

*Dietary nitrate supplementation increases acute mountain sickness severity and sense of effort during hypoxic exercise*

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1 **Dietary nitrate supplementation increases acute mountain sickness severity and sense of effort during**  
2 **hypoxic exercise**

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15 **Running head:** Nitrate supplementation increases AMS severity

16

17

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27 **ABSTRACT**

28 Dietary nitrate supplementation enhances sea level performance and may ameliorate hypoxemia at high altitude.  
29 However, nitrate may exacerbate acute mountain sickness (AMS), specifically headache. This study investigated  
30 the effect of nitrate supplementation on AMS symptoms and exercise responses with 6h hypoxia. Twenty  
31 recreationally-active men (mean(SD): age 22(4) years,  $\dot{V}O_{2max}$  51(6) mL·min<sup>-1</sup>·kg<sup>-1</sup>) completed this randomized  
32 double-blinded placebo-controlled crossover study. Twelve participants were classified as AMS- based on  
33 Environmental Symptom Questionnaire (AMS-C) score <0.7 in both trials, and five participants were classified  
34 as AMS+ based on AMS-C score ≥0.7 on placebo. Five days nitrate supplementation (70mL beetroot juice  
35 containing ~6.4mmol nitrate daily) increased plasma NO metabolites by 182μM compared to placebo but did not  
36 reduce AMS or improve exercise performance. After 4h hypoxia (F<sub>I</sub>O<sub>2</sub>=0.124) nitrate *increased* AMS-C and  
37 headache severity (visual analogue scale (VAS); whole sample Δ10[1,20] mm; *p*=0.03) compared to placebo. In  
38 addition, after 5h hypoxia, nitrate increased sense of effort during submaximal exercise (Δ7[-1,14]; *p*=0.07). In  
39 AMS- nitrate did not alter headache or sense of effort. In contrast, in AMS+ nitrate increased headache severity  
40 (Δ26[-3,56] mm; *p*=0.07), sense of effort (Δ14[1,28]; *p*=0.04), oxygen consumption, ventilation, and mean arterial  
41 pressure during submaximal exercise. On the next day, in a separate acute hypoxic exercise test (F<sub>I</sub>O<sub>2</sub>=0.141),  
42 nitrate did not improve time to exhaustion at 80% hypoxic  $\dot{V}O_{2max}$ . In conclusion, dietary nitrate increases AMS  
43 and sense of effort during exercise, particularly in those who experienced AMS. Dietary nitrate is therefore not  
44 recommended as an AMS prophylactic or ergogenic aid non-acclimatized individuals at altitude.

45

46 **KEY WORDS:** Altitude sickness, beetroot, headache, nitric oxide, rating of perceived exertion

47

48 **NEW AND NOTEWORTHY**

49 This is the first study to identify that the popular dietary nitrate supplement (beetroot) does not reduce acute  
50 mountain sickness (AMS) or improve exercise performance during 6 h hypoxia. The consumption of nitrate in  
51 those susceptible to AMS exacerbates AMS symptoms (headache) and sense of effort, and raises oxygen cost,  
52 ventilation, and blood pressure during walking exercise in 6 h hypoxia. These data question the suitability of  
53 nitrate supplementation during altitude travel in non-acclimatized people.

54 **INTRODUCTION**

55 Many people engage in altitude travel for work and leisure, with 300 million overnight stays in alpine regions  
56 each year (5) and hundreds of thousands of individuals traveling to other high altitude regions such as the  
57 Himalayas and Kilimanjaro (17). Approximately half of those that visit high altitude suffer from illnesses such as  
58 acute mountain sickness (AMS) (6). AMS can lead to high altitude cerebral edema (HACE) which if left untreated  
59 can be fatal (19). High altitude exposure also reduces exercise capacity (16). Finding interventions to counteract  
60 hypoxemia, the root-cause of these negative effects, is therefore important.

61  
62 Nitric oxide (NO) is a potent signaling molecule that modulates human physiological function via its role in the  
63 regulation of blood flow, muscle contractility and mitochondrial respiration (44). NO can be produced via the  
64 oxygen (O<sub>2</sub>)-dependent oxidation of L-arginine with the help of NO synthase (NOS) enzymes (31). However, it  
65 is now clear that NO can also be generated by the serial reduction of nitrate to nitrite and then NO (12). An  
66 increase in NO bioavailability has been observed after the consumption of dietary inorganic nitrate (49) and has  
67 been associated at sea level with a reduction in resting blood pressure (43), increased skeletal muscle blood flow  
68 (14), and enhanced exercise performance (21). Importantly, the reduction of nitrite to NO is increased in  
69 environments of low O<sub>2</sub> tension, and therefore dietary nitrate consumption may be a particularly effective method  
70 to increase NO bioavailability at altitude. During acute exposure to hypoxia ( $\leq 2$  h), nitrate supplementation has  
71 been shown to improve peripheral and muscle oxygenation during rest and exercise (33), and improve muscle  
72 energetics (46), lower the O<sub>2</sub> cost of submaximal exercise (24), and enhance exercise performance in some (e.g.  
73 (33, 42)) but not all (e.g. (2, 10)) studies. NO may also improve diffusion capacity within the lung, and enhance  
74 O<sub>2</sub> delivery (15), potentially ameliorating the root cause of AMS (hypoxemia). Because of these proposed  
75 beneficial physiological and exercise responses, some have recommended dietary nitrate for altitude travel (4,  
76 37), with a recent study concluding its use is safe and feasible at altitude (20).

