Nitric Oxide 50 (2015) 58–64

Contents lists available at ScienceDirect

Nitric Oxide

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

Acute dietary nitrate supplementation improves arterial endothelial function at high altitude: A double-blinded randomized controlled cross over study

Emily Bakker [a], Harald Engan [b, c, 1], Alexander Patrician [b], Erika Schagatay [b, d], Trine Karlsen [a, e], Ulrik Wisløff [a], Svein Erik Gaustad [a, *]

[a] K.G. Jebsen Center of Exercise in Medicine at the Department of Circulation and Medical Imaging, Norwegian University of Science and Technology, Faculty of Medicine, Trondheim, Norway
[b] Department of Health Sciences, Mid Sweden University, Östersund, Sweden
[c] LHL-Klinikkene Røros, LHL-Health, Norway
[d] Swedish Winter Sports Research Center, Mid Sweden University, Östersund, Sweden
[e] St. Olav's University Hospital, Trondheim, Norway

ARTICLE INFO

Article history:
Received 8 May 2015
Received in revised form
23 August 2015
Accepted 24 August 2015
Available online 29 August 2015

Keywords:
Nitric oxide
Beetroot juice
Field-study
FMD
Flow mediated dilation
Nepal
Altitude medicine

ABSTRACT

Introduction: Dietary nitrate (NO_3^-) supplementation serves as an exogenous source of nitrite (NO_2^-) and nitric oxide (NO) through the $NO_3^- - NO_2^- - NO$ pathway, and may improve vascular functions during normoxia. The effects of NO_3^- supplementation in healthy lowlanders during hypobaric hypoxia are unknown.
Purpose: Determine the effect of acute oral NO_3^- supplementation via beetroot juice (BJ) on endothelial function (flow mediated dilation; FMD) in lowlanders at 3700 m.
Methods: FMD was measured using ultrasound and Doppler in the brachial artery of 11 healthy subjects (4 females, age 25 ± 5 yrs; height 1.8 ± 0.1 m, weight 72 ± 10 kg) sojourning to high altitude. In a randomized, double-blinded crossover study design, FMD was measured 3 h after drinking BJ (5.0 mmol NO_3^-) and placebo (PL; 0.003 mmol NO_3^-) supplementation at 3700 m, with a 24-h wash out period between tests. FMD was also measured without any BJ supplementation pre-trek at 1370 m, after 5 days at 4200 m and upon return to 1370 m after 4 weeks of altitude exposure (above 2500 m). The altitude exposure was interrupted by a decent to lower altitude where subjects spent two nights at 1370 m before returning to altitude again.
Results: Ten subjects completed the NO_3^- supplementation. FMD (mean ± SD) pre-trek value was 6.53 ± 2.32% at 1370 m. At 3700 m FMD was reduced to 3.84 ± 1.31% ($p < 0.01$) after PL supplementation but was normalized after receiving BJ (5.77 ± 1.14% ($p = 1.00$). Eight of the subjects completed the interrupted 4-week altitude stay, and their FMD was lower at 4200 m (FMD 3.04 ± 2.22%) and at post-altitude exposure to 1370 m (FMD 3.91 ± 2.58%) compared to pre-trek FMD at 1370 m.
Conclusion: Acute dietary NO_3^- supplementation may abolish altitude-induced reduction in endothelial function, and can serve as a dietary strategy to ensure peripheral vascular function in lowland subjects entering high altitude environments.

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1. Introduction

At high altitude (HA) proper acclimatization is crucial to maintain physiological functions. Preservation of the vascular system is vital for this process, due to its importance for oxygen (O_2) delivery to the tissues and regulation of perfusion pressure to different organs. The definition of a normal vascular response to HA is unclear, and there is no general consensus in the literature of how HA affects

* Corresponding author. Norwegian University of Science and Technology, Department of Circulation and Medical Imaging, Prinsesse Kristinas gt. 3, 7006 Trondheim, Norway.
E-mail address: Svein.e.gaustad@ntnu.no (S.E. Gaustad).
[1] Joint first author.

