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Effects of Inorganic Nitrate Supplementation on Heart Rate and Ventilatory Responsiveness to Acute Hypoxia and Blood Pressure in Patients with Obstructive Sleep Apnea

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by

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A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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All requirements for graduation with Honors in the
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ABSTRACT

When individuals encounter periods of low oxygen, hypoxia, the peripheral chemoreflex works to restore saturation to normal levels. The peripheral chemoreceptors are responsible for sensing such decreases in oxygen and signaling consequent increases in ventilation and sympathetic-mediated cardiovascular (CV) responses. Aside from oxygen, other molecules like glucose, insulin, leptin, carbon dioxide, and nitric oxide (NO) have been shown to affect this reflex. NO, specifically, exhibits a reflex blunting effect demonstrated by various animal studies. Obstructive sleep apnea (OSA), a disease characterized by repeated mechanical occlusions of the upper airway during sleep, demonstrates both a pathophysiological sensitization of the peripheral chemoreflex and a noted decrease in bioavailable NO. This study's purpose was to analyze the effects of acute inorganic nitrate supplementation (beetroot juice), a dietary path for increasing bioavailable NO, on the heart rate and ventilatory response to acute hypoxia and brachial blood-pressure (BP) in subjects with OSA. We hypothesized that, inorganic nitrate would blunt chemoreflex responsiveness as observed through the ventilatory and cardiovascular (HR) biomarkers. The study followed a double-blind, randomized, placebo-controlled format in which 14 subjects with mild-to-moderate OSA, over the course of two study days, consumed both the placebo beetroot juice (BR_P) and the nitrate beetroot juice (BR_N;500mg inorganic-nitrate and 40mg nitrite). Peripheral chemoreflex responsiveness was analyzed as the absolute-change and the linear-regression against SpO_2 for HR and V_E. The ventilatory response was analyzed under two criteria: baseline to target 80% hypoxia (Bl-T) and baseline to peak response (Bl-P). The effects of BR_P and BR_N were analyzed as the comparison between the two bouts of acute hypoxia (pre-vs-post supplementation) conducted on each study day. The intervention successfully increased NO bioavailability (plasma-nitrate: pre:26±11μmol/L , post:292±90μmol/L, $p<0.01$; plasma-nitrite: pre:248±155nmol/L, post:738±427nmol/L, $p<0.01$). V_E responsiveness increased in the absolute-change Bl-P-BR_P (pre:3.1±1.2L/min, post:4.3±2.3L/min, $p=0.03$) but not BR_N (pre:4.3±2.6L/min, post:4.8±2.7L/min, $p=0.43$). The V_E Bl-T absolute-change ($p=0.11$), Bl-P linear-regression ($p=0.33$), and Bl-T linear-regression ($p=0.08$) also did not change. The HR response to hypoxia (linear-regression and absolute-change) was unaltered in either intervention ($p=0.12$, $p=0.54$ respectively). Systolic BP (SBP) increased under BR_P (pre:120±9, post:127±10, $p<0.01$), whereas the rise in SBP was not observed with BR_N (pre: 123±13 mmHg, post: 123±9 mmHg, $p=0.96$), DBP did not change in BR_P or BR_N ($p=0.08$). Holistically, these findings suggest no impact of nitrate supplementation on peripheral chemoreflex effectors (HR and ventilatory response) in OSA. Alternatively, the SBP response to nitrate supplementation may suggest inorganic nitrate can blunt diurnal increases in BP.