77  
78 However, nitrate supplementation may be harmful during hypoxia. Dietary nitrate may increase AMS by elevating  
79 headache. Not only the cardinal symptom of AMS, it has been suggested that high-altitude headache contributes  
80 to the accompanying AMS symptoms of gastrointestinal symptoms, fatigue, dizziness, lightheadedness, and poor  
81 sleep (27). A growing body of data supports the proposed pathophysiology of high-altitude headache as a result  
82 of arterial dilation causing elevated cerebral blood volume and intracranial pressure. This leads to increased  
83 arterial and intracranial pressure transmission, trigeminovascular sensitization, and pain sensation (27). Given that

84 NO is implicated in hypoxia-induced vasodilation (35), and directly stimulates the trigeminovascular system (3),  
85 supplementing with nitrate may exacerbate high-altitude headache pathophysiology. However, to date no  
86 laboratory studies have been completed to investigate the effects of dietary nitrate on AMS. Resolving the efficacy  
87 and safety of this dietary intervention, particularly in reference to the cerebral component of AMS, is of clinical  
88 and timely importance. In addition, the effect of dietary nitrate consumption on exercise responses during longer-  
89 duration hypoxic exposures (>2 h) is unknown. Since the physiological stimulus of exercise may increase AMS  
90 severity in hypoxia (11, 38), the effects of nitrate on exercise responses, whether positive or negative, could  
91 potentially alter nitrate's effect on AMS symptoms. Given that individuals travelling to high altitude will engage  
92 in exercise, it is important to investigate the effects of nitrate under conditions of exercise in hypoxia. In addition,  
93 AMS is a condition that is greatly affected by individual susceptibility (48). It is possible that nitrate may  
94 exacerbate AMS pathophysiology and symptoms in individuals who experience AMS, but have beneficial  
95 physiological and exercise performance effects in individuals who do not suffer AMS.

96

97 The primary aim of the present double-blind placebo-controlled crossover study was to determine the effect of  
98 five days of dietary nitrate supplementation on high-altitude headache and AMS symptom severity with a 6 h  
99 exposure to hypoxia. We hypothesized five days of dietary nitrate supplementation would decrease high-altitude  
100 headache and AMS symptom severity with a 6 h exposure to hypoxia. Secondary aims of the present study were  
101 to explore: the effect of five days dietary nitrate supplementation on submaximal exercise responses during 6 h  
102 hypoxia; and the interaction of AMS (AMS present or absent) and nitrate supplementation on high-altitude  
103 headache severity and submaximal exercise responses during 6 h hypoxia. In addition, due to equivocal findings  
104 in the literature (e.g. (10, 33)), we also determined the effect of six days dietary nitrate supplementation on exercise  
105 performance in acute hypoxia.

106 **METHODS**

107 *Participants*

108 Twenty recreationally-active men were recruited into the study (mean (SD); age, 22 (4) years; height, 180 (10)  
109 cm; body mass, 79 (11) kg;  $\dot{V}O_{2max}$ , 51 (6) ml·kg<sup>-1</sup>·min<sup>-1</sup>). Eleven participants (45%) had previously travelled to  
110 high altitude ( $\geq 2500$  m), and of these 11 participants, 2 (18%) reported previous AMS, and none had a history of  
111 HAPE or HACE. Participants had not travelled to altitude ( $\geq 1500$  m) in the preceding six months, and had no  
112 medical contraindications to maximal exercise testing. All participants provided written informed consent. Ethical  
113 approval was granted by the Ethics Committee of the School of Sport, Health, and Exercise Sciences at Bangor  
114 University, and the study was registered on [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (trial ID: NCT03101904).

115

116 *Study design*

117 The study followed a double-blinded placebo-controlled crossover design. Normoxic ( $F_{I}O_2 = 0.209$ ; sea level)  
118 and hypoxic ( $F_{I}O_2 = 0.141$ ; equivalent 3225 m) maximal exercise tests were conducted at baseline. Participants  
119 then completed two six-day supplementation periods, separated by a minimum ten-day washout. On day five of  
120 each supplementation period, a 6 h hypoxic exposure ( $F_{I}O_2 = 0.124$ ; equivalent 4219 m) was conducted to assess  
121 AMS and responses to submaximal exercise. On day six, a time to exhaustion test was conducted at 80%  $\dot{V}O_{2max}$   
122 reserve in acute hypoxia ( $F_{I}O_2 = 0.141$ ; equivalent 3225 m) to assess exercise performance. An overview of the  
123 protocol is depicted in Figure 1.