http://dx.doi.org/10.1016/j.niox.2015.08.006
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the arterial endothelial function and vascular tone. Conflicting reports of arterial vasoconstriction or vasodilation upon HA exposure are possibly due to variation in the study designs (population, type and duration of hypoxia) and measurement sites (pulmonary, cerebral, peripheral vasculature) [1,2]. Nevertheless, a key player in obtaining proper endothelial function is nitric oxide (NO). It has been suggested that natives of Tibet living at HA partly have adapted to altitude by NO related mechanisms, and it has been shown that they exhibit high levels of circulating bioactive NO products [4]. Moreover, the combination of high concentrations of circulating bioactive NO products and low hemoglobin is associated with increased forearm blood flow in this population [4]. In native lowlanders, the role of circulating vasoactive nitrogen oxides during acclimatizing to HA was assessed, showing that nitrate (NO_3^-), nitrite (NO_2^-) [5,6] and cyclic guanosine monophosphate (cGMP) [5], biomarkers of NO production and activity, were elevated upon HA acclimatization.

NO is formed through both the L-arginine pathway and the NO_3^--NO_2^--NO pathway. However in an O_2 depleted environment, the L-arginine pathway has a decreased ability to generate NO [8–10], which makes the NO_3^--NO_2^--NO pathway more important. Ingested inorganic NO_3^- is rapidly absorbed from the gut to the blood and then metabolized to NO_2^- by salivary bacteria [9]. This pathway can restore NO production in hypoxic conditions as there is increased generation and use of NO_3^- and NO_2^- [9]. This is due to increased reduction to NO_2^- and NO by deoxyhemoglobin and deoxymyoglobin [11–13], which are more available at HA when blood O_2 saturation is decreased.

At sea level dietary NO_3^- supplementation is effective for optimizing vascular function, resulting in a reduction of blood pressure (BP) [14–17] and improvement of endothelial function with 0.5–4% increase in flow-mediated dilation (FMD) in healthy subjects after acute supplementation [18,19]. Beetroot juice (BJ), known to contain high doses of NO_3^- [17,20], has been demonstrated to have a vasoprotective role [17] and improved FMD after a high fat meal [21]. Based on the positive effect of dietary NO_3^- supplementation on endothelial function at sea level, its' effectiveness in optimizing endothelial function under hypobaric hypoxic conditions should be explored. In this study, the primary aim was to investigate the effects of dietary NO_3^- supplementation on endothelial function during hypobaric hypoxia at HA in lowlanders. The secondary aim was to investigate the effect of prolonged HA exposure on endothelial function.

It was hypothesized that 1) FMD at 3700 m would be depressed compared to pre-trek FMD at 1370 m, 2) that dietary BJ NO_3^- supplementation normalizes FMD at 3700 m, and 3) FMD will remain suppressed compared to pre-trek FMD at 1370 m after a interrupted 4-week exposure to HA (above 2500 m).

2. Materials and methods

This study was performed in Kathmandu and the Rolwaling Valley, Nepal. It was ethically approved by Swedish Research Council and Nepal Health Research Council and performed according to the Helsinki declaration.

2.1. Subjects

A total of 11 healthy lowlanders (4 female, 25 ± 5 yrs, 1.8 ± 0.1 m, 72 ± 10 kg) participated in this field study. The subjects received oral information and read and signed an informed consent. None had been at altitude within 12 months prior to the expedition. A baseline measurement was completed for 10 of the subjects, as one did not follow subject restrictions prior to this measurement. A different subject did not participate in the BJ supplementation due to logistical reasons, 10 subjects completed BJ supplementation. Three subjects did not complete the expedition, rendering a total of 8 subjects for final measurement after 4 weeks at HA.

2.2. Study timeline

FMD was measured 5 times at 3 different altitudes within 39 days of the trekking expedition (Fig. 1). To avoid potential effects of chronobiology, all measurements were obtained in the morning. Pre-trek measure (Test 1) was taken at 1370 m in Kathmandu, one day after arrival. HA was considered as elevations above 2500 m (4 of the 5 weeks of the expedition).

2.2.1. Acute measurements

The NO_3^- supplementation protocol (BJ/placebo (PL)) took place on days 7 and 8 at 3700 m (Test 2/3), after the subjects had spent 3 days walking from 1525 m to 3700 m and after one day residing at 3700 m.

