Emily Bakker

Is dietary nitrate supplementation necessary to ensure proper endothelial function at altitude?

Master's thesis in Exercise Physiology and Sport Science

Trondheim, May 2013

Supervisors: Post doctors Svein Erik Gaustad and Trine Karlsen

Norwegian University of Science and Technology
Faculty of Medicine
Department of Circulation and Medical Imaging

NTNU – Trondheim
Norwegian University of
Science and Technology

ABSTRACT

Introduction: At altitude the body is exposed to systemic hypobaric hypoxia and adapts in order to optimize oxygen delivery to the tissue. Maintaining peripheral vascular function is essential in this process, tightly regulated through complex pathways involving local endothelium derived factors. Nitric oxide (NO) is the most important player in regulation of endothelial tone via endothelium dependent vasodilation. Dietary nitrate (NO_3^-) supplementation has been shown to increase NO bioavailability and improve vascular functions during normoxia, however the effects during hypobaric hypoxia are unknown. **Purpose:** To study the endothelial function in lowlanders in response to 1) administration of NO_3^-supplementation via beetroot juice (BJ) at altitude, and 2) short term and 4-week altitude exposure. **Methods:** Endothelial function was measured as flow-mediated dilation (FMD) using ultrasound and Doppler in the brachial artery of 11 healthy subjects (4 female, 24.7±5.0 years, outdoor management students) sojourning from sea level to altitude. In a randomized, double-blinded crossover design FMD measure was performed 3 hours after drinking BJ (5.0 mmol NO_3^-) and placebo (0.003 mmol NO_3^-) supplementation at 3700m (after 3 days above 2000m), with a 24-hour wash out period. FMD was also measured at low altitude (1370m), after 5 days at altitude (4200m) and upon return to 1370m after 4 weeks of altitude exposure (2825 -5330m). **Results:** The supplementation intervention was completed by 10 subjects, and the 4-week altitude stay by 8 subjects. FMD was 6.53±2.32% at low altitude (1370m) (mean±SD). At 3700m with PL supplementation FMD was 3.84±1.31% ($p=0.004$) and with BJ supplementation 5.77±1.14% ($p=1.00$). FMD was lower at 4200m (FMD 3.04±2.22%), and 1370m post-altitude exposure (FMD 3.91±2.58%), compared to baseline FMD at 1370m (mean±SD, $p<0.05$). **Conclusion**: Acute dietary nitrate supplementation may reverse the reduced endothelial function found in lowlanders at 3700m after 3 days of altitude exposure. FMD decreased 5 days into altitude exposure (at 4200m), and after a 4-week stay between 2825 -5330m compared to FMD baseline.

Key words: high altitude, FMD, dietary nitrate supplementation, hypobaric hypoxia, vascular function, nitric oxide

Preface

This master's thesis is a part of a Masters in Exercise Physiology and Sport Science, at the Norwegian University of Science and Technology, Trondheim.

This thesis would not have been possible without the help of support from others. I greatly appreciate the time and dedication of my supervisors Post doctors Svein Eirk Gaustad and Trine Karlsen. I would like to thank Professor Ulrik Wisløff and the Cardiac Exercise Research Group (CERG) whose contributions made this project possible. I would also like to thank post doctor Arnt Erik Tjønna for help in the learning of FMD measurement and analysis technique.

As this project was in co-operation with Mid-Sweden University I would like to thank Professor Erika Schagatay, for accepting me into this project, and PhD candidate Harald Engan, for his support along the way.

To all the students who participated and/ or helped with this project, the contribution of your time was irreplaceable.

Emily Bakker
May, 2014

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Abbreviations

AD: Artery diameter

AMS: Acute mountain sickness

BF: Blood flow

BP: Blood pressure

BJ: Beetroot juice

Ca^{2+}: Calcium molecule

cGMP: Cyclic guanosine monophosphate

DBP: Diastolic blood pressure

ECG: Electrocardiography

eNOS: Endothelial nitric oxide synthase

FMD: Flow-mediated dilation

HA: High altitude

HAPE: High altitude pulmonary edema

HAPE-S: High altitude pulmonary edema susceptible

HR: Heart rate

mmHg: Millimeter mercury

O_2: Oxygen

MAP: Mean arterial pressure

NO: Nitric oxide

NO_2^-: Nitrite

NO_3^-: Nitrate

NOS: Nitric oxide synthase

PL: Placebo

PO_2: Partial pressure of oxygen

ROS: Reactive oxygen species

SaO_2: Arterial oxygen saturation

SBP: Systolic blood pressure

Definitions

Altitude

Moderate Altitude: 1500-2500m

High Altitude: 2500-3500m

Very High Altitude: 3500-5800m

Extreme Altitude >5800m

Hypobaric hypoxia

When reduction in partial pressure of oxygen is due to a reduction in ambient barometric pressure with oxygen fraction constant

Normobaric hypoxia

When reduction in partial pressure of oxygen is due to reduction in oxygen fraction (e.g. % oxygen content) with ambient barometric pressure constant

Normoxia

When oxygen availability is near to that of sea level, supplying the physiologically adequate amounts of oxygen for normal cell function and activity throughout the body

Systemic hypoxia

When blood oxygen levels are reduced because of decreased oxygen intake, rather than a disruption of blood flow

Vascular Function

The regulation of vascular tone to enhance blood flow and distribution, based on local and neural factors, in order to optimize delivery of oxygen and nutrients and removal of waste at the tissue

1 INTRODUCTION

Throughout the world, high altitude regions (HA) (>2500m) receive millions of visitors for recreation, commercial, military purposes and are home to about 140 million permanent dwellers [1]. Exposure to the hypoxic environment of HA can result in severe medical complications due to reduction in oxygen (O_2) availability. When ascending to HA, barometric pressure decreases, resulting in corresponding reductions in the partial pressure of oxygen (PO_2). As the barometric pressure at sea level is 760 millimeters mercury (mmHg), with 21% O_2 content, PO_2 is 160 mmHg. The reduction in barometric pressure at HA causes inadequate O_2 delivery to the tissues, a state known as hypobaric hypoxia (21% O_2, decreased PO_2, at 3500m barometric pressure is 505 mmHg, 21% O_2, PO_2 is 106 mmHg). As physiological processes are dependent on adequate O_2 delivery, reduced PO_2 poses the greatest challenge for the body at HA [2].

The individual physiological responses and overall tolerance to HA is highly variable and it is still an enigma why some people are HA prone while others are not [3]. Up to 50% of individuals walking to above 4000m (using more than 5 days) develop symptoms of acute mountain sickness (AMS) including headache, nausea, insomnia, and anorexia [4, 5]. Although AMS is usually self-limiting, its incidence is unpredictable, pathogenesis is elusive and it is generally not foreseeable using physiological measures [2, 5].

Reduced PO_2 at HA induces physiological changes necessary for proper function, a process known as acclimatization. Physiologically the body acclimatizes at HA in order to optimize O_2 delivery to tissues, and changes in vascular tone occur due to increased sympathetic drive and local endothelium-derived factors [6]. Nitric oxide (NO) is the most important factor for the regulation of vascular tone via endothelium dependent vasodilation. In order to optimize O_2 delivery to the tissue, maintenance of vascular system function is vital. Vascular changes related to blood flow (BF) and vessel diameter play important roles in physiologic adaptation to the HA environment [7].

1.1 Vascular function

The vascular system, consisting of blood vessels throughout the body, is an active organ functioning to maintain circulatory homeostasis, and to transport O_2 and nutrients to the tissue and remove waste products [8, 9]. Biomechanical mediators including cytokines, hormones, neurotransmitters, biomechanical forces generated by BF and blood pressure (BP) control the functions of this system [8]. It is the ability of the vascular system to respond to these signals that regulates vascular tone, which effects BP, BF, and blood distribution. A key component for this process is the endothelium [10].

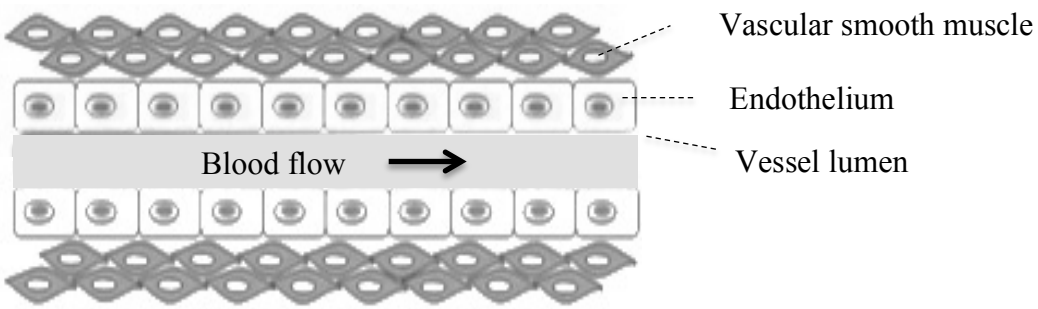

Figure 1. *Model of the vascular system components including the vascular smooth muscle (outmost layer), the endothelial cells making up the endothelium, the vessel lumen and the blood flowing within. Adapted from* [11].

1.1.1 Endothelial function

The endothelium consists of a monolayer of cells lining all blood vessels, situated between the vessel lumen and vascular smooth muscle (Figure 1) [11, 12]. A healthy endothelium regulates vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and vessel wall inflammation in response to various chemical and physical signals, including shear stress, via regulatory signals [13-16]. Although many regulatory signals exist, including endothelial-derived hyperpolarizing factor, vasodilatory prostaglandins and calcium molecules (Ca^{2+}), the most important is nitric oxide (NO) [15, 16].

With a healthy vascular system, blood vessels respond to increases in BF by endothelial dependent dilation that is primarily mediated by NO, termed flow-mediated dilation [10]. Endothelial dysfunction, with reduced endothelium dependent vasodilation through a depressed NO production, has been identified as a hallmark of many cardiovascular diseases including

hypertension, congestive heart failure, diabetes, and atherosclerosis, as well as High Altitude Pulmonary Edema (HAPE) [11, 12, 15, 17].

1.1.2 Assessment of vascular function

Although there are currently no measurement methods for vascular function that result in a definitive and complete assessment of vascular health [18], endothelium function measurements assess a crucial component of the vascular system. Based on dilatory response to shear stress, a technique for assessment of vascular function through the function of the endothelium was developed in 1992 [19]. It is now known as flow-mediated dilation technique (FMD), is considered the *'gold standard'* for non-invasive investigation endothelial function [14], and is predictive of cardiovascular events [14, 16, 20].