124

125 *Supplementation*

126 Participants were randomly assigned to receive six days of inorganic nitrate or placebo supplementation, separated  
127 by a minimum ten-day washout. Supplementation consisted of a daily nitrate-rich concentrated beetroot juice shot  
128 (nitrate; 70 mL/day containing ~6.4mmol NO<sub>3</sub><sup>-</sup>; Beet It Sport, James White drinks Ltd, Ipswich, UK) or a nitrate-  
129 depleted concentrated beetroot juice shot (placebo; 70 mL/day containing ~0.003 mmol NO<sub>3</sub><sup>-</sup>; James White drinks  
130 Ltd, Ipswich, UK) that was identical in appearance, taste, and texture. Placebo shots were created by passing the  
131 NO<sub>3</sub><sup>-</sup> rich concentrated beetroot juice through a Purolite A520E NO<sub>3</sub><sup>-</sup> selective ion exchange resin before  
132 pasteurization (26). Supplements were ingested 2.5 h before any experimental tests, and at the same time each day  
133 throughout both supplementation periods. Participants were phoned or texted to remind them to ingest the  
134 supplement and were asked to confirm ingestion by phone or return text.

135

136 To isolate nutritional effects of the intervention, participants avoided high  $\text{NO}_3^-$  food and drink throughout the  
137 study, and diet and physical activity was matched before each trial, confirmed by food and exercise diaries.  
138 Participants also abstained from using antibacterial mouthwashes as this has previously shown to lessen the  
139 reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  by commensal bacteria within the oral cavity (18).

140

141 Before (day 0) and on day five (in hypoxia) and day six (pre-hypoxia) of each supplementation phase, venous  
142 blood samples were collected into lithium heparin-coated 6 mL vacutainers (BD Vacutainer tubes; Becton,  
143 Dickinson and Company; New Jersey), immediately centrifuged at 4000 rpm and 4°C for 7 min before the plasma  
144 was extracted and stored at -80°C for later analysis of plasma NO metabolites (nitrate + nitrite [ $\text{NO}_x$ ]). To confirm  
145 participants were blind to the supplementation they were receiving and that the placebo was successful, a  
146 manipulation check was conducted after each supplementation was completed, by asking participants to guess  
147 what intervention they had received (possible responses were “beetroot”, “placebo”, or “don’t know”).

148

149 *Procedures*

150 Maximal aerobic capacity ( $\dot{V}\text{O}_{2\text{max}}$ )

151 All exercise tests consisted of loaded walking whilst carrying a 15 kg rucksack on a motorized treadmill (H-P-  
152 Cosmos, Sports & Medical GmbH; Nussdorf-Traunstein; Germany). At baseline, participants completed an  
153 incremental exercise test to exhaustion with simultaneous online pulmonary gas analysis (Cortex Metalyzer,  
154 Biophysik GmbH; Leipzig; Germany). The incremental exercise test protocol commenced at 5 km/h and 1%  
155 gradient with a ramped increase in gradient to 25% over 18 min. If 25% was reached, treadmill speed was  
156 increased by 0.66 km/h/min. Sense of effort during exercise (Rating of Perceived Exertion; RPE) was recorded  
157 each minute of the test using the Borg CR100 (9).  $\dot{V}\text{O}_{2\text{max}}$  was identified if all of the following criteria were met:  
158 volitional fatigue; a plateau in oxygen consumption ( $\dot{V}\text{O}_2$ ) despite an increase in workload; respiratory exchange  
159 ratio (RER)  $\geq 1.15$ . Participants also completed the same maximal exercise protocol in hypoxia ( $F_{\text{I}}\text{O}_2 = 0.141$ ,  
160 equivalent 3225 m), with the two tests separated by a minimum of 48 h.

161

162 Six-hour hypoxic exposure

163 On day five of each supplementation period, participants completed a six-hour poikilocapnic hypoxic exposure in  
164 a normobaric hypoxic chamber ( $F_{\text{I}}\text{O}_2 = 0.124$ ; equivalent 4219 m; Hypoxico, Inc. New York, NY). After fifteen  
165 min seated rest, participants completed the first of three 20-min bouts of submaximal exercise at 40% hypoxic



166  $\dot{V}O_{2\max}$  reserve. The submaximal exercise protocol during the 6 h exposure consisted of exercise bout one,  
167 completed between 15-35 min; exercise bout two, 140-160 min; and exercise bout three, 300-320 min. Blood  
168 pressure and heart rate measured by a stress BP system (Tango+; SunTech Medical, Inc., Morrisville, NC; USA),  
169 and oxygen saturation (SpO<sub>2</sub>) measured by pulse oximetry (9550 OnyxII; Nonin Medical Inc, Minnesota), were  
170 assessed every hour and pre- and post-each exercise bout throughout the 6 h exposure. Mean arterial blood  
171 pressure (MAP) was calculated to account for heart rate using the equation provided by Moran and colleagues  
172 (36):

$$173 \quad \text{MAP} = \text{DBP} + ((0.01 \times \exp(4.14 - 40.74 \div \text{HR})) \times (\text{SBP} - \text{DBP}))$$

174 Where MAP, Mean arterial blood pressure; DBP, Diastolic blood pressure; HR, Heart rate; SBP, Systolic blood  
175 pressure.

