{(2,0,26,1)} = 21.67$; $p < 0.001$), with *post hoc* tests revealing significantly higher blood lactate values in caffeine versus nitrate (mean difference: 2.28 mmol·L⁻¹; 95% likely range: 0.84 – 3.73 mmol·L⁻¹), caffeine versus placebo (mean difference: 2.04 mmol·L⁻¹; 95% likely range: 0.90 – 3.18 mmol·L⁻¹), caffeine + nitrate versus nitrate (mean difference: 2.74 mmol·L⁻¹; 95% likely range: 1.03 – 4.45 mmol·L⁻¹), and caffeine + nitrate versus placebo (mean difference: 2.50 mmol·L⁻¹; 95%

likely range: 1.14 – 3.85 mmol·L⁻¹). The supplement × split interaction was not significant ($F_{(9,117)} = 1.94$; $p = 0.053$).

Tissue oxygenation

The effects of supplementation on NIRS-derived measures of tissue oxygenation at rest, during warm-up, and during the time-trials are presented in Table 4. There were no significant effects of supplementation on any of the measures apart from [HHb] ($F_{(3,39)} = 5.34$; $p = 0.004$) during the time-trials. *Post hoc* analyses revealed significantly lower values for [HHb] in the caffeine + nitrate versus the nitrate trial only (mean difference: 3.90; 95% likely range: 0.84 – 6.96).

iEMG

There were five instances where electrodes became detached during the trials. As a result, iEMG responses during the time-trials are based on a sample size of $n = 10$. There was no significant effect of supplementation on the level of iEMG activity during warm-up ($F_{(3,39)} = 0.33$; $p = 0.81$) or during the time-trials ($F_{(3,27)} = 0.59$; $p = 0.63$) (see Table 5). There was, however, a significant effect of 5 km split ($F_{(1,4,12,5)} = 6.75$; $p = 0.016$), with the final 5 km split producing higher levels of activity (relative to warm-up) than the 5-10 km (mean difference: 11.1%; 95% likely range: 0.6 – 21.6%) and 10-15 km (mean difference: 10.0%; 95% likely range: 2.9 – 17.2%) splits. There was no supplement × split interaction for iEMG activity ($F_{(3,4,30,9)} = 1.97$; $p = 0.13$).

DISCUSSION

The aim of this study was to examine the acute supplementation effects of dietary nitrate, caffeine, and their combination on subsequent 20 km time-trial performance in well-trained, competitive athletes. Relative to placebo, the results showed a significant effect of caffeine, but no effect of nitrate. Moreover, while performance following caffeine + nitrate was not significantly

different from the caffeine only condition, it was not significantly different from the nitrate and placebo conditions.

The pre-trial plasma nitrate and nitrite concentrations observed in the caffeine and placebo conditions are commensurate with values previously reported for the same level of dietary nitrate restriction (30). Moreover, the increases in plasma nitrate and nitrite concentrations, relative to placebo, are consistent with those reported following similar acute nitrate dosing strategies (12,28). Nevertheless, nitrate supplementation had no effect on time-trial performance. Apart from the findings of Lansley et al. (28), and the final three of six 500 m rowing repetitions (no overall effect) used by Bond et al. (6), previous research, using various modes of exercise and time-trial durations (4.5 – 138 mins), have also found no significant effect of acute nitrate supplementation on endurance performance (12,13,33,36,43). Since the nitrate dosing strategies used by Lansley et al. (28) and Bond et al. (6) fall within the range used by the aforementioned investigations, and since all the studies listed used well-trained subjects, it is difficult to provide an explanation for these discrepancies.

Previous research has generally, but not always (4,5,13,36), reported a reduced oxygen cost of submaximal exercise following acute or chronic nitrate supplementation (1,2,11,27,29,30,33). In those studies that have examined time-trial performance, several have failed to evaluate performance relative to $\dot{V}O_2$ (6,11,12,13,36). Of those that have, Muggeridge et al. (33) reported a reduction in $\dot{V}O_2$, despite no change in performance; Lansley et al. (28) found no change in $\dot{V}O_2$ despite an increase in performance; and Wilkerson et al. (43), despite finding no change in $\dot{V}O_2$ or performance, found an increase in power output relative to $\dot{V}O_2$. In the present study, subjects completed the nitrate supplemented time-trials at a mean $\dot{V}O_2$ of approximately 83% of $\dot{V}O_{2max}$ (~ 95% of OBLA). As such, it is possible that the absence of an effect of nitrate on $\dot{V}O_2$ could be due to the intensity of the protocol. Indeed, Larsen et al. (30) found that nitrate supplementation only reduced $\dot{V}O_2$ at submaximal intensities of $\leq 80 \dot{V}O_{2max}$. Of course, it is also possible that the nitrate dose delivered

was insufficient to produce an effect. However, since plasma nitrate and nitrite concentrations were significantly elevated, relative to placebo, prior to each time-trial, and since the nitrate dose administered was only slightly less (~ 1 mmol) than the dose recently reported to optimise the effect of acute nitrate supplementation on submaximal $\dot{V}O_2$ and a time-to-exhaustion task (44), this suggestion seems unlikely.