2.2.2. Measurements during altitude exposure

The endothelial function was measured at 4200 m (Test 4) on day 10, after 5 days above 2500 m. Post-HA measures were performed one day after return flight to Kathmandu at 1370 m (Test 5) on day 39 of the trekking expedition, after 4 weeks of HA exposure (above 2500 m) which was interrupted by a decent to lower altitude where subjects spent two nights at 1370 m. Most travel between elevations occurred by walking, with the exception of nine hours bus transportation to the Rolwaling valley (1525 m) on day 2. Additional motorized transportation was used on day 21 (9 h bus from Rolwaling valley) and day 23 (1 h plane to Khumbu valley at 2860 m) due to extreme weather conditions preventing the subjects from climbing a mountain pass between these valleys (two days altitude interruption to 1370 m). The subjects were transported from the Khumbu valley to Kathmandu by plane (1 h) on the final day of the trek (day 38).

2.3. Tests and measurements

FMD was assessed in the brachial artery using a 12-MHz Doppler probe and ultrasound imaging (*Vivid* I, GE Healthcare, USA) following current guidelines [22]. All tests were performed according to a standardized procedure, and consisted of FMD technique to estimate NO-mediated vasodilation, as well as recording of heart rate (HR), BP, and arterial oxygen saturation (SaO_2).

2.3.1. Subject preparation

To avoid measurement bias, subjects avoided caffeine, tobacco and exercise on the testing days and food intake 3 h prior to measurements [22]. Subjects avoided mouthwash and tooth brushing on the BJ/PL test days in order not to wash out lingual bacteria important for NO_3^- reduction [23]. Prior to the measurement, subjects were questioned about their compliance to the study restrictions. In the field subjects were tested inside cabins, and a combination of wood ovens, propane heaters and down sleeping bags were used to control ambient temperature and keep subjects warm (BJ/PL test, room temperature 20 ± 2 °C).

2.3.2. FMD procedure

An occlusion cuff (SC10, D.E. Hokanson Co., Bellevue, USA) was placed on the non-dominant forearm distal to the measuring site and three lead ECG electrodes on shoulders and chest [24]. The measuring site for all FMD measures was above the anticubital fossa, with the arm extended [24]. Once the subject and measurement equipment were prepared, the subjects were requested to

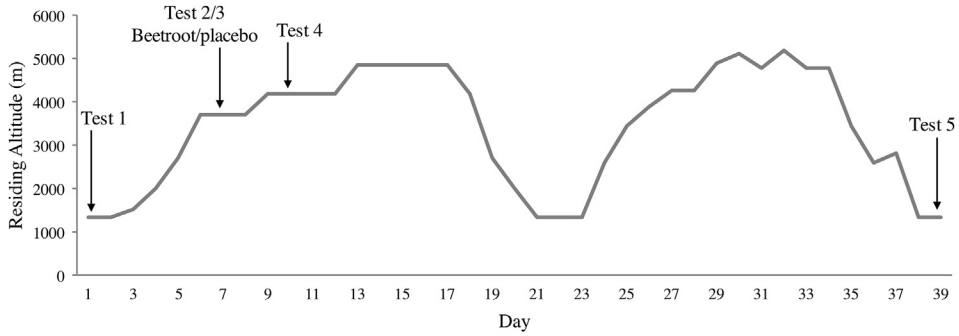

Fig. 1. Depiction of the residing altitudes (m) during the 39 days of the study, with specified days of FMD interventions indicated with arrows. The interventions are denoted as Test 1: measurement of pre-trek values (1370 m), Test 2/3: measurement (3700 m) following beetroot juice or placebo juice, Test 4: measurements at altitude (4200 m), Test 5: measurements (1370 m) 1-day after returning from 4-weeks at high altitude which was interrupted by 2 days decent to 1370 m.

relax in a supine position.

After 10 min total supine resting the FMD measurement began, and baseline diameter and blood velocity of the brachial artery were recorded. Once baseline measures had been recorded, the occlusion cuff was inflated to >250 mmHg for 5 min [22]. Upon release of the cuff occlusion the resultant peak in blood flow was recorded (within 10 s) using Doppler and the artery diameter image was recorded for 3 min in 30-s intervals.