176

#### 177 High-altitude headache and AMS

178 High-altitude headache was assessed every hour and pre- and post-each exercise bout throughout the six-hour  
179 exposure on day five of supplementation, using a 0-100 mm visual analogue scale (VAS) (23). Participants were  
180 asked to mark on the line the intensity/feeling that corresponded to their headache, where the beginning of the line  
181 indicated no perception/feeling at all and the end of the line indicated maximum perception/feeling. AMS was  
182 assessed by the Acute Cerebral Mountain Sickness (AMS-C) score calculated from the 11-item Environmental  
183 Symptoms Questionnaire (ESQ) (41). Participants rated the severity of each item from one to five, and the ratings  
184 were multiplied by their factorial loadings and summed. In order to investigate whether any effects of nitrate  
185 supplementation were affected by the presence or absence of AMS, participants were split into two groups  
186 depending on whether they had AMS on placebo. Participants with AMS-C < 0.7 in both trials were classified as  
187 AMS-, while participants with AMS-C ≥ 0.7 on placebo were classified as AMS+ (40). Participants with AMS-  
188 C < 0.7 on placebo, but AMS-C ≥ 0.7 on nitrate were included in the whole sample analyses, but were not included  
189 in AMS+/- sub-analyses.

190

#### 191 Submaximal exercise responses

192 Sense of effort during the three submaximal exercise bouts was assessed by RPE. RPE was recorded using the  
193 Borg CR100 (9) which asks participants to rate the intensity of the exercise sensation using numbers from 0-100+  
194 and verbal descriptors (e.g. “moderate”, equivalent to 25). Extensive evidence supports the use of RPE as a valid  
195 and appropriate method to record sense of effort and perceptual responses to exercise (13). RPE, heart rate and

196 SpO<sub>2</sub> were recorded at two-min intervals during the three exercise bouts. In addition to these variables, the study  
197 design targeted exercise bout two to examine extra physiological responses, including ventilatory parameters and  
198 exercising blood pressure, in an attempt to identify possible mechanisms of nitrate's effect on exercise and AMS  
199 before AMS was present. We did not collect this data during exercise bout one because this bout occurred too  
200 early, when variable responses such as the hypoxic ventilatory response (47) would likely mask any mechanistic  
201 physiological changes. We did not collect this data during exercise bout three because this bout occurred too late,  
202 when AMS was already present. To be consistent with a cause and effect relationship, any physiological changes  
203 must be observed before the onset of AMS; at exercise bout three it would not be possible to differentiate between  
204 cause and consequence of AMS. Thus, during exercise bout two exercising blood pressure was recorded every  
205 two min and ventilation ( $V_E$ ),  $\dot{V}O_2$ , RER, and end-tidal carbon dioxide ( $P_{ET}CO_2$ ) were measured using online gas  
206 analysis. To account for the hypoxic conditions, the gas analyzer was calibrated using ambient air (12.4% O<sub>2</sub>;  
207 0.03% CO<sub>2</sub>) and a FiO<sub>2</sub>-specific calibration gas (8% O<sub>2</sub>; 5% CO<sub>2</sub>; Industrial Gases, BOC ltd, Surrey, UK).

208

#### 209 Exercise performance (time to exhaustion; TTE)

210 On day six of each supplementation period, participants completed a time to exhaustion (TTE) test at 5 km/h and  
211 a gradient corresponding to 80% of their hypoxic  $\dot{V}O_{2max}$  reserve in acute hypoxia ( $F_{I}O_2 = 0.141$ , equivalent 3225  
212 m). Exercise performance was defined as TTE determined by the time from onset of test to task failure (volitional  
213 exhaustion or inability to maintain treadmill speed). During the exercise test SpO<sub>2</sub>, heart rate, and RPE were  
214 recorded each minute. Participants were blind to time elapsed and provided no verbal encouragement.

215

#### 216 *Plasma nitric oxide metabolites (NO<sub>x</sub>)*

217 All glassware, utensils, and surfaces were rinsed with deionised water to remove residue NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> before  
218 blood analysis. After thawing at room temperature, plasma samples were initially deproteinized using cold ethanol  
219 precipitation. Initially 0.5 mL of sample was placed in a chilled microcentrifuge tube, along with 1 mL of cold  
220 (0°C) ethanol; then the samples were vortexed and left to stand at 0°C for 30 min. Thereafter, samples were  
221 centrifuged at 14,000 rpm for 5 min, and the supernatant removed for subsequent analysis. NO<sub>x</sub> (nitrate + nitrite)  
222 in the deproteinized plasma samples was reduced to NO in the presence of 0.8% (w/v) vanadium trichloride in 1  
223 M hydrochloric acid. The production of NO was detected by a Sievers gas-phase chemiluminescence NO analyser  
224 (Sievers NOA 280i; Analytix, Duham, UK) and the NO<sub>x</sub> concentration was derived by plotting signal (mV) area  
225 against a calibration plot of 1-750 μM sodium NO<sub>3</sub><sup>-</sup>.

226

227 *Statistical analyses*

228 Differences between nitrate and placebo were determined by confidence intervals relating to *a priori* meaningful  
229 differences (22), supported by statistical differences testing by repeated measures analysis of variance (RM  
230 ANOVA) or t-tests as appropriate ( $p < 0.05$ ). For the primary analysis, (to determine the effect of dietary nitrate  
231 supplementation on high-altitude headache) a  $2 \times 4$  (Trial  $\times$  Time) RM ANOVA was used to compare high-  
232 altitude headache severity by VAS from 4 to 6 h between nitrate and placebo trials. The time course of 4 to 6 h  
233 was chosen for the primary analysis based on the expected time that AMS would develop from previous data from  
234 our laboratory at a similar  $FiO_2$  (28–30) that showed no incidence of AMS before 4 h, but an AMS incidence of  
235 50% after 6 h. A sample size estimation for the primary analysis indicated that 16 participants were needed to  
236 produce an 80% chance of obtaining statistical significance at the 0.05 level for a two-tailed design (45), based  
237 on a minimum important difference of 10 mm (23), a standard deviation of the difference of 18 mm, and an  
238 estimated average correlation of 0.4 (data from (29)). To determine the influence of AMS, all analyses were  
239 repeated including a factor for AMS presence or absence (AMS+ or AMS-), and interpreted on the basis of  
240 significant interactions.