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TABLE OF CONTENTS

INTRODUCTION

Obstructive Sleep Apnea: Obstructive sleep apnea (OSA), an increasingly prevalent disease in the United States [1, 2], is typified by repeated mechanical occlusions (apneas) of the upper airway during nightly sleep. These sleep associated apneas sporadically inhibit ventilation leading to periodic bouts of low oxygen (hypoxia) and high CO_2 (hypercapnia) [3]. Consequentially, individuals with OSA report decreased sleep quality, and sleepiness during waking hours [4, 2]. Long term, OSA places individuals at an increased risk for work/motor vehicle accidents, depression, metabolic disorders and cardiovascular diseases (CVD): hypertension, arrythmias, and stroke [1, 3, 4, 2, 5]. Degrees of severity for this disease are scaled according to the apnea hypopnea index (AHI), a metric assessing the average per hour number of apneas (ventilatory occlusions lasting more than 10 seconds) and hypopneas (periods of decreased airflow which lead to an arterial drop in O_2 saturation $\geq$3% (or periods of decreased airflow terminated by neural excitation)) during sleep [3]. Mild, moderate, and severe OSA are defined as an AHI of 5-14, 15-29, and 30+ events/hour, respectively [6]. Three major factors influence OSA susceptibility: increased collapsibility of the upper airway, decreased function of pharyngeal dilators, and irregular ventilation control [7]. Collapsibility is dictated by both the skeletal and soft tissue factors which define the length, diameter, and shape of the airway [1, 8]. In particular, longer, smaller and certain airway shapes increase one's risk for OSA. For these reasons, Asian (craniofacial structure) and obese (high adiposity) populations show and increased prevalence of OSA [9].

OSA and the Peripheral Chemoreflex: Hypoxia plays a significant role in the pathophysiology of OSA. Naturally, the body detects and resolves hypoxic disturbance through the carotid (i.e., peripheral) chemoreceptors and physiological effectors of the peripheral chemoreflex [4]. More specifically, effectors work to increase bioavailable oxygen by means of pulmonary and cardiovascular influence (increased respiratory rate (RR), tidal volume (V_T), heart rate (HR), and blood pressure (BP)) signaled by increased sympathetic activity and parasympathetic withdrawal [4, 10]. Of these physiological adjustments, the ventilatory changes are often referred to as the hypoxic ventilatory response, a common marker for peripheral chemoreflex activity/sensitivity. In subjects with OSA, peripheral chemoreflex sensitivity is potentiated [4]. This may be a consequence of the frequent activation of the reflex during nightly hypoxic apneas and the corresponding increased presence of reactive oxygen species (ROS) [2]. Additionally, the apneas themselves prevent ventilatory thoracic stretch afferents from inhibiting sympathetic outflow leading to unmitigated effector signaling [3]. In general, the overactivity of the peripheral chemoreflex in OSA may play a role in OSA's deleterious pathophysiology [11].

Nitric Oxide and OSA: Nitric oxide (NO), first discovered in 1987, serves as a vital molecule in both bronchodilation and vasodilation [12]. Endogenously mediated by a class of nitric oxide synthase enzymes (NOS), NO is produced from the aerobic metabolism of L-Arginine [12, 13]. On the vascular level, endothelial NOS (eNOS/NOS III) produces NO when acetylcholine signaling drives an increase in intracellular calcium stabilizing NOS's allosteric activator calmodulin [12]. Shear stress on endothelial cells can also increase intracellular calcium through action on ion channels, integrins, and G protein-linked receptors [14]. The other NOSs have roles in the nervous system (nNOS) and immune response (iNOS) [12]. OSA exhibits a

symptomatic decrease in bioavailable NO [15]. Three main components play into this deficit: NO oxidative consumption, eNOS disintegration, and asymmetric dimethylarginine (AMDA, analog of L-Arginine) inhibition. OSA leads to unmitigated overproduction of ROS leading to damage of important macromolecules through oxidative stress [15]. On one level, the presence of ROS consumes NO in a chemically favorable reaction that produces the molecule peroxynitrite ($ONOO^-$). This "scavenging" of NO can dramatically decrease NO's bioavailability when ROS levels are increased [12]. $ONOO^-$ presence also drives uncoupling of the NOS enzyme, further decreasing NO production [12]. Additionally, oxidative stress can reduce the availability of functionally necessary eNOS subunits, decreasing the normal synthesis of NO [15]. Third, AMDA levels, an NOS inhibitor, are elevated in subjects with OSA [15]. While most interactions between OSA and NO have been observed at the endothelial level, it must also be noted that rats exposed to chronic intermittent hypoxia, a preclinical model of OSA, saw a downregulation of nNOS [2]. Thus, a deficit in nNOS may also potentially play into the NO deficit in OSA individuals.