As with nitrate, post-supplementation plasma caffeine concentrations were similar to values previously reported for the same dosing strategy (18). Moreover, the effect of caffeine supplementation on time-trial performance adds to the considerable body of previous research supporting a positive effect of caffeine on endurance exercise (10,20). Although research into the effects of caffeine on time-trial performance, particularly in well-trained athletes, is less substantive; it generally, but not always (14,22) corroborates the results of the present study (8,9,32,39,42). Nevertheless, the increase in power output was not accompanied by a change in cadence, suggesting that the effect was due to an increase in force production rather than an increase in the frequency of each pedal stroke. However, this response was not reflected in an increase in iEMG activity. Since the key mechanism by which caffeine is believed to enhance exercise performance is via the antagonism of adenosine receptors, leading to increases in neural drive and pain suppression (25); the absence of an effect on iEMG suggests that the procedure may lack the necessary sensitivity to detect changes of the magnitude observed in the present study.

Although differences in protocol design make direct comparisons difficult, many of the physiological responses that accompanied caffeine supplementation are consistent across studies. For instance, caffeine has consistently been shown to alter perceptual responses either, as in the present study, by allowing a greater amount of work to be performed at the same perception of effort, or by reducing the perception of effort for the same exercise intensity (16). Similarly, several studies that have observed a caffeine-induced increase in performance have, as in the present study, also reported

a corresponding elevation in heart rate (8,26,39). While the suppressive effect of caffeine on RPE is most likely due to caffeine's antinociceptive effects (25); the increase in heart rate is most likely reflective of the caffeine-induced increase in power output coupled with a progressive increase in cardiovascular drift. While it is also possible that caffeine exerted a direct effect on heart rate, research into the effects of caffeine on fixed-intensity submaximal exercise generally shows no effect (24,26,39).

Given the normally strong positive association between heart rate and $\dot{V}O_2$ at submaximal intensities, it was surprising that the significant effect of caffeine on heart rate was not reflected in a corresponding effect on $\dot{V}O_2$. However, caffeine did have a significant effect on blood lactate, suggesting that the caffeine-induced increase in time-trial performance was due to an increased contribution from anaerobic metabolism. While this suggestion provides a fitting explanation for the above responses, Graham et al. (21) could not attribute caffeine-induced increases in blood lactate to an increase in lactate release from working muscles. Although further research is required to resolve this issue, the caffeine-induced increase in blood lactate, in the absence of any change in $\dot{V}O_2$, provides an explanation for the observed increase in RER via an increased buffering of associated hydrogen ions; although, it is difficult to resolve why this effect was not observed in the caffeine + nitrate trial or in other performance-based studies (9,24).

Typical daily caffeine consumption of the athletes in the present study was similar to that of the general population ($> 200 \text{ mg}\cdot\text{d}^{-1}$) (17). Though there is some evidence that caffeine habituation may reduce the effects of supplementation (3), the fact that the relationship between habitual daily caffeine consumption and the caffeine-induced increase in power output was small, adds support to the majority of studies that report findings to the contrary (15,18,41).

Despite the significant effect of caffeine on time-trial performance, the absence of a significant effect when caffeine was combined with dietary nitrate suggests a possible negative interaction between the supplements. Indeed, the lack of a significant difference in plasma nitrite concentration between the caffeine + nitrate and the caffeine conditions adds some credence to this idea. However, the effects on time-trial performance were similar between caffeine and caffeine + nitrate conditions; moreover, relative to placebo, heart rate and blood lactate responses for the combined condition were similar to those of caffeine, suggesting that the magnitude of this effect is small. Considering the substantial differences between the timings of nitrate and caffeine administration, it seems unlikely that any negative interactive effects occurred pre-absorption; however, since possible interactions between caffeine and nitrate in blood do not appear previously to have been investigated, further research is needed to confirm and explain this finding.