241

242 The effect of nitrate supplementation on exercise performance (TTE) was determined by paired samples t-test. A  
243 sample size estimation for this analysis indicated that 12 participants were needed to produce an 80% chance of  
244 obtaining statistical significance at the 0.05 level for a two-tailed design (8), based on a minimum important  
245 difference of 30 s, and a standard deviation of the difference of 33 s (data from (33)). To determine the effect of  
246 nitrate supplementation on i) RPE, ii)  $SpO_2$ , and ii) heart rate, a  $2 \times 5$  (Trial  $\times$  Isotime) RM ANOVA was used to  
247 compare nitrate and placebo trials at 0%, 25%, 50%, 75%, and 100% of isotime during the TTE test. Resting  
248 values taken immediately before commencing the TTE were recorded as 0% isotime. 100% isotime was defined  
249 as the last complete minute of the shortest TTE, and the corresponding minute in the longest TTE for each  
250 participant. The minute identified as 100% isotime was multiplied by 0.25, 0.5, and 0.75 and rounded to the  
251 nearest complete minute to give 25% isotime, 50% isotime, and 75% isotime, respectively.

252

253 Five participants did not complete the TTE on day six due to injury or technical reasons, e.g. cramp or tripping  
254 whilst on the treadmill, and one of these was also removed from analyses relating to RPE on day five due to failure  
255 to use the RPE scale consistently. All analyses were completed using SPSS version 23 (IBM Corp, Armonk; NY).

256 **RESULTS**

257 Participants were sufficiently blinded to the intervention since the manipulation check indicated participants  
258 guessed correctly in only 18% of trials, guessed incorrectly in 34% of trials, and were unable to distinguish  
259 between interventions in 48% of trials.

260

261 *Plasma nitric oxide metabolites (NO<sub>x</sub>)*

262 Subject compliance with the supplementation protocol was 100%. This self-reported compliance measure was  
263 confirmed by plasma NO<sub>x</sub> data. Plasma NO<sub>x</sub> was similar between trials at baseline (nitrate = 27 (9); placebo = 28  
264 (9); [-5, 3] μM;  $p = 0.7$ ). The nitrate supplementation effectively altered plasma NO<sub>x</sub> concentrations. Compared  
265 to placebo, nitrate supplementation increased plasma NO<sub>x</sub> on day five (mean diff [95% CI]: Δ182 [155, 208] μM;  
266  $p < 0.001$ ). Compared to placebo, nitrate supplementation also increased plasma NO<sub>x</sub> on day six in the 15  
267 participants that completed the time to exhaustion tests (Δ244 [201, 286] μM;  $p < 0.001$ ). By design, plasma NO<sub>x</sub>  
268 was not altered compared to baseline, with five (Δ-3 [-8, 2] μM;  $p = 0.3$ ) or six (Δ-3 [-10, 1] μM;  $p = 0.2$ ) days of  
269 placebo supplementation.

270

271 *High-altitude headache and AMS*

272 There was no headache at 0 h (pre-hypoxia) in either trial (nitrate = 1 (4); placebo = 4 (9); [-7, 2] mm;  $p = 0.2$ ).  
273 Although headache tended to increase after exercise bout one (Δ4 [-1, 9] mm;  $p = 0.08$ ) and bout two (Δ8 [0, 15]  
274 mm;  $p = 0.05$ ), there was no effect of nitrate (both  $p \geq 0.4$ ; Figure 2A). As expected, AMS and headache increased  
275 in the latter part of the trial ( $p < 0.05$ ). Unexpectedly this effect was exacerbated by nitrate. From 4 to 6 h, nitrate  
276 increased headache severity compared to placebo (Δ10 [1, 20] mm;  $p = 0.03$ ) and tended to increase AMS-C  
277 compared to placebo (Δ0.15 [-0.01, 0.31];  $p = 0.07$ ; Figure 2B).

278

279 *Physiological responses to 6 h hypoxia*

280 Resting physiological responses (heart rate, SpO<sub>2</sub>, and blood pressure) changed throughout the 6 h exposure, but  
281 were not affected by nitrate. Heart rate increased over the hypoxic exposure (Δ16 [11, 21] bpm;  $p < 0.001$ ), and  
282 after each exercise bout, but was not affected by nitrate (Δ-2 [-5, 2] bpm;  $p = 0.4$ ). SpO<sub>2</sub> decreased over the  
283 hypoxic exposure (Δ-14 [-17, -11] %;  $p < 0.001$ ), and after each exercise bout, but was not affected by nitrate (Δ0  
284 [-1, 1] %;  $p = 0.8$ ). Nitrate supplementation had no effect on any measure of resting blood pressure, with no

285 difference in systolic blood pressure (SBP;  $\Delta 1$  [-4, 5] mmHg;  $p = 0.7$ ), diastolic blood pressure (DBP;  $\Delta -1$  [-4, 2]  
286 mmHg;  $p = 0.4$ ), or mean arterial blood pressure (MAP;  $\Delta 1$  [-3, 4] mmHg;  $p = 0.8$ ).