The Peripheral Chemoreceptors and Possible Modulators: As mentioned earlier, the peripheral chemoreceptors play a key role in regulation of circulatory oxygen levels. More specifically, the glomus cells in the carotid bodies are responsible for this sensory role [16, 17]. Oxygen related chemosensory discharges from these cells result from the action of the constitutively expressed oxygen dependent heme oxygenase 2 (HO-2). When oxygen is present, HO-2 metabolizes heme to produce carbon monoxide (CO). During hypoxia, CO levels decrease leading to the downstream production of hydrogen sulfide (H_2S). H_2S in turn drives an increase in intracellular calcium leading to chemosensory signaling [17, 18]. Apart from oxygen, other

molecules can modulate glomus cell signaling and the coinciding peripheral chemoreflex. Low glucose levels (hypoglycemia), high CO_2 levels (hypercapnia), high lactate levels, and acidification can all stimulate signaling outflow of the glomus cells. Significant evidence also exists to support that insulin, leptin, and body temperature can also play roles in carotid body stimulus [16]. NO, another molecule that effects glomus cell activity, plays an inhibitory role in afferent signaling [16].

Both animal and clinical trials suggest that increasing bioavailable NO may decrease peripheral chemoreceptor sensitivity. Tryode dissolved NO profusion of in vitro cat carotid bodies resulted in decreases in both frequency and amplitude of chemosensory discharges [19]. Furthermore, inhibiting NO production through dosage of nitric oxide synthase blocker, L-N^G-nitroarginine methyl ester in vivo rats, significantly increased activity in the peripheral end of the carotid sinus nerve [20].

The Nitrate-Nitrite-NO Pathway and Nitrate Supplementation: The reduction of dietary nitrate in the nitrate- nitrite- NO pathway has been shown to increase NO bioavalibility independent of NOS enzymes [21]. In a process known as the enterosalivary nitrate circulation, nitrate consumed in the diet passes through most of the upper digestive track and is absorbed into the body's plasma at the proximal small intestine. Accounting for the amount not excreted in urine and sweat, blood circulation delivers the remaining 25% of this nitrate to the salivary glands where it is concentrated and reduced to nitrite by bacterial reductase enzymes of the oral microbiota [13]. Swallowed with saliva, nitrate-derived-nitrite can then progress through a number of metabolic processes to yield NO [13]. This natural pathway offers a therapeutic target

for mitigating deficits or increasing levels of bioavailable NO through inorganic nitrate supplementation. Specifically, beetroot juice, a supplement high in inorganic nitrate, has been shown to increase bioavailable NO presumably by this pathway [21]. In type 2 diabetics, a population commonly exhibiting low NO [22], nitrate supplementation produced a significant increase in NO bioavailability [21]. Other literature suggests that the therapeutic effects of nitrate supplementation can decrease blood pressure. Webb et al. [23] demonstrated that inorganic nitrate supplementation (via beetroot juice) significantly decreased both systolic (SBP) and diastolic blood pressure (DBP) in young individuals. Furthermore, this lowering effect was mitigated when subjects interrupted the enterosalivary reduction by spitting [23]. This BP lowering effect seems to be further increased in individuals with hypertension [21]. In a randomized clinical trial, Bock et al. [24] demonstrated that four weeks of daily nitrate supplementation decreased the hypoxic ventilatory response in older individuals. These findings suggest the blunting of the ventilatory response to hypoxia with inorganic nitrate may serve as a possible intervention for the sensitized ventilatory response in individuals with OSA, a population with a marked NO deficit [2]. Therefore, we hypothesized that acute inorganic nitrate supplementation by way of beetroot juice would attenuate the ventilatory and HR responses to acute hypoxia in individuals with OSA.

METHODS

Subjects: The 14 subjects aged between 30-70 with diagnosed mild-to-moderate OSA were recruited by means of the University of Iowa's Sleep Disorders Clinic and enrolled in the study. Subjects were excluded if exhibiting symptomatic heart disease, diabetes, autonomic disorders, severe OSA (AHI>30), central sleep apnea, kidney disease, allopurinol usage, continuous positive airway pressure (CPAP) usage, morbid obesity (body mass index $\geq$40 kg/m^2), hormone replacement therapy, or smoking (minimum of six months since cessation).