FMD method operates through measuring the brachial artery diameter (AD) before and after 5 minutes cuff occlusion [14]. The increased flow and shear stress that follows cuff occlusion results in vasodilation of the artery due to endothelial derived factors [21]. Age and gender specific FMD mean values range from 6.7% (women, 20-29 years) to 3.5% (women, 70-79 years) [22]. Endothelial dysfunction has been defined as FMD $\leq$ 0% [22]. Although FMD of the upper arm is a measure of peripheral conduit artery function, it has also been shown to be highly associated with pulmonary endothelial function including pulmonary artery pressure and pulmonary vascular resistance [23].

1.2 Endothelial function at high altitude

When exposed to systemic hypoxia, such as at HA, vascular adjustments depend on both activation of the sympathetic nervous system [24] and vasoactive substances from the endothelium [6, 25, 26]. In healthy individuals during short exposure to hypoxia (up to 3 hours at 4800 to 9000m), the balance between sympathetic and local endothelial produced factors typically results in net vasodilation in the peripheral vasculature [6, 24-26], whereas longer exposure (3 days or more at 3470 to 5330m) results in vasoconstriction [7]. Under hypoxia cerebral BF increases are between 0 and 33% [5, 27], while the pulmonary circulation displays

marked vasoconstriction (followed by increased vascular resistance) a mechanism from fetal development [28-31]. These responses are exaggerated in individuals vulnerable to HA complications. Overall, there is no general consensus in the literature on how HA effects the endothelial function and vascular tone. Some report vasoconstriction and others dilation due to variation in the study design (population, type of hypoxia, time of hypoxia) and measuring location (pulmonary, cerebral, peripheral vasculature). This thesis will focus on the peripheral vasculature, as a clear interpretation may be vital in understanding the role of the vascular system in adaptation to HA and health at HA.

1.2.1 Short exposure to hypoxia

In general, most studies have used normobaric hypoxia to examine the vascular response to short term exposure (up to 3 hours) to hypoxia and a NO-mediated vasodilation is reported by some, but not all, literature. Under normobaric hypoxia (PO_2=50mmHg) a vasodilation effect has been reported as 20-25% increases in peripheral BF (forearm BF baseline of 2.4 at normoxia to 3.0 $mL \cdot 100mL^{-1} \cdot min^{-1}$) [24, 26], reduced arterial stiffness (6% decrease in augmentation index), vasodilation [32] and decreased vascular resistance [33]. By comparing NO pathway blocked and normal vessel dilation under normobaric hypoxia, the conclusion drawn was that normobaric hypoxia invokes a NO-mediated vasodilatation in the peripheral vessels at simulated altitude where PO_2= 50mmHg [26].

Despite these literature reports NO-mediated vasodilation was not apparent while examining brachial arterial diameter (AD) or FMD. After 5 minutes of exposure to normobaric hypoxia (12.5% O_2 simulating 4500 m) no changes in AD and reductions in FMD were found (~6% decrease in ratio of FMD to nitroglycerin mediated dilation under hypoxia) in healthy controls [34]. To my knowledge this is the only short term exposure to hypoxia study that measured FMD in healthy individuals. The authors suggested the reduction in FMD was due to reduced bioavailability of NO, as demonstrated in intermittent hypoxia studies on animals (1 minute 5% O_2 hypoxia, 4 minutes normoxia 12 hr/day and normoxia 12hr/day for 14 days) [35]. There is no agreement within the literature on whether there is a NO based vasodilation or reduced NO bioavailability with short-term exposure to normobaric hypoxia.

1.2.2 Longer exposure to hypoxia

Upon longer exposure (3 or more days) to a systemic hypoxia, such as HA, sympathetic nervous system stimulation is increased, and the peripheral vessels tend to constrict [36]. Increases in BP are reported with a 5% increase in mean arterial pressure (MAP) after 3 days at 4559m, and 28% after 9 weeks at 5260m 5 [37, 38]. In limbs, systolic BP (SBP) is reported to increase by 14% in legs while not in the arms with ascent from sea level to 4100m [36], and BF in the brachial artery was 40% lower in healthy individuals at 4370m compared to sea level controls [39].

During ascent, from 1300m to 5300m over 25 days, conduit vessel flow and diameter in the brachial, common femoral, superficial femoral, and deep femoral arteries showed a decreased volumetric BF (25-45%), with a concurrent decreased vessel diameter (8-11%) when compared to sea level [7]. This vasoconstriction was maintained 24 hours after descent to lower altitude (1310m), although BF increased upon descent. At HA hypoxic stimulation of the kidneys leads to natruiresis and diuresis, and in combination with fluid loss from high respiratory rates, there is a potential of BF decrease due to reduction in plasma volume [40].

Although there are several studies that assess BP, BF, and AD, there are no studies that measure FMD during a HA expedition in healthy individuals. The closest corresponding study was performed on metabolic syndrome patients where after 3 weeks exposure to altitude (1700m) a significant reduction in FMD result (3.8% versus 7.4% baseline) and decreased baseline AD (4.3mm versus 4.5 mm baseline) were reported [41]. Upon return to lowland, FMD continued to be reduced from baseline values after 6 weeks, but recovered after 10 weeks at lowland.

1.2.3 High altitude complication vulnerable individuals

Individuals susceptible to HA complications may have an exaggerated vasoconstriction response to hypoxia [28], making physiological responses easier to observe in individuals prone to HAPE. Systemic vasculature in HAPE susceptible (HAPE-S) subjects have been studied during normoxia exercise and the conclusion drawn was that these subjects had an augmented flow-dependent vasoconstriction [42-44]. In 2005, Berger et al. measured forearm BF response to substances that induced endothelial dependent and independent vasodilation during normoxia and

normobaric hypoxia (12% O_2, corresponding to 4500m) in HAPE-S and healthy subjects. The authors reported attenuated vascular response to acetylcholine (induces endothelium dependent dilation) in HAPE-S subjects during hypoxia, as a result of decreased bioavailability of NO, and suggested that susceptibility to HA complications may partially be explained by endothelial function [17]. Numerous studies have reported a decrease in both exhaled and plasma NO products in HAPE victims compared to healthy subjects at altitude [17, 45-47].

1.3 Nitric oxide and endothelial function

In order to maintain cardiovascular homeostasis, the endothelium responds to various chemical and mechanical signals to adjust vascular tone. NO is the most important of these signals and acts to promote endothelium-dependent vasodilation via smooth muscle relaxation, decrease the vasoconstriction effect of Ca^{2+}, and reduce sympathetic outflow [12]. The production pathway and supply of NO becomes noteworthy at HA, where the vasodilation effects of NO could be advantageous in maintaining O_2 delivery to the tissues.

1.4 Nitric oxide production

Humans produce NO in the body through two mechanisms, the L-arginine pathway and the nitrate-nitrite-nitric oxide (NO_3^-- NO_2^-- NO) pathway. NO acts by stimulating the soluble gaunyltl cyclase, increasing concentrations of cyclic Guanosine Monophosphate (cGMP); which further acts on smooth muscle, causing relaxation and subsequent artery dilation [48]. NO is biologically unstable and is converted through oxidation for transport in the blood and tissues [49]. In sea level conditions, roughly half of plasma NO is L-arginine derived and half is from dietary sources [50]. The most important dietary source of NO_3^- is vegetables, accounting for approximately 80% [51]. When muscle is exposed to severe hypoxia (both acute ischemia, 1 hour, and chronic exposure to 1% and 5% O_2 for 24 hours) the L-arginine pathway NO production is inhibited, whereas the generation of NO from the NO_3^-- NO_2^-- NO pathway is enhanced [48, 52, 53].

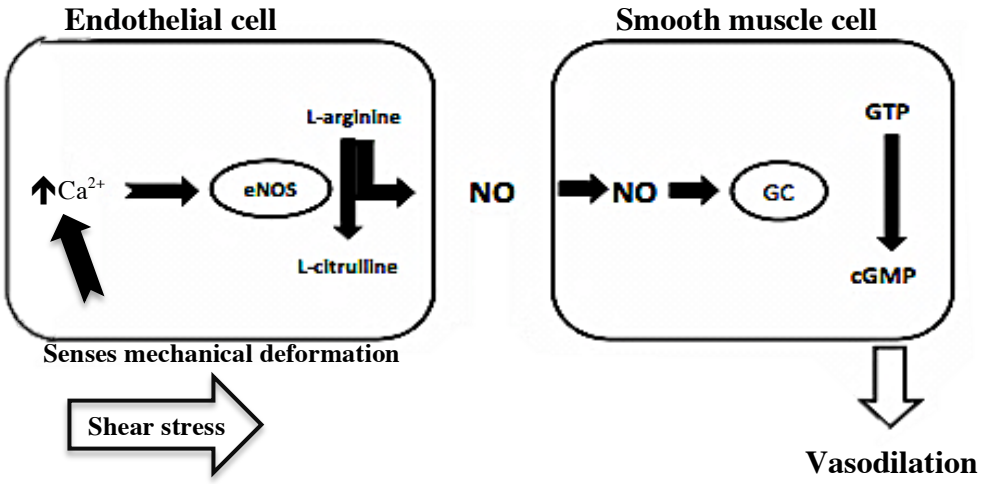

Figure 2. *Schematic of the L-arginine pathway stimulation, by shear stress or increased intercellular calcium concentrations, signalling nitric oxide production. Nitric oxide further acts on cyclic guanosine monophosphate and results in vasodilation. Where* $Ca^{2+:}$ *calcium, cGMP: cyclic guanosine monophosphate, eNOS: endothelial nitric oxide synthase, GC: guanylate cyclase GTP: guanosine triphosphate, NO: nitric oxide, adapted from [12, 16].*

1.4.1 L-arginine pathway

The most thoroughly described NO producing mechanism is the L-arginine-NO pathway; an O_2 dependent process that requires nitric oxide synthase (NOS) and L-arginine to produce NO and citrulline [54]. In relation to vascular function, endothelial NOS (eNOS) is recognized as having the important role in regulating blood vessel diameter [12]. NO production via eNOS occurs from the vascular endothelium [49]. This pathway increases NO production when stimulated directly by Ca^{2+} or shear stress, which increases intracellular Ca^{2+} concentrations (Figure 2) [8, 55].

The L-arginine pathway is altered in a hypoxic state and has a decreased ability to generate NO, due to the need for O_2 as a substrate [49, 56-58]. In hypoxia there is decreased eNOS mRNA transcription and altered eNOS function and hence, eNOS production [13]. This subsequently leads to a diminished NO production [59-61]. Demonstrated in animal tissue *in vitro* a reduction in tissue PO_2 from 150 to 40mmHg decreased NO production 52% [62], and in trabecular smooth muscle and cell culture *in vitro* a graded relationship has been described, where decreasing PO_2 corresponded to decreasing NO production [63, 64]. When entering an O_2 depleted environment, such as HA, the need for sufficient O_2 delivery to the tissues, along with a decreased ability to produce NO for vasodilation through the L-arginine pathway, renders the NO_3^-- NO_2^-- NO

pathway increasingly important [13].