287

### 288 *Submaximal exercise responses*

289 Dietary nitrate *increased* sense of effort compared to placebo during submaximal exercise in 6 h hypoxia (Figure  
290 3A). Nitrate elicited a small but significant increase in RPE for exercise bout one ( $\Delta 4$  [0, 8];  $p = 0.03$ ), and a larger  
291 increase for exercise bout three ( $\Delta 7$  [-1, 14];  $p = 0.07$ ), although had no effect on RPE for exercise bout two ( $\Delta 2$   
292 [-5, 9];  $p = 0.5$ ). In the nitrate trial, headache at 5 h (pre-exercise bout three) was positively correlated with change  
293 in RPE from exercise bout one to exercise bout three ( $r = 0.67$ ;  $p < 0.01$ ). When analyzed across the whole sample,  
294 nitrate had no effect on any physiological response to the three submaximal exercise bouts (Table 1).

295

### 296 *Influence of AMS presence or absence (AMS+/-)*

297 Twelve participants were classified as AMS- and five were AMS+ (see Methods: High-altitude headache and  
298 AMS). There were no differences in baseline characteristics or NO<sub>x</sub> concentrations at any point between AMS+  
299 and AMS- (all  $p > 0.5$ ; data not shown).

300

301 In AMS-, nitrate had no effect on high-altitude headache ( $\Delta 2$  [-5, 9] mm;  $p = 0.6$ ; Figure 2C) or AMS-C ( $\Delta -0.04$   
302 [-0.13, 0.06];  $p = 0.4$ ; Figure 2D). In AMS+, nitrate increased altitude illness severity from 4 h: in AMS+, high-  
303 altitude headache severity was more than doubled with nitrate compared to placebo ( $\Delta 26$  [-3, 56] mm;  $p = 0.07$ ;  
304 Figure 2E) and AMS-C was similarly increased ( $\Delta 0.46$  [-0.10, 1.02];  $p = 0.09$ ; Figure 2F). In AMS-, there was no  
305 difference in RPE between nitrate and placebo for any of the exercise bouts (Figure 3C). In AMS+, by the end of  
306 the exposure, dietary nitrate had increased sense of effort during submaximal exercise compared to placebo  
307 (Figure 3E;  $\Delta 14$  [1, 28];  $p = 0.04$ ).

308

309 Depending on whether participants were AMS- or AMS+, nitrate had opposite effects on physiological responses  
310 to submaximal exercise during the hypoxic exposure (determined by significant AMS-trial interactions). During  
311 exercise bout two, in AMS-, nitrate decreased ventilation in comparison to placebo ( $-3.0$  [-5.5, -0.4] L/min;  $p =$   
312  $0.03$ ; Figure 4A), and had no effect on  $\dot{V}O_2$  ( $\Delta -0.02$  [-0.13, 0.09] L/min;  $p = 0.6$ ; Figure 5A) or MAP ( $\Delta -1$  [-9, 6]  
313 mmHg;  $p = 0.7$ ; Figure 6A). In contrast, in AMS+, nitrate increased ventilation ( $\Delta 3.1$  [-0.7, 7.0] L/min;  $p = 0.1$ ;  
314 Figure 4B),  $\dot{V}O_2$  ( $\Delta 0.10$  [0.03, 0.17] L/min;  $p = 0.02$ ; Figure 5B), and MAP ( $\Delta 7$  [-3, 17] mmHg;  $p = 0.1$ ; Figure

315 6B). During exercise bout three, in AMS- nitrate increased SpO<sub>2</sub> compared to placebo from 12 min onwards ( $\Delta$  3  
316 [0, 4] %;  $p = 0.03$ ; Figure 3D). However, in AMS+, nitrate had no effect on SpO<sub>2</sub> for any exercise bout (Figure  
317 3F). The effect of nitrate on P<sub>ET</sub>CO<sub>2</sub> was not altered depending on the presence or absence of AMS (AMS-trial  
318 interaction;  $p = 0.2$ ).

319

320 *Exercise performance (time to exhaustion; TTE)*

321 Six days dietary nitrate supplementation had no effect on TTE in hypoxia ( $\Delta$ 10 [-103, 123] s;  $p = 0.9$ ). Dietary  
322 nitrate supplementation had no effect on heart rate, SpO<sub>2</sub>, or RPE at any isotime, or at exhaustion (Table 2).

323

324 Whether participants experienced AMS did not influence the effect of nitrate on exercise performance (TTE;  
325 group-trial interaction;  $p = 0.9$ ). Specifically, nitrate had no effect on TTE in AMS- ( $\Delta$ 16 [-115, 148] s;  $p = 0.8$ )  
326 or in AMS+ ( $\Delta$ 10 [-103, 123] s;  $p = 0.9$ ).