Data Collection & Study Day Format: Enrolled subjects, after completing a signed consent participated in two separate trial days (cross over design). Over the two trial days, nitrate dosed beetroot juice (BR_N) (500mg inorganic nitrate and 40mg nitrite), and a placebo beetroot juice (BR_P, devoid of nearly all nitrate and nitrite) were given in a double-blind randomized order. The powdered supplement was administered in 8floz of water. The following outcome measures were assessed: HR (three-lead electrocardiogram; Cardiocap Cardiocap/5, Datex-Ohmeda, Louisville, CO), resting SBP and DBP (automated brachial cuff; Cardiocap), respiratory rate (RR, Hans Rudolph Pneumotachometer, Shawnee, KS), tidal volume (V_T, pneumotachometer), oxygen saturation (SpO_2, finger pulse oximetry, Cardiocap), and end-tidal CO_2 (Cardiocap). Minute ventilation (V_E) was calculated by multiplying V_T and RR. Peripheral chemoreflex sensitivity was analyzed by regressing HR and V_E against SpO_2, with all data being binned into 7-breath averages. The 7-breath average seeks to minimize data variability due to fluctuations in individual breaths. Absolute change was assayed for HR and V_E under two protocol: baseline to peak response and baseline to target hypoxia. For V_E, the peak response was recorded as the greatest 7 -breath average and the target hypoxia V_E represented the last minute of hypoxia. Plasma nitrate and nitrite concentrations were gathered from analysis of venous blood samples.

Blood was collected before and two hours after beetroot juice consumption in tubes containing a lithium heparin anti-coagulant. Plasma separated by centrifugation at 3,000 rpm for 15 minutes was frozen in Eppendorf tubes at -80°C until analysis. Thawed samples were combined with a vanadium III chloride/hydrochloric acid solution at 90°C and a potassium iodide/acetic acid solution at room temperature during chemiluminescence NO analysis (NOA 280i: Sievers Instruments, Boulder, CO).

Over the course of each study day, subjects engaged in two rounds of acute isocapnic hypoxia (pre- and post-supplementation). Anesthesia gas blender titration of 21% and 10% FiO_2 reservoirs was used to drive oxygen saturations to a target hypoxia (80% SpO_2). In addition to the reservoir air, a partial rebreathe system aided in balancing carbon dioxide levels while providing potential access to ambient air. To prevent central chemoreflex interference on outcome measures, reservoir flow rate was titrated according to the end tidal CO_2 levels recorded at baseline.

The exact timing and outline of subject participation was as follows (Figure 1). The trial days began within one week of obtaining the written consent and were separated by a minimum of three days to ensure proper washout of the supplement and its metabolites. Each study day occurred under a fasted state to prevent dietary interference in variables of interest. Subjects also were instructed to abstain from exercise, caffeine, alcohol, COX inhibitors and prescriptions the morning of the study. For the two days prior to each study day, subjects were asked to follow a low-nitrate diet.