Although the caffeine + nitrate condition failed to significantly enhance time-trial performance, it did, relative to the nitrate condition, result in a significant reduction in [HHb]. It is difficult to reconcile this effect, particularly given the lack of a corresponding significant effect with caffeine, and the absence of a difference in [HHb] between the caffeine + nitrate and the placebo conditions. Bailey et al. (2) observed a significant reduction in [HHb] amplitude during moderate intensity exercise coupled with an increase in [HbO₂] at rest and during moderate intensity exercise following chronic (6.2 mmol·d⁻¹ for 6 days) nitrate supplementation. In contrast, the results obtained at rest and during warm-up in the current study, albeit using an acute dosing strategy, failed to show the same responses. However, Bailey et al. (2) observed no effect of nitrate on NIRS-derived indices of muscle oxygenation during 'severe' exercise, again supporting the idea that the lack of an effect of nitrate in most time-trial studies (12,13,33,36,43) is due to the intensity of the protocols.

The present study used competitive female athletes; first, because they were well-trained, and secondly, because there is a lack of research in this population. The busy training schedules of the

athletes, combined with the need to allow for sufficient recovery between trials, meant that possible circa-mensal effects could not be controlled. However, while there is some evidence to the contrary, and despite several methodological issues and a lack of research using well-trained athletes, many studies have found no effect of the menstrual cycle on cardio-respiratory measures or exercise performance (23,35). As such, and given the randomized order of supplement administration, this was not considered to be a major limitation to the investigation.

PRACTICAL APPLICATIONS

The results of the present study support the well-established beneficial effects of caffeine supplementation on endurance performance. In contrast, acute supplementation with dietary nitrate, in the form of a commercially available sport-specific beetroot supplement, had no effect on 20 km cycling time-trial performance or any associated physiological responses. For those athletes inclined to take combinations of supplements to enhance endurance performance, the results of this study suggest that acute supplementation with dietary nitrate adds nothing to the ergogenic benefits of caffeine supplementation and, though further research is required to confirm, may even have an antagonistic effect.

Acknowledgements

The authors would like to thank all the athletes who participated in this investigation for their enthusiasm and dedication to the project. In addition, the authors would like to thank Dr Philip James (Cardiff University School of Medicine, UK) and Bionox Ltd. for their help with plasma nitrate/nitrite analyses. Finally, the authors would like to thank the School of Sport, Health & Applied Science Research Fund (St Mary's University) for funding the project.

Conflict of interest

The authors have no conflicts of interest that are relevant to the content of this article.

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Table 1. The effects of dietary nitrate, caffeine, and their combination on plasma concentrations of nitrate, nitrite, and caffeine prior to a 20 km cycling time-trial. Values are means \pm standard deviation.

Supplement	Plasma concentration		
	Nitrate ($\mu\text{mol}\cdot\text{L}^{-1}$)	Nitrite ($\text{nmol}\cdot\text{L}^{-1}$)	Caffeine ($\mu\text{g}\cdot\text{ml}^{-1}$)
Placebo	48.2 \pm 21.6 ^{a,c}	91.7 \pm 22.6 ^{a,c}	0.32 \pm 0.46 ^{b,c}
Nitrate	350.0 \pm 156.4 ^d	296.8 \pm 140.4 ^d	0.29 \pm 0.37 ^{d,e}
Caffeine	41.4 \pm 14.5 ^f	103.4 \pm 53.4	4.70 \pm 3.01
Caffeine + Nitrate	192.5 \pm 181.7	206.3 \pm 132.1	4.83 \pm 2.89

Note: Superscripted letters indicate significant ($p < 0.05$) differences between: ^aPlacebo vs Nitrate; ^bPlacebo vs Caffeine; ^cPlacebo vs Caffeine + Nitrate; ^dNitrate vs Caffeine; ^eNitrate vs Caffeine + Nitrate; ^fCaffeine vs Caffeine + Nitrate.

Table 5. The effects of dietary nitrate, caffeine, and their combination on the level of integrated electromyographic activity, relative to a fixed intensity warm-up (100 W), during a 20 km cycling time-trial. Values are means \pm standard deviation.

Supplement	iEMG activity (%)			
	0-5 km	5-10 km	10-15 km	15-20 km
Placebo	139.5 \pm 25.9	148.2 \pm 39.9	152.4 \pm 38.0	164.5 \pm 43.2
Nitrate	138.5 \pm 41.2	132.8 \pm 36.4	131.6 \pm 38.4	147.3 \pm 48.5
Caffeine	148.3 \pm 41.7	150.1 \pm 38.6	148.4 \pm 37.2	158.2 \pm 39.1
Caffeine + Nitrate	148.9 \pm 50.9	144.5 \pm 58.6	147.6 \pm 61.7	150.1 \pm 60.4

Note: iEMG = integrated electromyography.