327 **DISCUSSION**

328 The primary findings of this study were that dietary nitrate supplementation did not reduce AMS severity,  
329 specifically high-altitude headache, or improve exercise performance in hypoxia. When assessed over the whole  
330 sample, nitrate had no effect on any physiological response to hypoxia. However, opposing effects of nitrate were  
331 observed in those with and without AMS. In participants who did not develop AMS (AMS-), nitrate decreased  
332 ventilation and improved SpO<sub>2</sub> during exercise but this did not translate into reduced AMS symptoms or an  
333 improvement in exercise performance. In contrast, in AMS+, nitrate increased ventilation and O<sub>2</sub> cost of exercise,  
334 headache and AMS severity, and sense of effort during submaximal exercise in 6 h hypoxia.

335

336 The increase in headache and AMS that occurred in the latter part of the trial was exacerbated by nitrate. It is clear  
337 this was not due to nitrate increasing exercise-induced headache, since there was no effect of nitrate on headache  
338 post exercise bout one or two that occurred earlier in the hypoxic exposure, before AMS was present. In those  
339 that developed AMS dietary nitrate induced a 26 mm increase in headache, which is of sufficient magnitude to  
340 have clinical relevance (23). This finding is in agreement with a previous study that reported greater headache at  
341 altitude after L-arginine supplementation (32). However it contrasts the null finding reported by Hennis and  
342 colleagues in the field (20); the only previous study to investigate the effects of chronic dietary nitrate  
343 supplementation on AMS. This difference in results is most likely due to differences in study design. Specifically,  
344 in Hennis and colleagues' study (20) biochemical confirmation of the nitrate supplementation was not performed  
345 and therefore it was not possible to determine if the nitrate supplementation was effective. This is of particular  
346 concern since the authors reported poor compliance. Further, as AMS is determined by subjective responses, the  
347 use of a non-taste matched placebo means placebo or nocebo effects cannot be dismissed. In contrast, we  
348 completed a double-blind placebo-controlled study, and report 100% compliance to the supplementation protocol,  
349 with confirmation of successful blinding and increased plasma NO<sub>x</sub> in the nitrate trial.

350

351 When activated in the brain, NO directly stimulates the trigeminovascular system, responsible for headache pain  
352 sensation (3). In addition, an increase in cerebral blood flow and resultant increase in intracranial pressure can  
353 also result in trigeminovascular system activation, and is suggested to cause high-altitude headache and AMS  
354 (27). Therefore increasing the bioavailability of NO through dietary nitrate supplementation is likely to directly  
355 stimulate trigeminovascular afferents, and concurrently elevate hypoxia-induced cerebral vasodilation. This  
356 provides a possible mechanism for the increase in high-altitude headache and AMS observed herein. Further

357 support for this explanation is provided by previous studies that have utilized NO-synthase inhibitors (e.g. L-  
358 NMMA) (3) and artificial vasoconstriction (25) to successfully reverse headache.

359

360 Nitrate supplementation did not benefit exercise performance as assessed by time to exhaustion in acute hypoxia.  
361 This finding is in agreement with the only previous randomized controlled trial (RCT) to assess exercise  
362 performance in acute hypoxia (< 2 h) following six days dietary nitrate supplementation (10). Bourdillon et al.  
363 (10) found dietary nitrate supplementation did not alter hypoxic pulmonary vasoconstriction or 15 km time trial  
364 performance in acute hypoxia ( $F_{I}O_2 = 0.11$ ). A further novel approach of the current study was to examine exercise  
365 responses during 6 h hypoxic exposure after dietary nitrate supplementation. Contrary to our hypothesis, nitrate  
366 did not improve submaximal exercise performance even in AMS- where nitrate improved  $SpO_2$  during exercise  
367 (3% after 5 h in hypoxia). Further, in those that developed AMS+, nitrate actually impaired exercise by increasing  
368 sense of effort; participants had to invest more effort to achieve the same exercise output in the nitrate trial,  
369 compared to placebo. In those that developed AMS (AMS+), nitrate also increased the  $O_2$  cost of fixed workload  
370 exercise, which may have been driven by an increase in ventilation. Increased ventilation is associated with greater  
371 respiratory muscle demand and dyspnea (1), which are important contributing factors of sense of effort (7). As  
372 exercising sense of effort, ventilation, and  $O_2$  cost of exercise were increased and present before AMS, nitrate  
373 should be considered the cause of the negative effects observed in AMS+, rather than an effect of AMS symptoms.  
374 In addition, the elevated AMS symptoms may have contributed to the increase in sense of effort in the final bout  
375 of exercise, as the increase in sense of effort was proportional to headache severity immediately before exercise.  
376 By the end of the final exercise bout sense of effort was increased by 54% in those who experienced AMS,  
377 equivalent to an entire verbal descriptor (from “moderate”, to “somewhat strong”). Since the exercise typically  
378 completed at altitude is often long-duration and submaximal, this finding has great importance for those travelling  
379 to altitude for work and recreation as increased sense of effort is associated with poorer mood, and increased  
380 fatigue (39), which is an important risk factor at altitude (15).

381

382 This study is limited by the absence of a direct measure of cerebral blood flow to support the proposed mechanistic  
383 interpretation. However, the conclusion of cause and effect is supported by the use of a strong experimental design,  
384 and a theoretical explanation backed by a wealth of existing literature (27). This study was also limited by the  
385 duration of exposure (6 h), and is therefore unable to conclude the effects of nitrate on physiological and perceptual  
386 responses with more chronic exposure to altitude, for example over many days or weeks. Another limitation of



387 this study is that all participants were men, and thus the findings may not be applicable to women. In addition, the  
388 study design did not allow separation of the effects of nitrate in hypoxia *per se* from any interaction with exercise,  
389 although the effects of nitrate supplementation with exercise in normoxia have been studied in detail in previous  
390 literature (34).