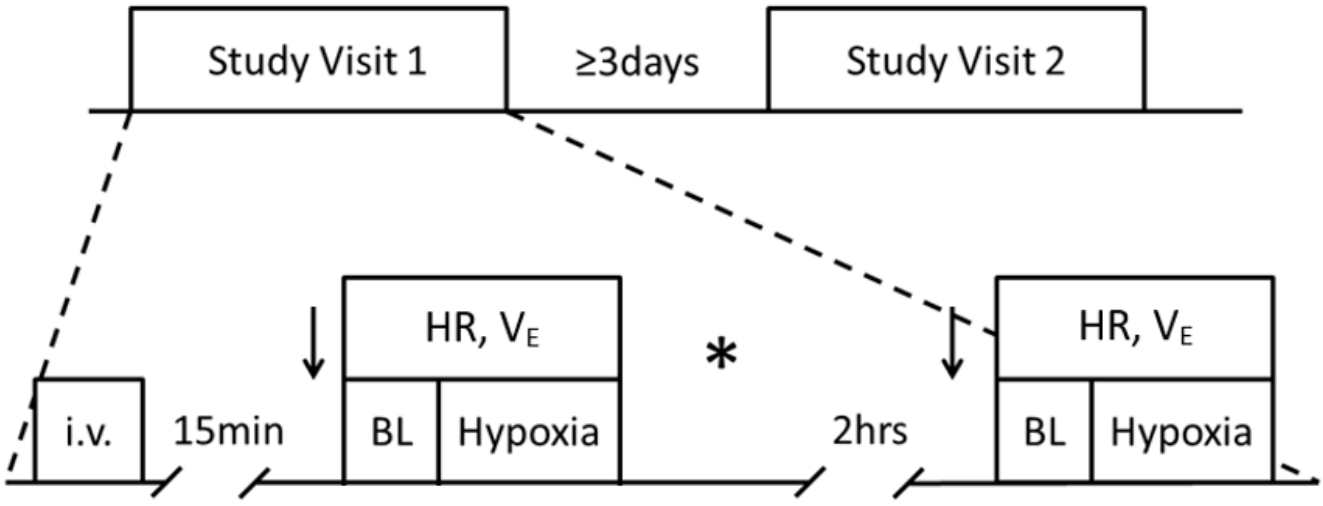

Figure 1. Temporal organization of study. i.v., venous catheter placement; BL, baseline on 21% oxygen compressed gas; Hypoxia, acute hypoxic exposure (titrated hypoxic gas to 80%SpO_2); HR, heart rate; V_E, minute ventilation. Brachial blood pressure and venous blood draws are denoted by ↓. * marks consumption of beetroot juice (placebo/nitrate).

Study days began with a venous catheterization followed by a 15-minute rest period during which the first venous blood draw and baseline BP were taken. After setup for the pneumotachometer, pulse oximeter, and electrodes for EKG, the first two-minute baseline followed under the administration of the 21% O_2 reservoir. Post-baseline, the subject's pulse oxygen saturation was lowered to the 80% threshold over the course of 2-5 minutes (variable according to inter-subject variability) in the manner discussed above. Measurements were continued eight breaths after reaching the 80% threshold and were followed by re-exposure to ambient air. After recovery to baseline oxygen saturation, subjects drank the beetroot juice supplement and waited two hours for nitrate and nitrate concentration maximums [25]. In the manner described above, a second round of acute hypoxia (post-supplementation) after the second venous draw and BP measurement.

Statistical Analysis: Data was compared using a repeated measure analysis of variance (RM-ANOVA) and are reported as mean ± SE (with the exception of demographic data being reported as mean ± SD) In this manner, each set of regressions (HR/SpO_2 and V_E/SpO_2) were compared to asses potential significant group (BR_P/BR_N) by time (pre/post) interactions. Absolute changes from baseline to target hypoxia (Bl-T) in HR and V_E were also analyzed in this manner. To avoid possible under reporting of the ventilatory response due to a nonlinear trend, ventilatory regression and absolute change were also analyzed from baseline to peak ventilatory response (Bl-P). The effects of BR_P and BR_N on brachial cuff measures of SBP and DBP were also analyzed for a group by time interaction. All significant interactions were further analyzed using Tukey's post-hoc which assessed for between and within group differences. Statistical analysis was done at a significance level $\alpha \leq .05$ using Sigma Plot 11.0 (Systat Software Inc., San Jose, CA).

RESULTS

Demographics: Baseline demographics collected on subject's first study day are displayed in Table 1. 18 subjects were recruited for the study, 14 completed the protocol.

Table 1: Research Subject's Demographic Values (n=14)

Characteristic:	Value
Age, years	53 ± 10
Sex, male/female	8/6
BMI, kg/m^2	29.2 ± 5.8
SBP, mmHg	123 ± 14
DBP, mmHg	76 ± 9
MAP, mmHg	91 ± 9
AHI, events/hr	18.8 ± 7.5
Nadir SpO_2, %	86 ± 3

Data are means ± SD. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; AHI, apnea hypopnea index; Nadir SpO_2, lowest SpO_2 recorded during sleep test.