1.4.2 Nitrate-nitrite-nitric oxide pathway

As NO is a biologically unstable molecule NO_3^- and nitrite (NO_2^-), oxidized NO, are measurable compounds found in the blood and tissue [49]. Since the mid-80's NO_3^- and NO_2^- have been known to be vasodilators and cytoprotectors [50]. Despite the reduced NO production from the L-arginine pathway during decreased O_2 availability, there is increased generation and consumption of NO_3^- and NO_2^- as demonstrated by measurement in brachial arteries during hypoxia and ischemic hearts [65, 66]. Due to increased reduction of NO_3^- to NO_2^- during hypoxia, NO_3^-- NO_2^-- NO pathway is optimized in a hypoxic state [49, 50].

Both supplementation, such as sodium NO_2^- [67], and whole foods, such as root vegetables [51], are effective exogenous sources of NO. To describe the circulation of inorganic NO_3^- and NO_2^- from whole foods the entero-salivary pathway can be used [49]. Beginning in the oral cavity, up to 25% of NO_3^- is reduced to NO_2^- in the saliva [49, 68]. The NO_3^- and NO_2^- absorbed during this cycle can be found in the blood stream and tissues and subsequently reduced to NO when needed to act as vasodilator via cGMP [49].

1.5 Nitric oxide at high altitude

NO is highly reactive with other substances including reactive oxygen species (ROS). Mitochondrial production of ROS is inversely related to O_2 availability, as demonstrated in endothelial cells *in vitro* [69] and microvascular system *in vivo* [70]; with decreasing PO_2, ROS production increases. These ROS react with NO to form peroxynitrite and act to reduce NO availability [70].

It is the combination of the increased NO breakdown by ROS and the reduction of NO from the L-arginine pathway under hypoxia that may decrease NO availability at HA. It should be noted that despite a molecular mechanism, the effect of hypoxia exposure on whole body NO substrates is unclear [17, 71-73]. It has been demonstrated that individuals with higher circulating NO

substrate levels are healthy and have better function at HA (NO_2^- 116 versus 103 $nmol \cdot L^{-1}$ for control and HAPE-S subjects respectably) [17, 74]. Increasing NO availability using L-arginine infusion, in combination with NOS injection, is reported to improved SaO_2 (5.3% 15 to 45 minutes post infusion) and AMS score, without affecting BP, in healthy subjects (36 hours of exposure to 4350m) [75]. Other vasodilators are also shown to be important at HA, where pharmalogicals [76], as well as inhaled nitric oxide [77, 78] have been effective in treating HA illnesses.

1.6 Nitric oxide supplementation

Following the NO_3^-- NO_2^-- NO pathway, organic low cost vegetables can provide NO for vasodilation. Certain foods have been found to have particularly high concentrations (>250mg/100g) of inorganic NO_3^- including beetroot and spinach [51]. Beetroot juice (BJ) has been shown to be effective in the reduction of BP at sea level (10.4±3.0 mm Hg SBP, 8.1±2.1 mmHg diastolic BP (DBP) with 22.5 mmol NO_3^- intake) [79-82]. Dietary NO_3^- has been effective in improvement of endothelial function at low altitude, with 0.5 to 4% increases in FMD after acute supplementation [83, 84]. BJ specifically has demonstrated a vasoprotective role, protecting FMD function during ischemia [82], and improves FMD after the intake of a high fat meal (containing 56.6 g fat) [85].

During normoxic hypoxia BJ has been effective in improvement of SaO_2 and exercise tolerance [73, 86]. After multiple doses of BJ (5 applications of 1 mmol NO_3^- dose per day, for 6 day) or control, 15 healthy subjects (males, mean 21 years) were exposed to short term (2 hour) normobaric hypoxia (11% O_2) [86]. During hypoxia resting SaO_2 was approximately 77% without BJ and improved 3.5% with BJ, and during exercise SaO_2 was 68% with the control juice and improved by 2.7% with BJ. A single dose of BJ (9.3 mmol NO_3^-) 24 hours prior to 14.5% O_2 normoxic hypoxia is shown to restore exercise tolerance to normoxia levels (477 seconds BJ hypoxia, 393 placebo (PL) hypoxia, 471 seconds normoxia) in 10 healthy (2 female, mean 28 years) subjects [73].

Although vasodilators have demonstrated a positive effect on some HA illnesses and endothelial

function at low altitude, the effect of dietary NO_3^- supplementation on endothelial function at HA has not been studied before.

2 PURPOSE

In this study, we investigated the effects of dietary NO_3^- supplementation on endothelial function during hypobaric hypoxia in lowlanders. To gain a greater understanding of the vascular modifications in lowlanders during hypobaric hypoxia, we investigated the significance of the endothelium in this process. The specific aims of this study were:

1) To assess the effect of drinking BJ as a dietary NO_3^- supplementation on FMD, an estimate of NO-mediated vasodilation, at HA.

2) To investigate the effect of continued HA exposure on FMD, through measuring FMD pre-ascent to HA to create an altitude baseline measure, at HA (4200m, after 5 days at HA), and after 4 weeks at HA.

It was hypothesized that 1) at 3700m of altitude dietary NO_3^- supplementation via ingested BJ will ensure a HA FMD comparable to the norm in lowland natives at sea level (age 20-29 males: 5.4%, females 6.7%), and 2) continued exposure to HA (at 4200m, after 5 days at HA) will decrease FMD in relation to altitude baseline, as measured before ascent to HA, and FMD will continue to be reduced after 4 weeks in HA (2825 -5330m).

3 METHOD AND MATHERIALS

This study was performed during the spring of 2013 in Kathmandu and the Rolwaling Valley, Nepal. It was ethically approved by Swedish Research Council and Nepal Health Research Council and done according to the Helsinki declaration.

3.1 Subjects

Subjects were students of the Outdoor and Adventure Management program in Åre, Sweden, and read and signed the informed consent before participating in the study. A total of 11 healthy male and female lowlanders participated in this study, whereof 10 subjects participated in the BJ supplementation study due to logistical reasons. With regards to the FMD over time one subject's pre-expedition measure was excluded because of non-compliance to pre-measurement restrictions. All 11 subjects were included at altitude. There were 3 subjects that did not complete the expedition and, therefore, had insufficient stay at altitude. These 3 subjects were excluded from the post-expedition measurement, making a total of 8 subjects part of post-expedition measurements. Subject demographics data is displayed in Table 1.

Table 1. Subject demographic data (n=11, male=7, female=4).

	Age (years)	Height (m)	Weight (kg)
Mean±SD	24.7±5.0	1.76±9.78	71.6±9.92

3.2 Study timeline

FMD was measured 5 times at 3 different altitudes within 39 days of this expedition. This timeline, with respect to the altitude the subject resided at and measurement days, is demonstrated on Figure 3. Most movement between elevations occurred by walking, except transportation from Kathmandu to the beginning of the trek (1525m) by bus (day 3). The group was unable to climb over a high pass into Khumbu Valley due to weather constraints and, therefore, made a brief return to lower altitude in Kathmandu by bus (stayed days 21-23) and

subsequently traveled to and from Khumbu Valley by plane (Figure 3, days 24, 38) where they resumed the expedition.

Altitude baseline measure was taken at 1370m (Test 1) on the first day of the study. The BJ supplementation aspect of this study took place on days 7 and 8 at 3700m (Test 2), after the subjects had spent 3 days walking from 1525m to 3700m and 1 day residing at 3700m before the investigation. The altitude testing took place at 4200m (Test 3) on day 10, after 5 days above 2500m. At this point, the group formed smaller groups, some returning to Kathmandu, some subjects continuing on to climb mountains (6119 m) and others continuing to trek between altitudes (2825 -5330m). Therefore, each subject's exposure to altitude varied from day 12 to 38, with most sleeping at altitudes between 2900m and 5330m. Post-HA measures were performed 1 day after return to 1370m (Test 4) on day 39.

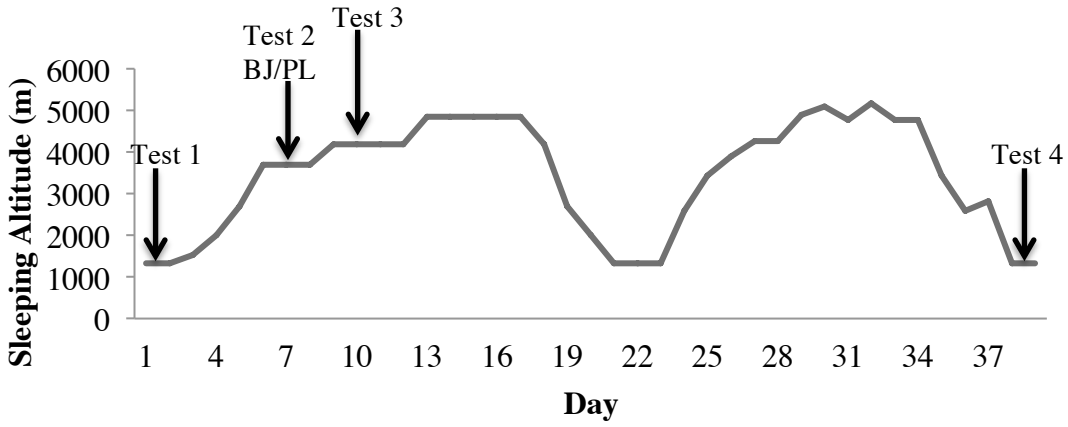

Figure 3. *Residing altitude (m) profile example in relation to days of the study and including testing days; Test 1: FMD altitude baseline values (1370m), Test 2: Nitrate supplementation (3700m) BJ (5.0 mmol NO_3^-) or placebo (PL) juice (0.003 mmol NO_3^-), Test 3: FMD at altitude (4200m), Test 4: FMD 1-day after 4 weeks altitude expedition (1370m).*

3.3 Testing and measurements

In this study, FMD was performed in the brachial artery using a 12-MHz Doppler probe and ultrasound imaging (*Vivid* I, GE Healthcare, USA) following current guidelines [10, 87]. All tests were performed in the same manner following a standardized procedure (Figure 4). Each test consisted of FMD technique to estimate NO-mediated vasodilation, as well as recording of heart rate (HR), BP, and SaO_2 as to be described below.