2.3.3. Blood pressure, heart rate and oxygen saturation procedure

Before FMD measurements, and after 5 min of supine rest, 3 BP and HR measurements were taken on the dominant arm in 1-min intervals using an automatic BP cuff (Omron Healthcare, Lake Forest, USA). During the vascular occlusion and reactive hyperemia phases of the FMD measurement, SaO_2 and HR were manually recorded on 1-min intervals using a pulse oximeter (OnyxVantage, Nonin Medical, Plymouth, USA), for a total of 8 recordings.

2.3.4. Beetroot/placebo juice supplementation procedure

In a double-blinded randomized-cross over manner, subjects drank 70 ml organic concentrated beetroot juice shots (James White Drinks Ltd., Suffolk, UK) containing either 5.0 mmol NO_3^- (BJ) or NO_3^- depleted beetroot juice shots (PL) containing 0.003 mmol NO_3^- on two consecutive days, with a 24-h washout period between doses [17]. In the PL juice, NO_3^- was removed via ion-exchanging resin, while the color, taste, smell and texture remained indistinguishable from the BJ servings [25]. The FMD measurement period commenced 3 h after supplementation, in order to assess FMD during anticipated peak NO_3^- and NO_2^- concentration [14,17].

2.3.5. Dehydration scoring

The subjects self-assessed dehydration by scoring urine color from 0 to 8. A score of 3 or less indicated hydrated state and a score greater than 3 indicated dehydration. Urine color is closely correlated with urine osmolality, and an acceptable measure in field studies [26].

2.4. Data analysis

The FMD measurements were evaluated blinded from the intervention. Artery diameter analysis (intima to intima) was performed using caliper measurement (0.1 mm resolution) using the peak of the ECG R wave of the cardiac cycle for timing. Three repetitions were made per cardiac cycle and 3 cardiac cycles per time point. The mean calculated to represent the artery diameter and each subject's peak artery diameter post occlusion was chosen individually based on maximum dilation [22]. Baseline and peak blood flow ($mm \cdot s^{-1}$) were estimated from Doppler waveform for velocity.

The FMD (%) was calculated from the difference in peak and baseline artery diameter (maximum dilation) divided by baseline. Shear rate (s^{-1}) was estimated by dividing peak blood flow by peak artery diameter, and further used to account for mechanical stress with a resultant FMD to shear stress ratio (normalized FMD; nFMD) [27]. The mean FMD was calculated as the sum of the FMD (%) from 30 s to 180 s post-occlusion divided by the total number of time points [19]. The flow stimulus received by the artery during reactive hyperemia was expressed as the ratio of peak blood flow to baseline blood flow [17].

An average of the two most similar values was used for calculation of BP and HR, while for SaO_2 calculations an average of the 8-recorded values was used.

2.4.1. Statistical analysis

For statistical analysis IBM *SPSS 21* statistics software (SPSS Inc, Chicago, USA) was used, and Graph Pad *Prism 6* (GraphPad Software Inc., San Diego, USA) for graph figures. Normality was tested using the Shapiro–Wilk test. For dietary NO_3^- supplementation data repeated measures ANOVA with Bonferroni correction was used to compare means of baseline, BJ and PL. Liner Mixed Models with Compound Symmetry using pairwise comparison of estimated marginal means with Bonferroni correction was used for comparison of Test 1, 4, and 5. For all tests significance was set to $p < 0.05$ and a trend denoted when $p < 0.1$.

3. Results

3.1. Endothelial function at altitude after acute supplementation of beetroot juice

At HA of 3700 m FMD was reduced after PL supplementation to 3.84 ± 1.31% in comparison to the 1370 m pre-trek levels at 6.53 ± 2.32% ($p < 0.01$). At 3700 m, BJ supplementation restored FMD to 5.77 ± 1.14%, similar to pre-trek levels at 1370 m ($p = 1.00$) (Fig. 2A). Similar increase from BJ supplementation was found examining nFMD, where nFMD was 0.025 ± 0.007%·s at 1370 m pre-trek, a lower value of 0.015 ± 0.004%·s with PL ($p < 0.01$), and restored to 0.023 ± 0.009%·s with BJ ($p = 1.00$) (Fig. 2B). Mean FMD, the average dilation over 3 min, was 1.46 ± 1.81% lower with PL (1.51 ± 1.78%) supplementation compare to BJ supplementation (2.91 ± 2.11%; $p < 0.01$).