Inorganic Nitrate Supplementation and Plasma Nitrate and Nitrite: Due to technical complications with either venous catheter placement or analysis of blood samples, plasma concentrations were not available from all subjects. Therefore, data presentation reflects the following subject numbers for each measured variable: BR_P nitrate and nitrite (n=13), BR_N nitrate (n=12), and BR_N nitrite (n=11). Dietary nitrate supplementation by way of BR_N increased plasma nitrate and nitrite concentrations (both $p<0.01$). BR_P yielded no change in either nitrate or nitrite levels (nitrate: p=0.33, nitrite: p=0.87, Figures 2A and B, respectively).

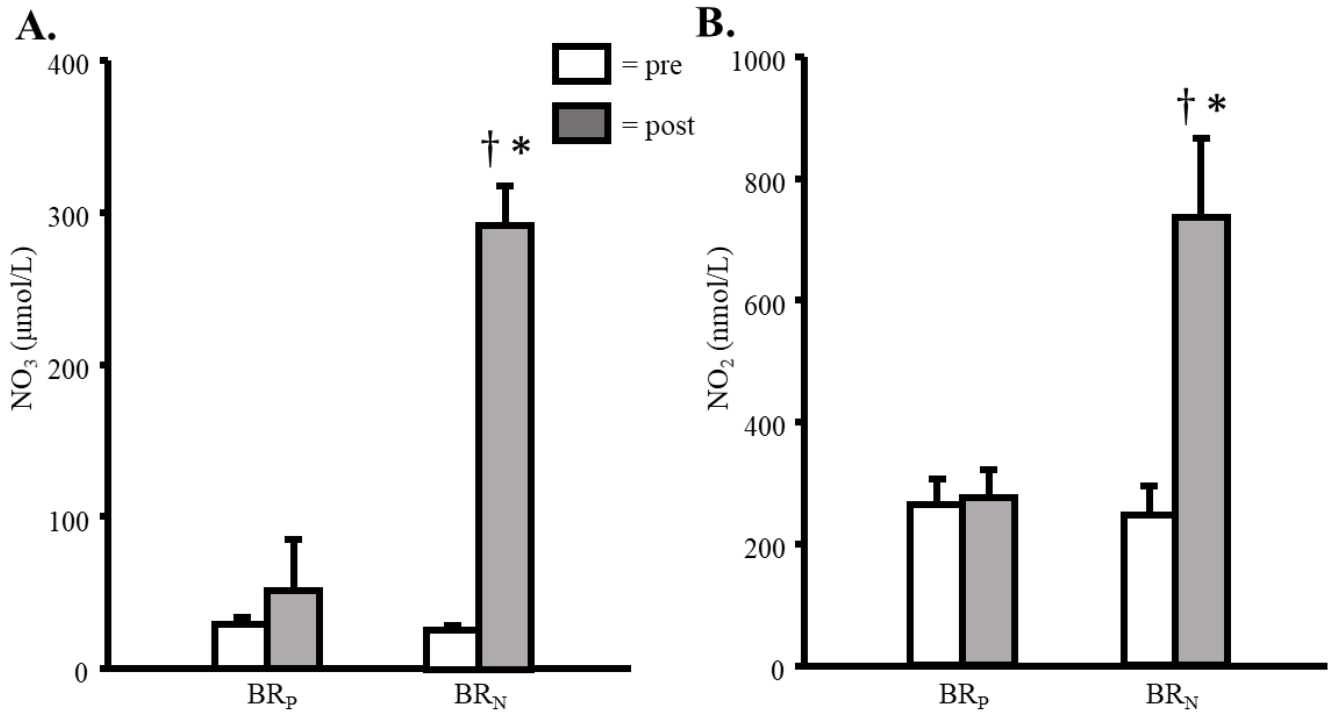

Figure 2. Plasma concentrations of A) nitrate (NO_3) and B) nitrite (NO_2) before and after supplementation of the placebo beetroot supplement lacking nitrate (BR_P) or the beetroot containing inorganic nitrate and nitrite (BR_N). Data are means ± SE. Pre and post reflect baseline and two hours after supplementation respectively. **†** $p<0.01$ vs pre. ***** $p<.01$ vs BR_P post.