3.3.1 Subject preparation

As FMD is a sensitive technique to obtain a reliable measure of endothelial function there were several subject-specific factors taking into account. These included temperature, fasting state, caffeine, tobacco, exercise and pre-measurement rest for acclimatization [87]. Although this was a fieldwork study, these factors were optimized through the following methods. A combination of wood ovens, propane heaters and down sleeping bags were used to control ambient and subjects temperature (BJ/PL test, room temperature 20°±2 C). This is because low ambient temperature cools the body, activating sympathetic outflow, resulting in peripheral vasoconstriction and reduced BF, with corresponding reductions in FMD [88].

Subject were instructed to restrain from food, caffeine, or tobacco intake 3 hours and exercise 2 hours prior to measurements, as these factors also affect FMD results [87]. With respect to dietary NO_3^- supplementation subjects were advised to avoid mouthwash and tooth brushing that day in order not to wash out lingual bacteria important for NO_3^- reduction [89]. Prior to measurement, subjects were questioned about their compliance to study restrictions.

3.3.2 Procedure

Preparation for FMD of the brachial artery included removal of arm-restrictive clothing, the placement of an occlusion cuff distal to the measuring site and electrocardiography (ECG) electrodes. ECG electrodes were place on the skin, one on each shoulder and left chest (mid clavicular line of the 5^{th} intercostal space) [22]. The measuring site for all FMD measures was above the anticubital fossa, with the arm extended [22]. An automatic BP cuff was placed on the dominant arm, and the non-dominant arm was prepared for FMD measurement as to reduce the effect of the occlusion during the BP measurement on the baseline FMD recorded. Once the subject and measurement equipment was prepared (2-3 minutes), the subjects were requested to relax in a supine position, avoiding moving and talking, and the 10-minute FMD rest period began.

After 5 minutes of supine rest, 3 BP and HR measurements were taken on 1-minute intervals using an automatic BP cuff. During the entire vascular occlusion and reactive hyperemia phases

of the FMD measurement SaO_2 was recorded and HR was monitored on 1-minute intervals, to a total of 8 recordings. This was performed manually using a pulse oximeter (OnyxVantage, Nonin Medical, Plymouth, USA) that had been placed on a finger of the dominant hand.

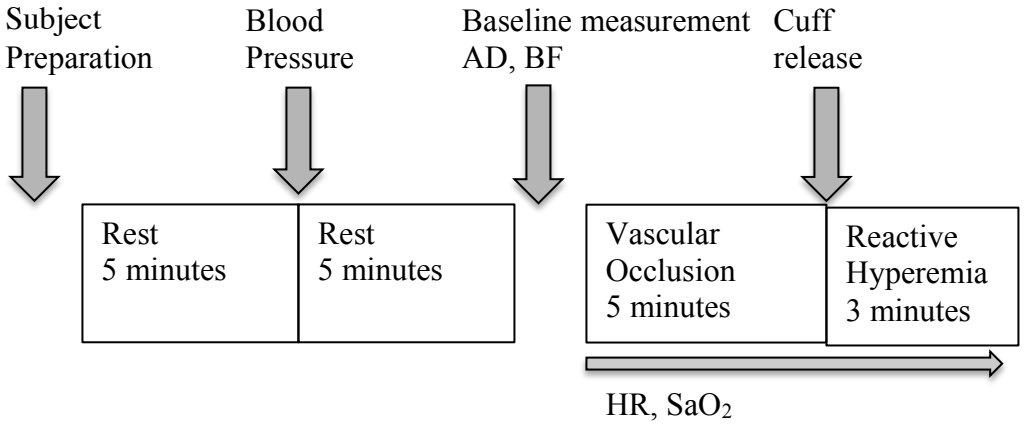

Figure 4. *Timeline for measurement procedure; beginning with subject preparations, followed by a resting period with blood pressure measurement. After 10 minutes total resting baseline measurements of artery diameter (AD) and blood flow (BF) were taken, followed by a 5 minutes vascular occlusion, cuff was released and reactive hyperemia ensued. During vascular occlusion and reactive hyperemia, heart rate (HR) and arterial oxygen saturation (SaO_2) were recorded every minute.*

3.3.3 Flow-mediated dilation procedure

After 10 minutes total supine resting time the FMD measurement began, while the subject continued to lie supine with the non-dominant arm extended. As FMD compares peak dilation to resting status, resting status measurement was assessed before occlusion and considered the baseline. Before image acquisition, a vessel image with clear upper and lower interfaces between the vessel wall and the intima was obtained [90]. The brachial AD image was saved in order to obtain a baseline artery status, and baseline blood velocity was recorded using Doppler, as this is important in relation to shear stress [87].

Once suitable baseline measures were recorded, a manual sphygmomanometer cuff (SC10, D.E. Hokanson Co., Bellevue, USA) was inflated to > 250mmHg on the forearm. This was to create an occlusion distal to the measuring site (Figure 5), as when the cuff is placed distally the result best reflects NO-mediated dilation [20, 21]. Time of occlusion has been shown to affect FMD result, and for consistency and subject comfort a 250mmHg cuff occlusion for 5 minutes was

performed as recommended by the literature [87]. The forearm occlusion restricts BF to the hand and upon release BF increases, causing shear stress, the stimulus for the resultant dilation [91].

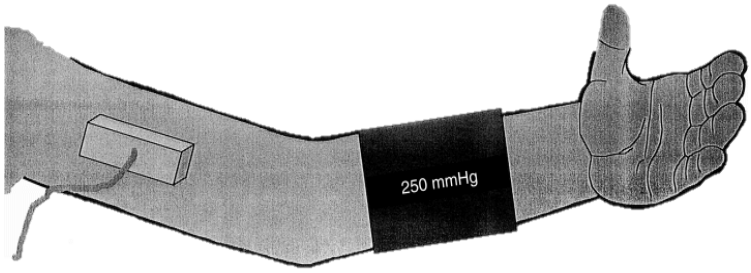

Figure 5. *Drawing displaying occlusion cuff, and ultrasound probe position on non-dominant arm, adapted from [10].*

The cuff was released abruptly, BF increased and the reactive hyperemia phase followed. Using Doppler, the resultant peak in BF was recorded within 10 seconds of cuff release, and used to estimate shear rate. The ultrasound device used in this study did not have duplex mode, therefore, total shear rate could not be calculated [87, 91]. In order to follow the vasodilation of the artery that was resultant of the increased BF and shear stress, the AD image was recorded for 3 minutes on 30-second intervals (30, 60, 90, 120, 150, 180 seconds post-occlusion). The peak AD post occlusion typically occurs at 50±11seconds for young individuals [92], and a minimum 2 minutes post-occlusion of AD assessment is recommended [87]. The amount of dilation, as shown by increase in AD post-occlusion is NO mediated and reflective of NO levels [21].

3.3.4 Beetroot/placebo juice supplementation protocol

In a blinded randomized-cross over manner, subjects drank a *Beet-it* juice (J. White Drinks Ltd., Suffolk, UK) shot (70mL) containing either BJ (5.0 mmol NO_3^-) or PL juice (0.003 mmol NO_3^-) on two consecutive days. Randomization of supplementation was done by creating two containers with paper slips; the slips in one container contained each subject number and the other containing an equal number of slips referring to supplement type, half written BJ and half PL. A name was drawn from one container and a supplement type from the other.

The subjects drank the supplementation in a pre-determined random order on 30-minute intervals, as this was the time needed to complete each measurement procedure. The FMD measurement

period commenced 3 hours after supplementation. The 3-hour schedule was chosen based on previous literature, in order to assess FMD when the concentration of NO products in the body peaks [79, 82]. As FMD is a reflection of NO levels, and the NO pre-cursor NO_3^- was the only component different between the two supplementations, the greatest differences were apparent at this time. There was a 24-hour washout period between doses, as to minimize the affect of the previous supplementation on the next. This time-period has been shown to render the plasma NO products differences between BJ and PL insignificant [82].

3.3.5 Dehydration scoring

At HA, there is a high potential for dehydration as hypoxic stimulation of the kidneys leads to natruiresis and diuresis, with additional fluid loss from high respiratory rates [40]. Mild dehydration has been demonstrated to reduced endothelial function and FMD result [93]. The subjects self-assessed dehydration on specific mornings, upon waking. This was done using a urine chart as shown in Appendix I, and a white 60 mL measuring cup. The score recorded from a value of 1 very pale yellow to 8, brown yellow. Urine color has been shown as closely correlated with urine osmolality, and an acceptable measure in field studies [94].

3.4 Data analysis

The principle investigator performed all FMD data collections and analysis to decrease observer bias. The principle investigator was blinded from the intervention until after the analysis process was completed. The analysis of AD (intima to intima) was performed using caliper measurement on the ultrasound image (0.1mm resolution) (Figure 6). For each HR cycle 3 repetitions of AD analysis were performed, and 3 HR cycles for each time point (for example 30 seconds post cuff occlusion). The mean of these 9 AD measurements was calculated to represent the AD of the time point.

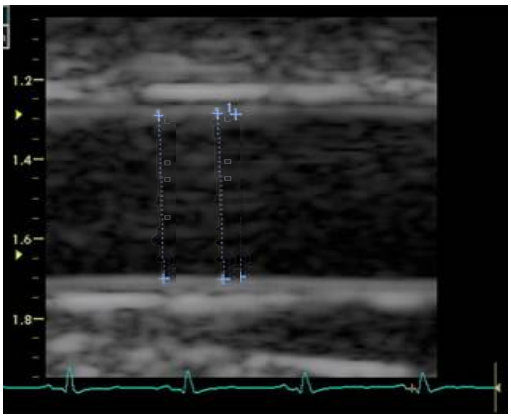

Figure 6. *A sample image of the brachial artery, as seen during ultrasound imaging process. This image includes three measurement calipers (intima to intima) and ECG recording of HR cycle.*

With respect to measurement timing, the peak of the ECG R wave of the cardiac cycle was used to reduce error due cyclic changes in AD, as indicated in the literature [95]. Resultant AD was the average of 9 measures for every time point (3 measures per cardiac cycle, 3 cycles per time point). Both baseline and peak BF ($mm \cdot s^{-1}$) were estimated from Doppler waveform for velocity. As time course of artery dilation varies for individual subjects, each subjects peak AD post occlusion was chosen individually rather than the traditional 60 second time point [92]. FMD and shear rate were calculated from AD and BF as shown by the equations below [96].

$$\text{FMD (\%)} = \frac{\text{(post-occlusion artery diameter (mm)-baseline artery diameter (mm))}}{\text{baseline artery diameter (mm)}} \cdot 100 \qquad \textit{(eq. 1)}$$

$$\text{Shear rate } \left(s^{-1}\right) = \frac{\text{peak blood flow } (mm \cdot s^{-1})}{\text{peak artery diameter (mm)}} \qquad \textit{(eq. 2)}$$

$$\text{nFMD (\%} \cdot \text{s)} = \frac{\text{FMD (\%)}}{\text{shear rate } (s^{-1})} \qquad \textit{(eq. 3)}$$

$$\text{Mean FMD} = \frac{\sum \text{FMD (\%) 30 to 180 seconds}}{\text{number of timepoints}} \qquad \textit{(eq. 4)}$$

$$\text{Stimulus ratio} = \frac{\text{peak BF } (mm \cdot s^{-1})}{\text{baseline BF } (mm \cdot s^{-1})} \qquad \textit{(eq. 5)}$$

The FMD (%) for all time points were calculated by post occlusion AD minus baseline AD, divided by baseline AD (*eq. 1).* All FMD (%) used in the results section were the highest calculated FMD (%), computed using peak post occlusion AD. As blood viscosity was not measured, shear rate (s^{-1}) was calculated by dividing peak velocity by peak AD (*eq. 2*). To account for mechanical stress, FMD was normalized (nFMD) with a resultant FMD to shear

stress ratio (*eq. 3)*. The mean FMD was calculated as the sum of the FMD (%) from 30 seconds to 180 seconds post-occlusion divided by the total number of time points (*eq. 4*) [84]. The flow stimulus the artery received during reactive hyperemia is expressed as the ratio of peak BF to baseline BF *(eq. 5)* [82].