The flow stimulus tended ($p = 0.085$) to be larger during BJ supplementation, with a difference of 15.8 ± 24.2% compared to PL at 3700 m (Table 1). No differences were observed when comparing

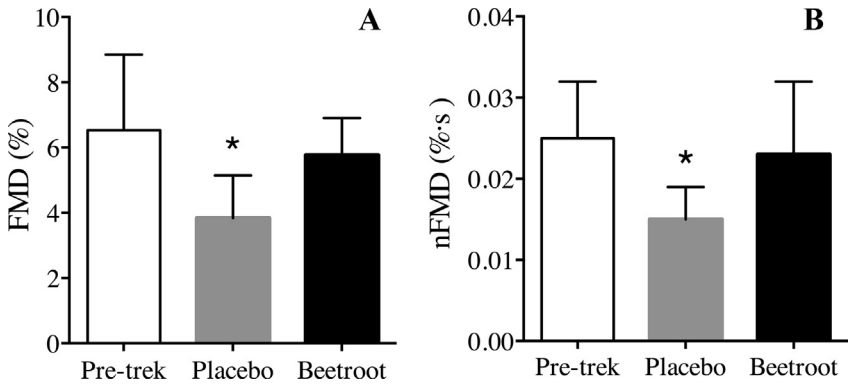

Fig. 2. A) Peak flow-mediated dilation (FMD) in percent (%), B) normalized flow-mediated dilation (nFMD) in %·s at pre-trek at 1370 m (white bars) and during supplementation of placebo (grey bars) and beetroot juice (black bars) at 3700 m. Values presented as mean ± standard deviation. * indicates significant difference from baseline and beetroot juice ($p < 0.01$).

Table 1
Acute vascular function with beetroot or placebo supplementation at 3700 m.

	Placebo	Beetroot
Baseline artery diameter (mm)	3.86 ± 0.56	3.84 ± 0.62
Peak artery diameter (mm)	4.00 ± 0.57	4.06 ± 0.63
Baseline blood flow (mm s^{-1})	808.2 ± 169.0	743.4 ± 159.5
Peak blood flow (mm s^{-1})	1001.0 ± 196.2	1060.6 ± 281.5
Flow stimulus	1.25 ± 0.18	1.43 ± 0.28
Shear rate (s^{-1})	250.7 ± 40.3	268.9 ± 86.8

Data are presented as mean ± standard deviation. mm = millimeters; s = seconds.

BJ and PL supplementation at 3700 m to baseline blood flow, peak blood flow, shear rate, or baseline and peak artery diameter (Table 1).

3.2. Endothelial function during altitude exposure

Compared to pre-trek at 1370 m, FMD remained reduced during altitude exposure at 4200 m, and when measured at 1370 m after 4 weeks of interrupted high (>2500 m) altitude exposure ($p < 0.05$, estimated marginal mean ± SD, Table 2). The nFMD reflected similar decreases (Table 2). There was a trend of reduction ($p = 0.091$) in baseline blood flow from the first day of the expedition at 1370 m (Test 1) to post-expedition measurements at 1370 m (Test 5; Table 2).

3.3. Heart rate, blood pressure and arterial oxygen saturation

The mean HR at 4200 m was 65 ± 11 beats·minutes^{-1}, which was significantly higher than at all other test points. Pre-trek HR at 1370 m was 58 ± 11 beats·minutes^{-1}, 12 ± 15% lower than the HR at 4200 m and 14 ± 18% higher than the post-expedition HR at 1370 m ($p < 0.05$). Mean SaO_2 at 4200 m was 84.9 ± 2.9%, and it was reduced by 12.4% from both pre-trek and post-expedition values, at 97.3 ± 2.9% and 97.3 ± 3.2%, respectively ($p < 0.01$). No differences were observed comparing BJ and PL supplementation at 3700 m with regard to the basic physiological measures, HR, SaO_2, systolic blood pressure, diastolic BP and mean arterial pressure (Table 3).

3.4. Dehydration score

Mean dehydration score was similar at all test locations, whereas individual subject values ranged from 1 to 8. Dehydration scores were not completed for all testing days/locations. Mean values were 3.4 ± 2.0 at 3700 m (day 8), 3.8 ± 2.0 at 4200 m (day 10) and 3.8 ± 2.2 post-expedition (day 38).