Table 2. The effects of dietary nitrate, caffeine, and their combination on performance time and cadence during a 20 km cycling time-trial. Values are means \pm standard deviation.

Supplement	Time (minutes)					Cadence (rpm)				
	0-5 km	5-10 km	10-15 km	15-20 km	0-20 km	0-5 km	5-10 km	10-15 km	15-20 km	0-20 km
Placebo	9.06 \pm 0.48	8.91 \pm 0.49	8.84 \pm 0.45	8.55 \pm 0.38	35.37 \pm 1.70 ^a	97.7 \pm 8.2	99.0 \pm 8.7	97.6 \pm 8.8	97.3 \pm 10.7	97.9 \pm 8.6
Nitrate	9.01 \pm 0.45	8.90 \pm 0.43	8.82 \pm 0.40	8.59 \pm 0.36	35.33 \pm 1.50 ^b	96.8 \pm 4.7	98.0 \pm 5.3	96.1 \pm 6.9	95.5 \pm 8.1	96.6 \pm 5.8
Caffeine	8.84 \pm 0.43	8.67 \pm 0.34	8.64 \pm 0.34	8.46 \pm 0.30	34.62 \pm 1.26	97.8 \pm 8.0	98.3 \pm 8.6	97.4 \pm 9.3	96.8 \pm 10.8	97.6 \pm 8.8
Caffeine + Nitrate	8.82 \pm 0.38	8.73 \pm 0.36	8.70 \pm 0.39	8.51 \pm 0.32	34.76 \pm 1.31	98.2 \pm 6.4	99.3 \pm 7.1	98.8 \pm 8.1	98.3 \pm 10.3	98.7 \pm 7.4

Note: rpm = revolutions per minute. Superscripted letters indicate significant ($p < 0.05$) differences between: ^aPlacebo vs Caffeine; ^bNitrate vs Caffeine

Table 3. The effects of dietary nitrate, caffeine, and their combination on oxygen uptake and ratings of perceived exertion during a 20 km cycling time-trial. Values are means \pm standard deviation.

Supplement	Oxygen uptake ($L \cdot min^{-1}$)					RPE			
	0-5 km	5-10 km	10-15 km	15-20 km	0-20 km	0-5 km	5-10 km	10-15 km	15-20 km
Placebo	2.49 \pm 0.47	2.62 \pm 0.44	2.64 \pm 0.35	2.77 \pm 0.44	2.63 \pm 0.41	13.5 \pm 1.5	15.1 \pm 1.5	16.5 \pm 1.7	18.6 \pm 1.0
Nitrate	2.53 \pm 0.33	2.69 \pm 0.33	2.68 \pm 0.34	2.77 \pm 0.35	2.67 \pm 0.32	13.0 \pm 1.8	15.1 \pm 1.5	16.6 \pm 1.3	18.3 \pm 1.3
Caffeine	2.62 \pm 0.33	2.84 \pm 0.35	2.80 \pm 0.37	2.83 \pm 0.32	2.77 \pm 0.32	13.5 \pm 1.5	15.5 \pm 1.6	17.0 \pm 1.7	18.9 \pm 1.2
Caffeine + Nitrate	2.56 \pm 0.46	2.69 \pm 0.49	2.72 \pm 0.41	2.79 \pm 0.36	2.69 \pm 0.42	13.7 \pm 1.9	15.4 \pm 2.0	17.0 \pm 1.5	18.5 \pm 1.2

Note: RPE = rating of perceived exertion.

Table 4. The effects of dietary nitrate, caffeine, and their combination on tissue oxygenation (located at the *vastus lateralis*) during a 20 km cycling time-trial as derived from near-infrared spectroscopy. Values are means \pm standard deviation.