An average of the two closest values were used for calculation of BP and HR, while for SaO_2 calculations average of the 8-recorded values was used. MAP was calculated from DBP and SBP *(eq. 6)*.

$$\text{MAP (mmHg)} = \frac{2\text{DBP (mmHg)} + \text{SBP (mmHg)}}{3} \qquad \textit{(eq. 6)}$$

3.4.1 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 Student's t-test was used to compare means of BJ and PL.

In tests 1, 3 and 4 there were some missing data points because not all subjects partook in all testing days (as described above) and equipment error, as the SaO_2 occasionally did not register on the pulse oximeter. This resulted in longitudinal unbalance data, with time as a cofactor, suited for Linear Mixed Models analysis [97].

Linear Mixed Models creates a subject-specific mean trajectory over time based on population characteristics and subject-specific effects. Using these predicted individual subject curves, this model estimates missing values to create an estimated marginal mean. Therefore, Liner Mixed Models with Compound Symmetry using pairwise comparison of estimated marginal means with Bonferroni correction was used for comparison of Test 1, 3, and 4.

For all tests significance was set to $p< 0.05$ and a trend denoted a $p<0.1$.

4 RESULTS

4.1 Beetroot juice supplementation

FMD

BJ supplementation at 3700m significantly increased FMD to 5.77±1.14% in comparison to PL supplementation FMD at 3.84±1.31% ($p<0.01$) (Figure 7A). Similar increases from BJ supplementation were found examining nFMD, where nFMD was 0.023±0.009 %·s with BJ and a lower value of 0.015±0.004 %·s with PL ($p<0.01$)(Figure 7B). Mean FMD was increased by 1.46±01.81% with BJ supplementation, where BJ mean FMD was 2.91±2.11% and PL was 1.51±1.78% ($p<0.01$). Results of the dietary NO_3^- supplementation are displayed in Table 2 and graphically in Figure 7 (mean±SD, $p<0.01$).

Blood flow

The flow stimulus tended ($p<0.1$) to be 15.8±24.2% larger during BJ supplementation (Table 3). No differences were observed comparing BJ and PL with respect to baseline BF, peak BF, or shear rate (Table 2).

Artery diameter

No differences were observed comparing BJ and PL with respect to baseline AD or peak AD (Table 3).

Basic physiological measures

Basic physiological measures (HR, SaO_2, SBP, DBP, MAP) displayed no significant differences (Table 3).

Table 2. Vascular variables during FMD measurement post-supplementation, dietary NO_3^- PL and BJ.

	PL	BJ
Baseline AD (mm)	3.86±0.56	3.84±0.62
Peak AD (mm)	4.00±0.57	4.06±0.63
Baseline BF ($mm \cdot s^{-1}$)	808.2±169.0	743.4±159.5
Peak BF ($mm \cdot s^{-1}$)	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 is presented as mean±standard deviation, PL: placebo, BJ: Beetroot juice, AD: artery diameter, BF: blood flow

Table 3. Cardiovascular variables during post-supplementation, dietary NO_3^- PL and BJ.

	PL	BJ
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 is presented as mean±standard deviation, PL: placebo, BJ: Beetroot juice, SaO_a: arterial oxygen saturation SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure.

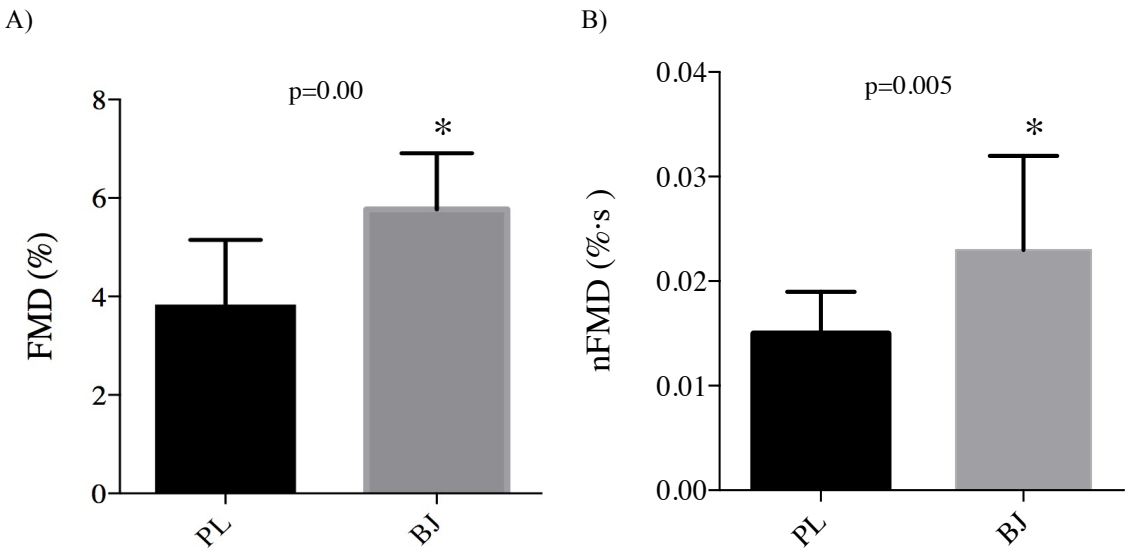

Figure 7. *Effect of beetroot juice supplementation (BJ, shown in grey) in comparison to placebo (PL, shown in black), on A) flow-mediated dilation (FMD), B) normalized flow-mediated dilation (nFMD). Values presented as mean±standard deviation. * indicates significant difference between the two supplementations (p<0.05).*

4.2 Endothelial function and high altitude exposure

FMD

The FMD was 6.53±2.32% at altitude baseline (Test 1, 1370m), and significantly reduced to 3.04±2.22% at HA ($p<0.01$, Test 3, 4200m) and was still reduced post expedition with 3.91±2.58% ($p<0.05$, Test 4, 1370m) (estimated marginal mean ±SD, Figure 8A). The nFMD reflected similar changes, as nFMD of Test 1 (1370m) 0.025±0.007%·s was significantly reduced at HA (Test 3, 4200m) to 0.011±0.007%·s ($p<0.01$) and displayed a trend of reduction ($p<0.1$) to 0.016±0.010%·s post-expedition (Test 4, 1370m) (Figure 8B). The individual variations in FMD are presented in Figure 9.

Blood flow

There was a trend of reduction ($p<0.1$) in baseline BF from altitude baseline of 773.1±177.6 $mm \cdot s^{-1}$ to post-expedition (Test 4, 1370m) of 644.3±192.2 $mm \cdot s^{-1}$ (Table 4). There were no significant differences in peak BF, stimulus ratio, or shear rate (Table 4).

Artery diameter

There were no significant differences in baseline AD or peak AD at any altitude (Table 4).

Basic physiological measures

The altitude baseline (Test 1) HR was 12±15% lower than the HA (Test 3) HR, and 14±18% higher than the post-expedition (Test 4) HR ($p<0.05$) (Table 2). HA HR (Test 3) was significantly higher than all other test points. SaO_2 at HA (Test 3) was 84.9±2.9%, a 12.4% reduction from all other locations, and post-expedition (Test 4). DBP and MAP were decreased in relation to HA (Test 3) value (Table 5). There were no significant differences in SBP.

Table 4. Vascular variables from FMD testing procedure.

	Test 1 (1370m) n=10	Test 3 (4200m) n=11	Test 4 (1370m) n=8
Baseline AD (mm)	3.78±0.56	3.72±0.56	3.67±0.57
Peak AD (mm)	4.02±0.56	3.83±0.55	3.82±0.57
Baseline BF ($mm \cdot s^{-1}$)	770.5±177.6	722.9± 172.0	644.6±192.2
Peak BF ($mm \cdot s^{-1}$)	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, analysed using linear mixed models, $p \leq 0.05$, * differ from Test 1, AD: artery diameter, BF: blood flow

Table 5. Cardiovascular variables from FMD testing procedure.

	Test 1 (1370m) n=10	Test 3 (4200m) n=11	Test 4 (1370m) n=8
Heart rate (bpm)	58±11†	65±11*	50±12*†
SaO_2 (%)	97.3±2.9†	84.9±2.9*	97.3±3.2†
SBP (mmHg)	118±12	118±12	111±13
DBP (mmHg)	70±11	73±11	66±11†
MAP (mmHg)	86±11	88±10	81±11†

Data is presented as mean±standard deviation, using linear mixed models, $p \leq 0.05$, * differ from Test 1, †differ from Test 3, SaO_a: arterial oxygen saturation, SBP: systolic blood pressure, DBP: diastolic blood pressure, MAP: mean arterial pressure