4. Discussion

The principal finding of this investigation was that dietary NO_3^- supplementation with BJ restored FMD of the brachial artery to pre-trek levels by enhanced NO-mediated dilation in healthy

Table 3
Physiological variables with beetroot and placebo supplementation at 3700 m.

	Placebo	Beetroot
Heart rate (bpm)	70 ± 18	68 ± 16
SaO_2 (%)	87.9 ± 4.0	87.3 ± 4.8
SBP (mmHg)	115 ± 15	115 ± 13
DBP (mmHg)	70 ± 14	73 ± 14
MAP (mmHg)	86 ± 13	86 ± 14

Data are presented as mean ± standard deviation. SaO_2: arterial oxygen saturation SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure.

Table 2
Vascular function at 1370 m and 4200 m.

	Test 1 (1370 m) n = 10, day 1	Test 4 (4200 m) n = 11, day 10	Test 5 (1370 m) n = 8, day 39
FMD (%)	6.53 ± 2.32	3.04 ± 2.22*	3.91 ± 2.58*
nFMD (%·s)	0.025 ± 0.007	0.011 ± 0.007*	0.016 ± 0.010
Baseline artery diameter (mm)	3.78 ± 0.56	3.72 ± 0.56	3.67 ± 0.57
Peak artery diameter (mm)	4.02 ± 0.56	3.83 ± 0.55	3.82 ± 0.57
Baseline blood flow (mm s^{-1})	770.5 ± 177.6	722.9 ± 172.0	644.6 ± 192.2
Peak blood flow (mm s^{-1})	1042.4 ± 205.6	1039.7 ± 199.7	958.4 ± 221.0
Flow stimulus	1.40 ± 0.3	1.46 ± 0.3	1.52 ± 0.3
Shear rate (s^{-1})	264.3 ± 59.2	273.5 ± 58.1	254.0 ± 61.9

Data is presented as estimated marginal mean ± standard deviation, * indicates significance ($p \leq 0.05$) from test 1 at 1370 m (pre-trek). FMD = flow mediated dilation; nFMD = flow mediated dilation normalized for share rate, mm = millimeter; s = seconds.

lowlanders while at HA. The secondary finding was that NO-mediated dilation of the brachial artery was reduced during altitude exposure and after returning from HA.

4.1. Improved endothelial function after dietary nitrate supplementation

The application of NO_3^- rich BJ at 3700 m abolished the hypobaric hypoxia induced endothelial reduction seen with PL supplementation, and restored FMD to 1370 m pre-trek values (Fig. 2). The improved FMD after dietary NO_3^- supplementation at HA is in line with several studies conducted under normobaric conditions, of subjects with both normal and reduced baseline FMD [17–19,21]. To the authors knowledge this is the first study to describe this effect under hypobaric hypoxic conditions during an expedition with healthy lowlanders. Dietary NO_3^- supplementation may therefore represent a promising strategy for maintaining endothelial function in native lowlanders at HA.

The BJ dose used in this study was within the range of previous studies [18,19,28,29]. After application of 5 mmol NO_3^- (BJ) in this study, mean FMD increased by 1.5 ± 0.2% which is slightly more than a 0.5% increase in mean FMD found with smaller dose (2.94 mmol NO_3^-) in healthy subjects at sea level [19]. In addition, the peak FMD increased by 1.9 ± 1.0% in the present study, which is less than a 4% increase in peak FMD after application of a larger dose (12.45 mmol NO_3^-) in healthy subjects at sea level [18]. Thus, it might be inferred that the dose used in this study was above a potential threshold to elicit a detectable response, but too low to elicit a potential maximal response. However, it should be noted that even if there seems to be a dose dependent relationship of dietary NO_3^- supplementation on vascular function and plasma NO_2^- at sea level [14], it is not known whether such a dose–response relationship is evident at HA. The increased FMD found 3 h after NO_3^- supplementation may imply that this time frame is sufficient to induce detectable changes in endothelial function. This is also indirectly supported by other studies showing increased plasma NO_3^- and NO_2^- concentrations and cardiovascular changes 1,5- 3 h following NO_3^- supplementation [14,17,21].