Supplement	Rest			Warm up			20 km time-trial		
	TSI (%)	[HbO ₂]	[HHb]	TSI (%)	[HbO ₂]	[HHb]	TSI (%)	[HbO ₂]	[HHb]
Placebo	70.2 \pm 3.0	2.10 \pm 2.57	0.38 \pm 1.68	62.7 \pm 4.4	-6.71 \pm 3.46	6.67 \pm 4.48	59.1 \pm 5.4	-5.61 \pm 5.94	11.01 \pm 5.51
Nitrate	70.9 \pm 4.1	0.63 \pm 1.15	0.33 \pm 1.24	63.1 \pm 4.8	-6.42 \pm 4.11	7.22 \pm 4.16	60.2 \pm 5.8	-4.85 \pm 5.16	11.64 \pm 5.52 ^a
Caffeine	70.1 \pm 2.2	-0.82 \pm 3.53	0.47 \pm 2.37	64.1 \pm 2.8	-7.90 \pm 3.62	5.76 \pm 3.72	59.5 \pm 4.9	-5.98 \pm 4.77	9.34 \pm 4.78
Caffeine + Nitrate	70.5 \pm 1.6	-0.48 \pm 2.29	-0.33 \pm 1.44	65.0 \pm 3.1	-5.86 \pm 2.76	4.61 \pm 3.66	61.2 \pm 4.4	-7.77 \pm 7.48	7.74 \pm 4.22

Note: TSI = tissue saturation index; [HbO₂] = oxyhemoglobin concentration change; [HHb] = deoxyhemoglobin concentration change. Units for [HbO₂] and [HHb] are arbitrary. Superscripted letter indicates significant ($p < 0.05$) difference between: ^aNitrate vs Caffeine + Nitrate.

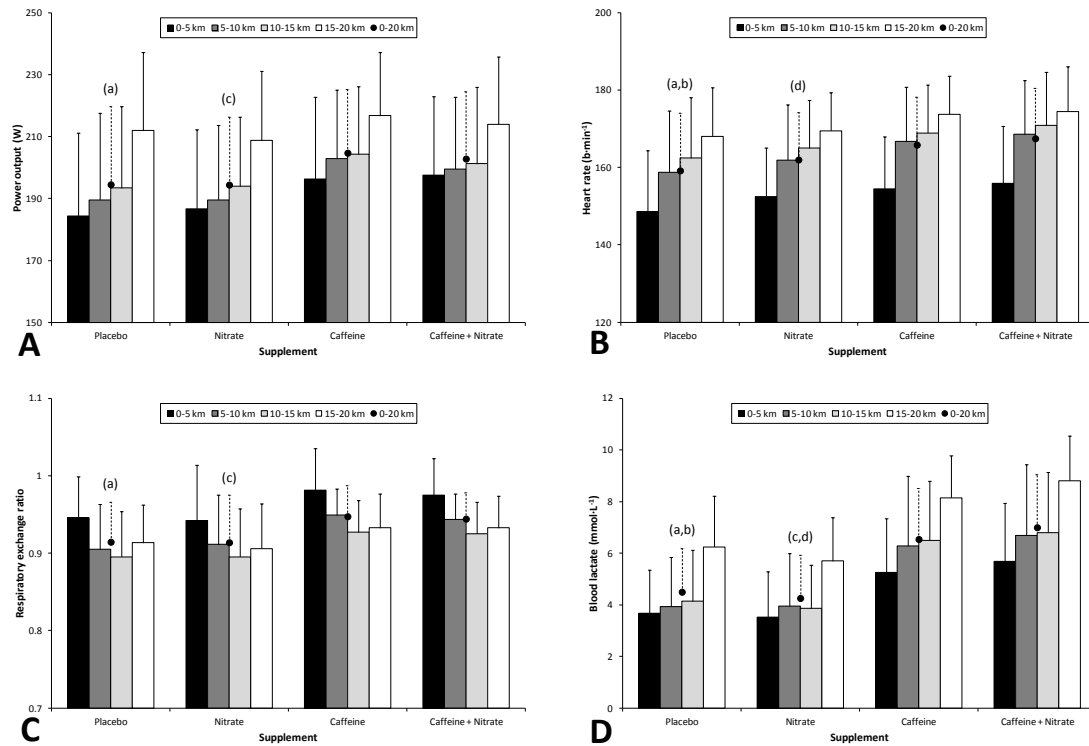


Figure 1. The effects of dietary nitrate, caffeine, and their combination on power output (A), heart rate (B), respiratory exchange ratio (C), and blood lactate (D), during a 20 km cycling time-trial. Values are means; bars are standard deviations. Closed circles (●) represent mean responses during each trial. Superscripted letters indicate significant ($p < 0.05$) differences between: ^aPlacebo vs Caffeine; ^bPlacebo vs Caffeine + Nitrate; ^cNitrate vs Caffeine; ^dNitrate vs Caffeine + Nitrate.