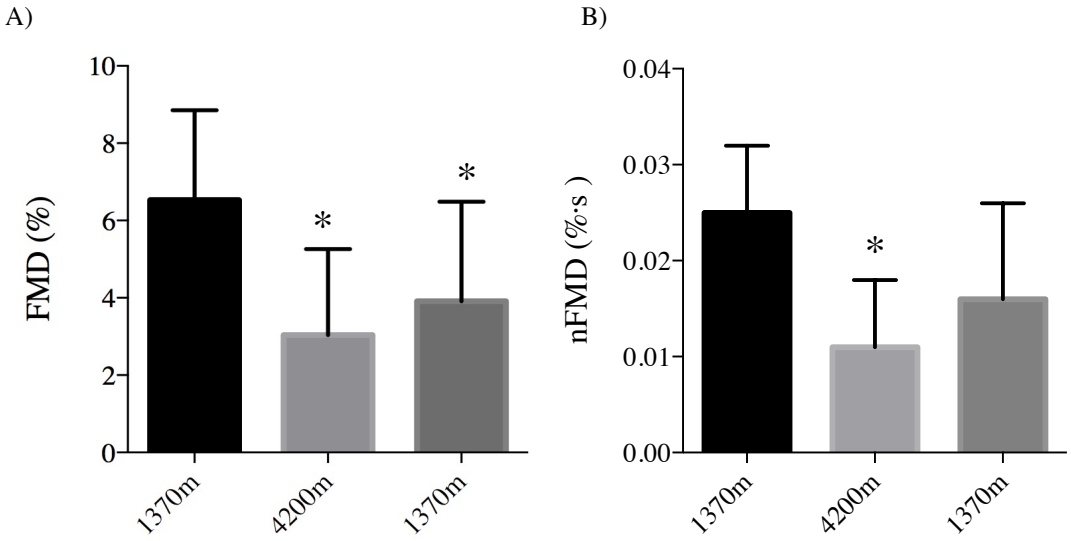

Figure 8. *Measurements based on FMD procedure at three time points, where the time points represent altitude baseline at 1370m (n=10), at altitude 4200m (n=11), and post high altitude exposure at 1370m (n=8). The measurements include A) flow-mediated dilation (FMD), B) normalized flow-mediated dilation (nFMD). Data presented as estimated marginal means±standard deviation.* indicates a significant difference from altitude baseline measurement (1370m) (p<0.05).*

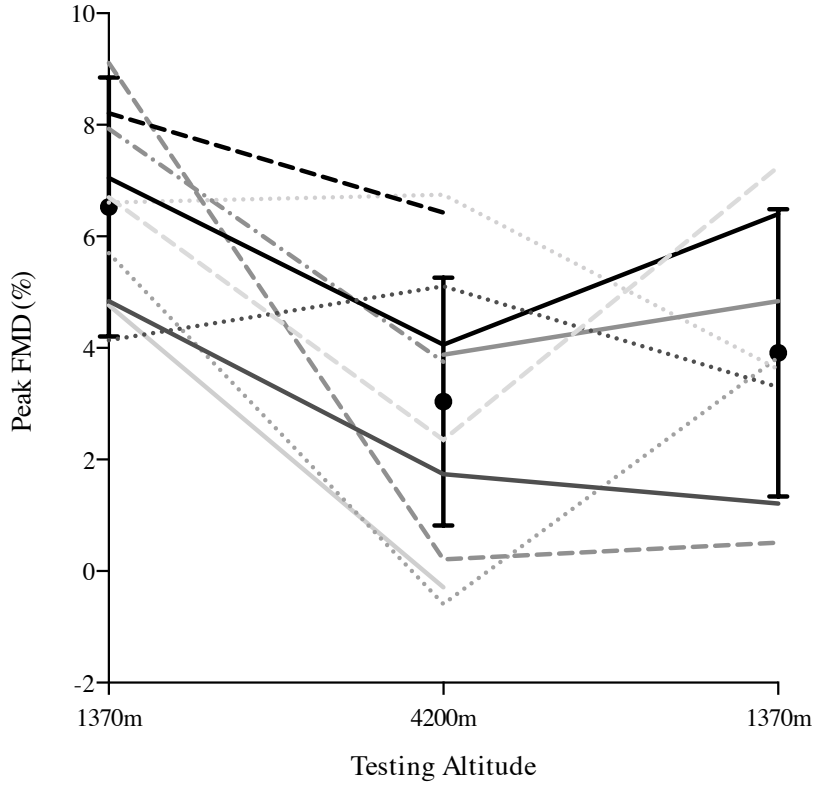

Figure 9. *Individual variation in FMD (%) at different altitudes (from pre-altitude measure at 1370m on the left, at altitude 4200m, post-altitude 1370m farthest right), lines represent individual subject data and black data marker represents estimated marginal means±standard deviation.*

4.3 Dehydration score

Mean dehydration score was not significantly different at any test location, whereas individual subject values ranged from 1 to 8. Dehydration scores were not complete for all testing days/locations. At 3700m (day 8), 4200m (day 10) and post-expedition (day 38) values were complete (Table 6).

Table 6. Dehydration scores at altitude and post expedition.

Day (altitude)	Day 8 (3700m)	Day 10 (4200m)	Day 38 (1370m)
	n=10	n=10	n=8
Dehydration score	3.4±2.0	3.8±2.0	3.8±2.2

Data is presented as estimated marginal mean ±standard deviation using linear mixed models

5 DISCUSSION

The principal finding of this investigation was that dietary NO_3^- supplementation with BJ restored FMD of the brachial artery, to pre-expedition altitude baseline level, by enhanced NO-mediated dilation while at altitude. The secondary finding was that NO-mediated dilation of the brachial artery was reduced during 4 weeks of HA exposure and after returning to moderate altitude.

5.1 Improved endothelial function after dietary nitrate supplementation

The application of NO_3^- rich BJ at 3700m increased FMD in comparison to PL supplementation. Supporting the hypothesis (1), the BJ FMD was not significantly reduced when compared to age matched lowlanders at low altitude (5.88% for population group made up of 7 men, 4 women in population group 20-29 years) [22] and similar to the altitude baseline FMD from this study (Test 1). The positive effect of dietary NO_3^- supplementation on FMD is in line with several previous studies at sea level [82-85], and to the authors knowledge this is the first study to describe this effect at altitude.

After application of 5 mmol NO_3^- in this study, the mean FMD increase of 1.46±0.24% can be compared to the smaller change, 0.5%, in mean FMD (over 4 minutes versus the 3 minutes in this study) found with smaller dose (2.94 mmol NO_3^-) at low altitude [84]. The FMD increase of 1.94±0.99% in this study can be compared to the larger increase, 4%, after application of a larger dose (12.45mmol NO_3^-) found at low altitude [83]. The distal occlusion FMD in this study resulted in a primarily NO-mediated dilation that reflects NO bioavailability [21]. This supports the suggested dose dependency of dietary NO_3^- supplementation on NO bioavailability at low altitude [79].

Most recent studies use 10 mmol NO_3^- when using BJ (corresponding to a double dose of BJ used in this study) which would cause a greater increase in NO availability and, therefore, FMD response [85]. The increased FMD found 3 hours post supplementation supports the 1.5-3 hours time needed for increasing plasma NO_3^- and NO_2^- after application of dietary NO_3^-

supplementation [79, 82, 85]. Combining the dose dependency and FMD as a reflection of NO availability, the proposal that NO_3^- is the significant component in the BJ supplementation is reinforced.

It should be mentioned that dietary NO_3^- supplementation does not seem to always improve FMD [98]. However, the effect on endothelial function seems to be more prevalent in individuals already experiencing a dysfunction ([85, 99]), as well as for protecting against ischemia induced dysfunction [82]. This implies that dietary NO_3^- supplementation may be effective for individuals showing reduced vascular function during altitude exposure, such as in this study where FMD was reduced at 4200m, compared to at 1370m. In addition, despite the improved FMD with dietary NO_3^-supplementation, an effect was not seen on other measurements. Dietary NO_3^- has been shown to increase SaO_2 (through improved peripheral oxygen efficiency) during 2-hour exposure to normobaric hypoxia (simulating 500m) [86]. This study differed in that the dietary NO_3^- was administered in multiple doses over 6 days, the time in hypoxia was much shorter (2 hours versus 3 days) and it was normobaric rather than hypobaric hypoxia.

5.1.1 Mechanism of improved endothelial function after dietary nitrate supplementation

5.1.1.1 Endothelial Function

The increased FMD after dietary NO_3^- supplementation is likely be related to NO bioavailability. The endothelium responds to increased BF, as experienced post distal cuff occlusion, with NO-mediated dilation [20, 21]. NO is formed through both the L-arginine and NO_3^-- NO_2^--NO pathway, and at low altitude derivation of NO is roughly half from each pathway [50]. In hypoxic conditions production of NO through the L-arginine-pathway, an O_2 dependent process [54], is likely decreased [49, 56-58]. This decreased L-arginine derived NO renders an imbalance in NO homeostasis at HA. Prior to dietary NO_3^- rich BJ supplementation, at HA, our subjects likely had decreased NO availability.

Although L-arginine production of NO may be impaired, dietary NO_3^- can supply NO through the NO_3^-- NO_2^- - NO pathway. Beginning in the mouth, ingested inorganic NO_3^- is rapidly

absorbed and metabolized to NO_2^- by salivary bacteria [49]. Further reduction of ingested NO_2^- to NO occurs through a variety of mechanisms including eNOS mediated reduction of NO_2^- to NO [81]. eNOS is a key enzyme in L-arginine pathway for production of NO (Figure 2). Although eNOS availability may be reduced at HA [13], eNOS reduction of dietary NO_3^- is 6 times more effective than from the L-arginine pathway [81]. In addition, deoxyhaemoglobin [49, 100] and deoxymyoglobin [101] have both been shown to be important for the reduction of dietary NO_3^-. At HA haemoglobin saturation decreases [102] (SaO_2 (to 84.9%) at 4200m) increasing the availability of deoxyhaemoglobin. This suggests a potential for increased reduction of ingested NO_3^- to NO_2^- at HA, regulated by the decreased haemoglobin oxygenation [100]. The overall increased reduction of dietary NO_3^- to NO optimizes the NO_3^-- NO_2^-- NO pathway in hypoxic conditions [49, 50]. In this study the intake of NO_3^- rich BJ supplementation likely increased previously reduced NO bioavailability.

Increased NO availability may affect endothelial function through several pathways. Primarily, increased bioavailability of NO can act as a vasodilator via cGMP for endothelium dilation and create smooth muscle relaxation (Figure 2) [103]. The increased FMD found after dietary NO_3^- supplementation supports the increased potential for endothelium dilation and smooth muscle relaxation. Increased availability of NO may also affect ROS production. This is important as ROS production is increased at HA [104, 105], and ROS further reduce NO supplies by reacting to form peroxynitrite [103]. Peroxynitrite is a powerful oxidant that damages molecules, proteins, DNA, and the endothelium [106]. This increased oxidative stress not only decreases NO availability, but ROS also increase vascular inflammation and leukocyte adhesion [70]. NO_3^- has been shown to inhibit mitochondria ROS production [107], attenuating ROS levels [70] and may in fact protect L-arginine production of NO [108]. The reduction of ROS by dietary NO_3^- may therefore act to increase FMD, as found after BJ supplementation in this study.