It should be mentioned that dietary NO_3^- supplementation does not seem to always improve FMD [30]. However, the effect on endothelial function seems to be more prevalent in individuals already experiencing a dysfunction [21], as well as for protecting against ischemia induced dysfunction [17,31]. In the current study, subjects with normal FMD at baseline measure had reduced FMD within days of exposure to HA, implying that dietary NO_3^- supplementation may be effective for individuals showing reduced vascular function during HA exposure. In contrast to studies conducted at sea level [14–17], NO_3^- supplementation did not lower BP in our study. The lack of BP reduction despite the increased FMD might be related to less sensitivity of the BP method. While FMD is mostly dependent on NO-activity, BP is also affected by several other factors at altitude such as alteration of sympathetic activity and elevated circulating catecholamine [32] and endothelin levels [33].

BJ NO_3^- supplementation has become a popular strategy to improve exercise capacity at sea level [34] and lately there are studies that have addressed the effect of dietary BJ NO_3^- supplementation on exercise performance during hypobaric [35] -and normobaric hypoxia [36]. The latter study showed that NO_3^- supplementation did not enhance altitude running performance in well trained athletes. Whether the FMD response to NO_3^- supplementation at altitude is dependent on exercise level remains unclear, and should be addressed in future studies.

4.1.1. Mechanism of improved FMD after BJ supplementation

4.1.1.1. Endothelial function. The increased FMD after dietary NO_3^- supplementation is likely related to increased plasma NO_3^- and NO_2^- concentrations [17]. Hypoxic conditions decreases NO synthesis from L-arginine [8–10], which likely occurred in our subject at HA, while the $NO_3^- – NO_2^-$ –NO pathway is likely facilitated at low O_2 tension levels [9]. After ingestion of dietary NO_3^-, the conversion NO_2^- to NO occurs through a variety of mechanisms including eNOS and xanthine oxidoreductase mediated reduction of NO_2^- to NO [16,37]. In addition, deoxyhaemoglobin and deoxymyoglobin [9,11,13] have been shown to be important facilitators for the reduction of dietary NO_3^-. The decreased hemoglobin oxygenation at HA (SaO_2 84.9% at 4200 m in the present study) increases the content of deoxyhaemoglobin, thereby likely facilitating the reduction of NO_3^- to NO_2^- [11]. In sum, during hypoxic conditions dietary NO_3^- supplementation is optimized due to increased reduction of dietary NO_3^- to NO [7,9], and the increased NO availability for smooth muscle action likely results in vasodilation [9,23].

4.1.1.2. Stimulus differences. The second potential mechanism for FMD increase after BJ supplementation is an altered FMD shear stimulus. However, cuff occlusion was identical for both supplementations. In addition, there were no differences in baseline or peak blood flow, or shear rate, but there was a trend of increased flow stimulus ratio following BJ supplementation compared to PL. BJ may affect blood flow [20,30,38], therefore acting as a potential difference for FMD stimulus. As urine color, indicating hydration status, was not analyzed both test days, any differences in hydration may have affected the FMD result in addition to a potential effect of BJ on vascular volume.

4.2. Endothelial function and high altitude exposure

The reduced FMD during altitude exposure found in this study is in line with earlier reports of altered FMD during both short (hours) exposure to normobaric hypoxia [39,40] and longer (days) exposure to HA [41]. However, in previous findings, subject groups displaying endothelial dysfunction were those previously determined to be vulnerable to HA [39–41]. Therefore a novel finding in this study is that FMD is reduced in healthy lowlanders at HA. Moreover, the subjects of our study displayed a FMD at 1370 m that was comparable to age-matched lowlanders at sea level [24]. In the literature there is there is no data directly comparable to our results, but interestingly it was shown that FMD in metabolic syndrome patients was reduced from 7.4% to 3.8% after 3 weeks at 1700 m [41]. However, it is not known if disease or altitude specific mechanisms could be involved in this reduction.

The decreased FMD measured at HA was apparent after descending from HA (Test 5), suggesting a loss of endothelial function. The recovery time for normal NO availability is unknown, and whether HA creates long term endothelial damage initiating development of cardiovascular disease, or if damages recover after complete rehydration or some months at lowland [41], should be investigated in long-term studies. In long-term studies, various cardiovascular parameters should also be taken into account, since it is known that long-term HA exposure is associated with prolonged elevated sympathetic activity [42] and transient changes of cardiac mass, function and energy metabolism [43] after return to sea level. Additionally, it should also be noted that levels of circulating vasoactive nitrogen oxides have been shown to increase during acclimatization to HA [5,6], and it remains to be investigated how this relates to endothelial function in the long-term in native lowlanders.