391

392 In conclusion, dietary nitrate increases AMS symptom severity, specifically headache, and sense of effort during  
393 submaximal exercise, particularly in those who experience AMS. Therefore, dietary nitrate is not recommended  
394 as an AMS prophylactic or ergogenic aid in non-acclimatized individuals at altitude.

395

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400

#### 401 **DISCLOSURES**

402 No conflicts of interest, financial or otherwise, are declared by the authors.

403

#### 404 **AUTHOR CONTRIBUTIONS**

405 G.M.K.R., J.H.M., S.J.L., B.W., V.N., H.E.D., and S.J.O conception and design of research; G.M.K.R., J.H.M.,  
406 L.J.W., S.J.L., B.W., V.N., K.A.H., R.B., H.E.D., and S.J.O performed experiments; G.M.K.R and L.J.W.  
407 analyzed data; G.M.K.R., J.H.M., L.J.W., S.J.L., K.A.H., R.B., and S.J.O interpreted results of experiments;  
408 G.M.K.R prepared figures; G.M.K.R., J.H.M., L.J.W., B.W., V.N., and S.J.O drafted manuscript; G.M.K.R.,  
409 J.H.M., L.J.W., and S.J.O edited and revised manuscript; G.M.K.R., J.H.M., L.J.W., S.J.L., B.W., V.N., K.A.H.,  
410 R.B., H.E.D., and S.J.O approved final version of manuscript.

411

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532

533

534 **FIGURE LEGENDS**

535 **Figure 1. Overview of trial protocol.** Supplementation was taken at the same time each day, 2.5 h before any  
536 exercise tests. Participants completed a 6 h exposure to hypoxia on day five, and an acute hypoxic exercise to  
537 exhaustion test on day six. All exercise intensities are set at a proportion of hypoxic  $\dot{V}O_{2max}$  reserve. Additional  
538 physiological data was obtained by online gas analysis and a stress blood pressure system during exercise bout  
539 two. Filled black arrows indicate headache and AMS data included in the primary analysis.

540  
541 **Figure 2. High-altitude headache and AMS during 6 h in hypoxia ( $F_{I}O_2 = 0.124$ ) on day five.** Grey shaded  
542 bars indicate submaximal exercise bouts (40%  $\dot{V}O_{2max}$  reserve). From 4 to 6 h, in the whole sample, nitrate  
543 increased headache by visual analogue scale (VAS; Panel A), and tended to increase AMS-C score calculated  
544 from the Environmental Symptoms Questionnaire (Panel B). In AMS- (participants with AMS-C score < 0.7 in  
545 both trials) dietary nitrate had no effect on headache (Panel C), or AMS-C (Panel D). In AMS+ (participants with  
546 AMS-C  $\geq 0.7$  on placebo), nitrate increased headache (Panel E) and AMS-C (Panel F). \*nitrate significantly  
547 higher than placebo ( $p < 0.05$ ).

548  
549 **Figure 3. Sense of effort (RPE) and oxygen saturation (SpO<sub>2</sub>) during submaximal exercise.** In the whole  
550 sample, dietary nitrate increased RPE (Panel A), but had no effect on SpO<sub>2</sub> (Panel B). In AMS- (participants with  
551 AMS-C score < 0.7 in both trials), dietary nitrate had no effect on RPE (Panel C), but increased SpO<sub>2</sub> during  
552 exercise bout three (Panel D). In AMS+ (participants with AMS-C  $\geq 0.7$  on placebo), dietary nitrate increased  
553 RPE (Panel E), but had no effect on SpO<sub>2</sub> (Panel F). \*nitrate significantly higher than placebo ( $p < 0.05$ ).

554  
555 **Figure 4. Ventilation during submaximal exercise (exercise bout two).** Dietary nitrate decreased ventilation  
556 during exercise bout two in AMS- (participants with AMS-C score < 0.7 in both trials; Panel A), and increased  
557 ventilation in AMS+ (participants with AMS-C  $\geq 0.7$  on placebo; Panel B). \*nitrate significantly higher than  
558 placebo ( $p < 0.05$ ).

559  
560 **Figure 5. Oxygen cost ( $\dot{V}O_2$ ) of submaximal exercise (exercise bout two).** Dietary nitrate had no effect on  $\dot{V}O_2$   
561 during exercise bout two in AMS- (participants with AMS-C score < 0.7 in both trials; Panel A), but increased  
562  $\dot{V}O_2$  in AMS+ (participants with AMS-C  $\geq 0.7$  on placebo; Panel B). \*nitrate significantly higher than placebo ( $p$   
563 < 0.05).

564

565 **Figure 6. Mean arterial pressure (MAP) during submaximal exercise (exercise bout two).** Dietary nitrate  
566 had no effect on MAP during exercise bout two in AMS- (participants with AMS-C score < 0.7 in both trials;  
567 Panel A), but increased MAP in AMS+ (participants with AMS-C  $\geq$  0.7 on placebo; Panel B). Nitrate significantly  
568 higher than placebo (\* $p$  < 0.05; \*\* $p$  < 0.01).