5.1.1.2 Stimulus Differences

The second potential mechanism for FMD increases after BJ supplementation is an altered FMD shear stimulus. However, cuff occlusion was identical for both supplementations (time, intensity, location), and there were no statistical differences in baseline BF, peak BF, or shear rate, but there was a trend of difference in flow stimulus ratio of BJ and PL. Supporting the greater

potential for increased flow (flow stimulus ratio), BJ has been shown to improve vascular compliance by increased BF [98, 103]. The larger flow stimulus for FMD during reactive hyperemia seen after BJ supplementation can result in larger dilation [10]. Despite the differences in flow stimulus with NO_3^- supplementation, the changes in nFMD, normalized for mechanical stress, reflected FMD changes. As urine color, indicating hydration status, was not analyzed both test days, the hydration status of the subjects may have affected the FMD result.

When comparing both BJ and PL vascular measures with all other testing days, it can be noted that the baseline AD of both PL and BJ (3.86±0.18, 3.84±0.20 respectively) tended larger than altitude baseline (Test 1 3.78±0.17) and significantly larger than at 4200m 3.72±0.17. This BJ/PL data does not fit the previously reported trend where AD decreases with increasing altitude, beginning as low as 1310m [7]. As AD was not measured before supplement application, whether or not this was affected by the supplementation can only be speculated. Both supplementations were made from beetroot, one NO_3^- depleted, and the red/purple pigment of beetroot is known as betalains [103]. Betalains, in combination with carotenoids and ascorbic acid contained in the beetroot render this juice (BJ and PL) with a high antioxidant capacity [109]. A 70mL concentrated beetroot supplementation, as used in this study, has been shown to increase total antioxidant capacity (98 μmol) [109]. These antioxidants act to balance ROS production under oxidative stress, such as at HA [103]. This increased antioxidant capacity may have influenced vascular homeostasis and acted to increase baseline AD.

5.2 Endothelial function at high altitude

The reduced FMD during altitude exposure found in this study is in line with previous reports of altered FMD during both short (hours) [17, 34] and longer (days) [41] exposure to hypoxia. In previous findings, subject groups displaying significant endothelial dysfunction were those previously determined to be vulnerable to HA including HAPE-S, HA natives with chronic mountain sickness [39], and individuals with cardiovascular and genetic risk factors for endothelial dysfunction [6, 34, 110]. A novel finding in this study is that longer exposure to HA alter FMD in lowlanders healthy at baseline while at altitude. The decrease in FMD is

comparable to reports from Frick et al. (2006) where FMD in metabolic syndrome patients was reduced from 7.4% to 3.8% after 3 weeks at 1700m [41].

The subject of this study displayed a baseline altitude FMD was comparable to age-matched lowlanders at low altitude [22]. Interestingly Frick et al. (2006) presented similar baseline FMD values metabolic syndrome patients, a disease where endothelial dysfunction is prevalent [111]. These patients were considered metabolic syndrome based on pre-set obesity, dyslipidemia, hyperglycemia, and hypertension criteria, and despite meeting these criteria this subject group did not display endothelial dysfunction. Approximately 10% of population 20-29 exhibits endothelial dysfunction, based on the dysfunction definition of FMD $\leq$ 0% [22]. The FMD of the subject group did not reach a FMD level considered dysfunction. Whereas, some individuals were on a level considered dysfunction, as shown by Figure 9.

5.2.1 Mechanisms for reduced endothelial function during altitude exposure

The mechanism for reduced endothelial response to the shear stimulus imposed during FMD technique could be related to NO bioavailability, and a possible underlying imbalance of vasoconstrictors and dilatators [103]. This potential imbalance is due to the alteration in endogenous production of NO through the L-arginine pathway and increased NO breakdown due to increased oxidative stress [105, 112], which decreases the bioavailability of NO at HA This study may corroborate that NO availability is in a graded relationship to O_2 availability, as has been described *in vitro* [63, 64]. With increases in altitude, decreases in FMD were found, 6.53±2.32% at 1370m, 3.84±1.31% at 3700m and 3.04±2.22% at 4200m. Reduced bioavailability of NO is further supported by the positive effect of the BJ supplementation, which increases the levels of NO products, has on FMD while at HA.

There are several other mechanisms, not addressed in this study, which may have contributed to the reduced endothelial dilation. As HA FMD was taken within 5 days of travelling above 2500m, the erythropoietin (EPO) production by the kidneys that occurs with hypoxia exposure peaks within 24-48hours (normalized with 3-weeks at altitude) may still have been increased [40]. EPO is a hormone produced by the kidneys that stimulates red blood cell production [113].

High doses (3000 units, a dose common in clinical practice) of infused EPO have been shown to impair endothelial dependent vasodilation in humans [114]. Although this rendered blood EPO approximately 30 times higher levels than seen after HA exposure (1 day at 3450m) [115], it cannot be ruled out that increased EPO levels could contribute to the impaired FMD in this study. In addition, hypoxic conditioned up-regulation of the vasoconstrictor endothelin [17, 116] may have contributed to the decreased FMD measured.

Another possible mechanism for reduced FMD is a result of possible differences in BF. The combination of altitude induced diuresis (decreasing water content) and altitude stimulated increased erythropoiesis (increasing red blood cells) may create more viscous blood and, therefore, change BF properties [40, 41]. No significant changes in velocity BF were found at in this study. This is similar to previous findings for velocity BF, on the contrary volumetric BF ($mL \cdot min^{-1}$) is reported as decreased in the brachial artery at HA, compared to both sea level and 1310m [7, 39]. The altitude baseline at 1370m, may have already have altered BF, AD, as reported previously [7]. The significant differences displayed also in nFMD (%·s) at HA, supports that it is not mechanical stress differences that were the reason for the FMD reductions.

5.3 Endothelial function upon descent

The decreased FMD measured at HA was apparent after descending from HA suggesting a loss of endothelial function that was persistent at low altitude. Despite the overall reductions in FMD, nFMD only trended as a reduction, indicating potential differences in mechanical stress. Baseline BF trended as decreased. Although there are no differences in urine color score post-expedition compared to any other testing point, the value of 3.8±0.7 indicates that the subjects were moderately dehydrated [94]. Taking into consideration the urine color score with the decreased BF, DBP, MAP and FMD, but not nFMD, the subjects were likely dehydrated.

Notably, Dumais et al. (2011) did not find significant changes in velocity BF upon descent. Volumetric BF ($mL \cdot min^{-1}$) was reported as increased, as compared to at 5330m, and suggested a relative hemodilution related to reversal of HA fluid shift. The current study was of longer duration with more time at altitude and may have created greater cumulative dehydration taking

more than 24 hours for recovery, although both studies reached similar altitudes and descended (by foot and plane) in a similar manner.

In addition to dehydration, there may have been alteration in endothelial response to shear stimulus. The sensitivity to endothelin, a powerful vasoconstrictor that may be unregulated at HA, is unknown. As well as possible depletion of antioxidants and NO production capacity of the L-arginine pathway after HA exposure. Considering the endothelial dysfunction present upon descent from HA found in this study, some clinical implications should be considered. Endothelial dysfunction the hallmark of many cardiovascular diseases [11, 12, 15, 17], and this study may imply a risk factor for lowland populations traveling at HA. Whether HA creates permanent endothelial damage initiating development of cardiovascular disease, or if damages recover after rehydration or some months at lowland [41], must therefore be investigated in long-term studies.

5.4 Endothelial function and health at high altitude

Populations adapted to HA living have demonstrated a better vascular function compared to lowlanders at 5050m, demonstrating a better ability to increase femoral BF after occlusion of the leg [117]. A pilot study measuring FMD (n=5) was conducted on local residents of Kathmandu (48.4±12.8 years) (Appendix II, Table 1, 2). FMD was measured to be 9.7±3.8 %. Although statistically not comparable, these individuals had a greater FMD than the group of much younger individuals from this study in Kathmandu (Appendix II, Figure 1). These Nepal locals also had a greater FMD when compared to age matched Scandinavian lowlanders, where in 40-49 year old males FMD peak was 4.3±3.8% [22]. The higher FMD may be due several factors including genetic and environmental factors (altering NO bioavailability [72]), diet (intake of dietary NO_3^- and NO_2^-) and EPO and ROS concentrations.

The Nepal locals had a lower baseline BF than the Caucasians in this study. This could be related to age differences as a 26% reduction limb BF has been previously reported in older (63 years) as compared to younger (28 years) males [118]. The combination of a lower baseline BF and a greater increase in flow resulted in a higher flow stimulus ratio in the Nepal locals, and greater

stimulus could be the reason for greater FMD [96]. Greater ability to increase femoral BF, after leg occlusion, has been reported in HA natives [117], supporting the differences in conduit artery function found in this study. Previous literature describes that among HA natives those with better vascular function had a higher SaO_2 and less symptoms of chronic mountain sickness [119].

Hypoxia triggers a set of physiological responses in order to regulate vascular tone [33]. Vasoconstriction in the peripheral circulation may be important to maintain arterial pressure and flow to the dilated cerebral and coronary vessels, but many HA complications are initiated by an exaggerated vascular response [120]. Improper regulation of NO may be responsible for the exaggerated vascular response of HA illnesses. NO is indicated to play a role in the HA headache (AMS) [5] and pulmonary vasoconstriction (HAPE) [121].

When lowlanders are exposed to HA, the body often responds by constriction in the peripheral and pulmonary circulation, reduced peripheral endothelial function, and dilation in the cerebral vessels, possibly a defense mechanism for survival in this hypoxic environment. Consuming a dietary NO_3^- supplementation at HA would increase bioavailability of NO throughout the body, along with the vascular potential for dilation. Although this may counteract the constriction in the pulmonary and peripheral systems, it may further increase dilation in the dilated cerebral vessels; the overall consequences on health at HA are unknown. In addition to the effects on the vascular system, NO may act as an important single molecule during adaptation to hypoxia, and NO supplementation may activate the physiological responses for acclimatization [71]. Dietary NO_3^- supplementation will manipulate NO metabolism, increasing NO availability, but whether or not this is a helpful at HA must be further addressed.

6 STUDY STRENGTHS AND LIMITATIONS

The randomized crossover design used is 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. On the contrary, the main limitation of this study is lack of 12 hours fasting prior to the FMD measurements. Furthermore modifications in diet were not taken into account. Change in diet including fat, antioxidant, NO_3^-, and NO_2^- intake may affect FMD [87]. An additional limitation in this study was the lack of baseline sea level measurement. The menstrual cycle of the females involved (n=4) was not followed, and may have been an additional factor affecting FMD [87]. The secondary aim of this study (FMD with relation to altitude exposure) was further limited by the missing data from some locations rendering a small sample size. A protocol was design to minimize environmental strains affecting FMD (including temperature [88], fluid status [7, 40] and physical activity [122]), yet we were dependent on self reports from subjects of adherence to this protocol.