4.2.1. Mechanisms for reduced FMD with altitude exposure

The positive effect of dietary NO_3^- supplementation on FMD at HA (Test 2) supports the possibility of an underlying imbalance of

vasoconstrictors and dilatators as discussed above [20]. Although not addressed in this study, there are other mechanisms that may have contributed to reduced endothelial dilation, including erythropoietin (EPO), up-regulation of the vasoconstrictor endothelin, and antioxidant status [40,44]. A factor that plays a critical role in a number of genetic responses controlling physiological effects in response to HA is the O_2-sensitive transcription factor hypoxia-inducible factor HIF-1α [45]. The role of HIF-1α in the decreased FMD seen at 4200 m in conjunction with the significant reduction in SaO_2 is unclear, but Janocha et al. [6] reported that a fall in oxyhemoglobin saturation was consistent with an increase in HIF upregulated proteins, endothelin-1 and EPO. In Tibetan highlanders, low levels of hemoglobin are reported to be associated with HIF-2α expression [46], and increased forearm blood flow [4]. Since the same population also exhibits high levels of circulating bioactive NO products [4], NO related mechanisms seems to play an important role in adaption to HA.

Another possible mechanism for reduced FMD may be differences in blood flow. It could have been that the combination of altitude induced diuresis and altitude induced erythropoiesis may have changed blood flow properties [41]. However, no significant changes in velocity of blood flow during rest were found in this study. This is similar to previous findings for velocity of blood flow, however is in contrast to volumetric blood flow ($mL \cdot min^{-1}$) that is reported as decreased in the brachial artery at HA, compared to both measurements at sea level and at 1310 m [1,47].

5. Study strengths and limitations

The double-blinded randomized controlled crossover design is a key in the overall strength of this study. With this design the bias was reduced, as each subject served as his or her own control. This was especially important due to the high variability in response to altitude and supplement interventions. Due to the field study design a strong external validity is present, while some limitations exist in experimental design. A limitation of this study is lack of 12 h complete fasting prior to the FMD measurements, but with subjects serving as their own control, this effect is limited. Furthermore overall modifications in diet (versus pre-expedition) were not taken into account, which could have effects on FMD [22]. Another limitation of this field study is the lack of blood plasma samples that were not obtained due to logistical reasons. This could have enabled a correlation analysis of FMD values with plasma NO_3^- and NO_2^- levels. Finally, since baseline measurements were not conducted at sea level prior to the expedition, the present study did not evaluate if FMD at 1370 m in Kathmandu would differ from sea level values.

6. Conclusion

This study showed that acute dietary NO_3^- supplementation abolished the reduction in endothelial function found at HA in healthy subjects. Dietary NO_3^- supplementation may therefore represent a promising strategy for maintaining endothelial function in native lowlanders at HA. It should however be noted that since the reduction in FMD was found at 4200 m 2–3 days after NO_3^- supplementation, that the duration of the positive effects from BJ supplementation in HA remains unclear. Since FMD after returning from four weeks of HA was reduced, potential prolonged effects on endothelial function from long-term HA exposure deserves further investigation.

6.1. Clinical implications

The results from this study imply a reduced capacity for peripheral blood flow regulation in healthy native lowlanders at HA which may impact cardiovascular health during and after HA exposure. The potential clinical implications of continued reduction in vascular function at altitude and upon descent should be further investigated. Ingestion of NO_3^- rich vegetables may represent a low-cost dietary strategy to improve vascular function for altitude dwellers.

Funding source

The Department of Circulation and Medical Imaging, K. G Jebsen Center for Exercise in Medicine, the Global Health Program at the Faculty of Medicine at NTNU, and the Swedish Centre for Research in Sports (CIF).

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

The authors would like to thank the volunteers for participating in the study despite straining conditions. We would also like to thank the guides and staff of Rolwaling Excusions for the arrangements and support while in Nepal, Dr Arjun Karki at Patan Academy, Nepal for his kind help to set up the study.

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