7 CONCLUSION

This study shows that acute dietary NO_3^- supplementation during hypobaric hypoxia (at 3700m after 3 days of altitude exposure) restored FMD from the PL value of 3.84±1.31% to 5.77±1.14%. Dietary NO_3^- supplementation may therefore represent a promising strategy for maintaining endothelial function in native lowlanders at altitude. Additionally, FMD was reduced 5 days into exposure to hypobaric hypoxia (at 4200m), and FMD continued to be reduced after descent from a 4-week altitude stay (2825 - 5330m) when compared to FMD altitude baseline. Reductions in FMD may have resulted from a combination of decreased endothelial function and dehydration causing reduced shear stimulus. These results imply a reduced capacity for peripheral BF regulation at HA, that may impact HA health, and the potential clinical implications upon descent must be further investigated. We conclude that the impaired endothelial function found in native lowlanders whilst at altitude can be improved by the ingestion of inorganic NO_3^-.

7.1 Perspectives of dietary nitrate supplementation at high altitude

Medical complications at HA are not only worries of the lowlander traveling, but also are major health concerns in HA populations [19]. Dietary NO_3^-supplementation with beetroot could provide a low-cost treatment and prevention strategy to maintain healthy vascular function at HA, and possibly in prevention and treatment of some HA medical complications. The cold environment at HA does not make easy growing conditions for many vegetables, but beetroot and spinach are both viable in these climates and rich in inorganic NO_3^- [123]. Therefore, dietary NO_3^-supplementation may represent an affordable solution to reduce morbidity for hundreds of thousands of HA populations and improve the wellbeing of the HA traveler [124]. Further studies should focus on the hemodynamic and metabolic role of dietary NO_3^- supplementation at different altitudes and of native HA populations and lowlanders.

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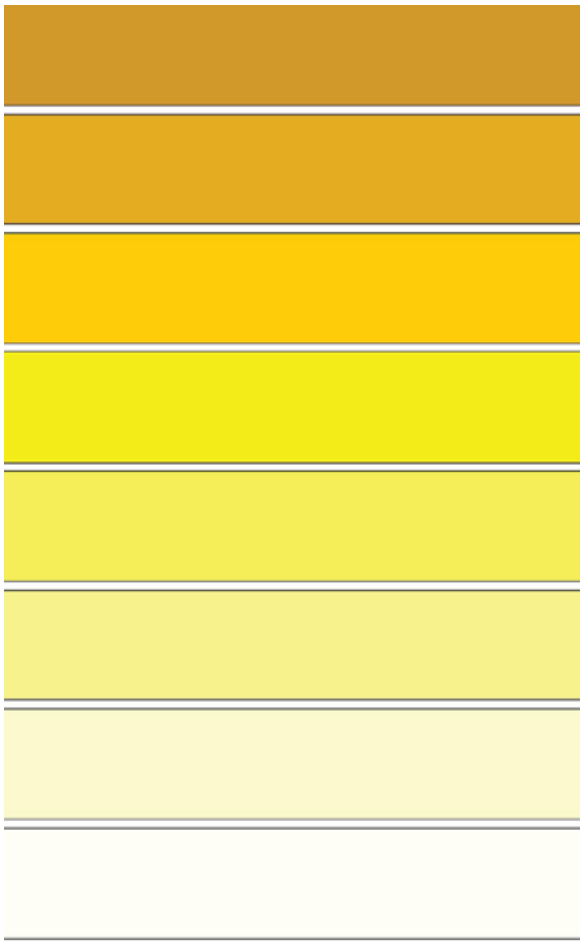
Appendix I

Urine color chart for dehydration assessment



Appendix II

Local Residents of Kathmandu, FMD measurement at 1370m

Table 1. Subject characteristics for endothelial function measurements in local residence of Kathmandu, male (n=5).

	Age (years)	Height (m)	Weight (kg)
Mean	48.4±12.8	1.54±0.06	51.1±5.4
Range	29-61	1.47-1.61	48.5-60.78

Data presented as mean±SD

Table 2. Cardiovascular parameters of local residence of Kathmandu taken during FMD measurement protocol at 1370m.

	Mean	Range
Baseline AD (mm)	3.79±0.40	3.37-4.33
Peak AD (mm)	4.14±0.40	3.63-4.65
baseline BF ($mm \cdot s^{-1}$)	596.7±150.8	453.2-837.3
peak BF ($mm \cdot s^{-1}$)	927.8±181.6	741.7-1098.1
stimulus ratio	1.60±0.31	1.23-1.94
shear rate (s^{-1})	223.6±18.2	201.3-245.1
nFMD (%·s)	0.042±0.015	0.030-0.070

Data presented as mean±SD, where AD: artery diameter, BF: blood flow FMD: flow-mediated dilation, nFMD: normalized flow-mediated dilation

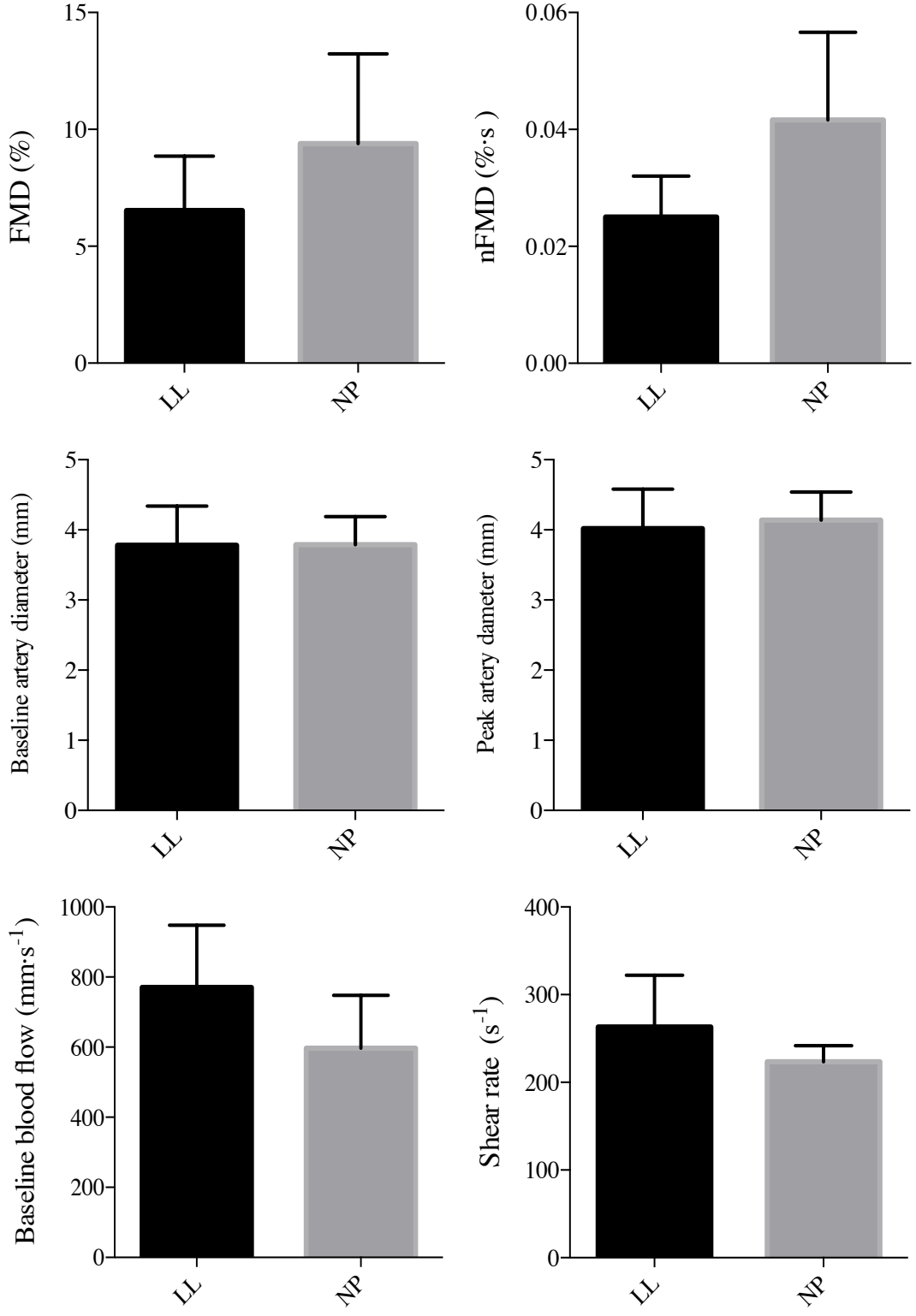


Figure 1. *Comparison of lowlanders (LL) (n=10) to Nepalese residence (NP) (n=5) measurements taken during FMD measurement procedure at 1370m, where black represents LL and grey represents NP. Data presented as mean±standard deviation.*

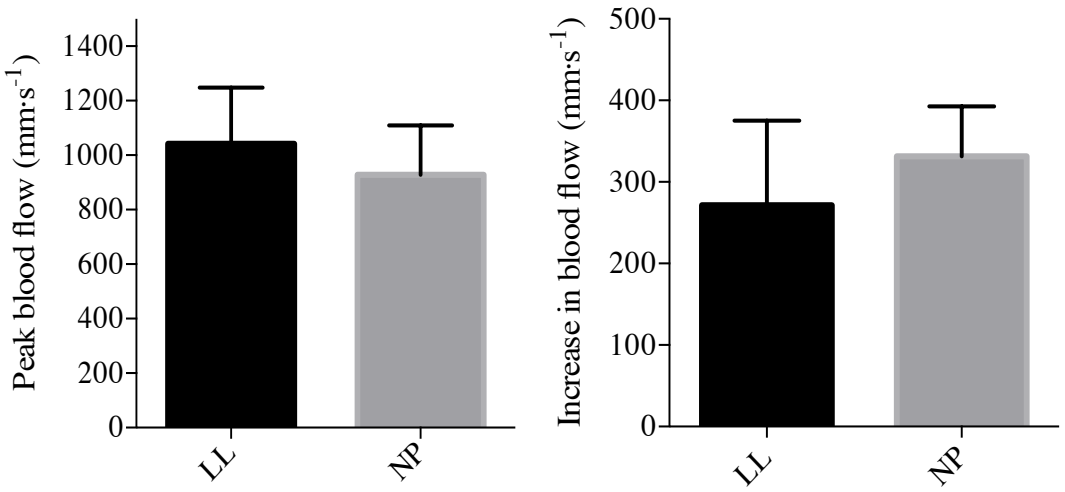


Figure 2. *Comparison of lowlanders (LL) (n=10) to Nepalese residence (NP) (n=5) measurements taken during FMD measurement procedure at 1370m, where black represents LL and grey represents NP. Data presented as mean±standard deviation*.