Hemodynamics: Brachial artery SBP and DBP responses to BR_N and BR_P are shown in Figure 3. There was an increase in SBP during the BR_P condition ($p<0.01$), whereas SBP did not change in the BR_N condition ($p=0.96$, Figure 3A). Furthermore, post SBP was greater in the BR_P condition compared to BR_N. No condition x time interaction was observed for DBP ($p=0.08$, Figure 3B).

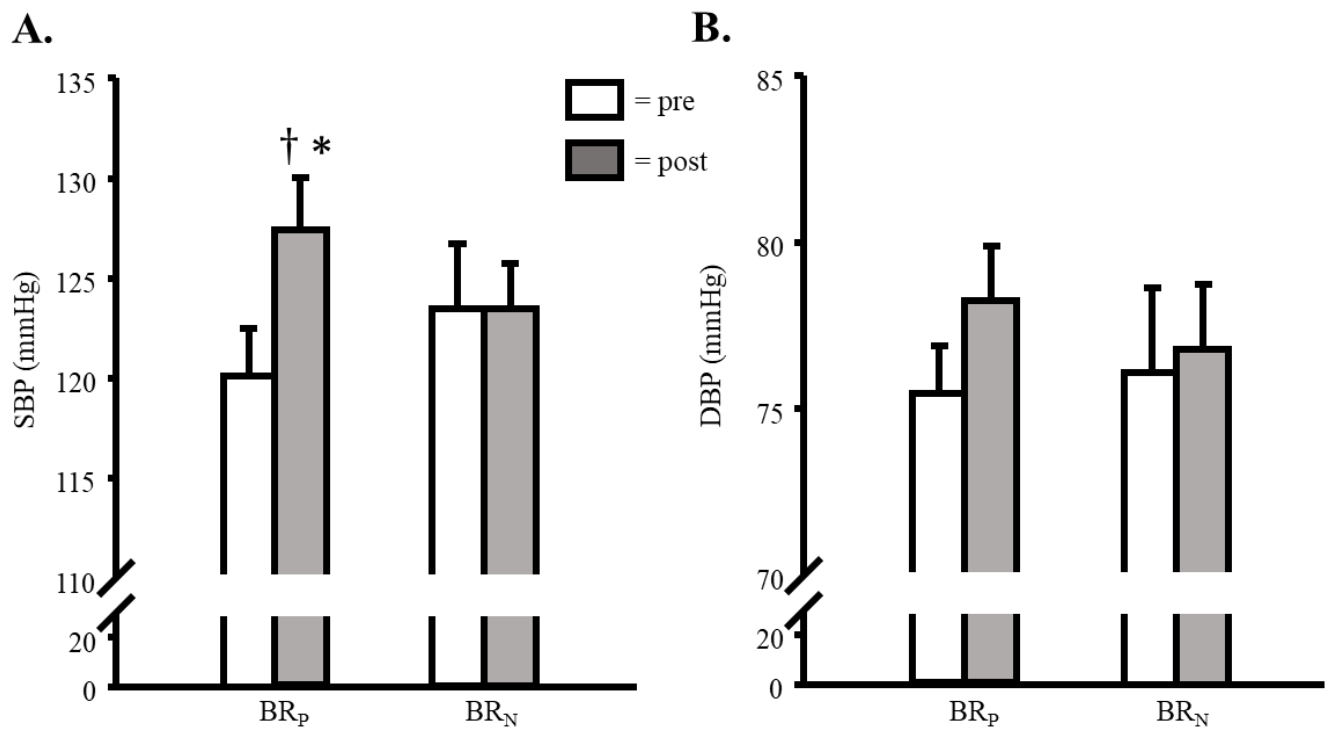

Figure 3. A) Systolic and B) Diastolic blood pressure before and after supplementation of the placebo beetroot supplement lacking nitrate and nitrite (BR_P) and the beetroot containing inorganic nitrate and nitrite (BR_N). Data are means ± SE. Pre and post reflect baseline and two hours after supplementation respectively. † $p<0.01$ vs pre. *****$p<0.05$ vs. BR_N post.

HR responses to hypoxia before and after BR_N and BR_P are shown in Figure 4. There were no condition x time interactions for the HR regression ($p=0.12$, Figure 4A) or the absolute change in HR ($p=0.54$, Figure 4B).

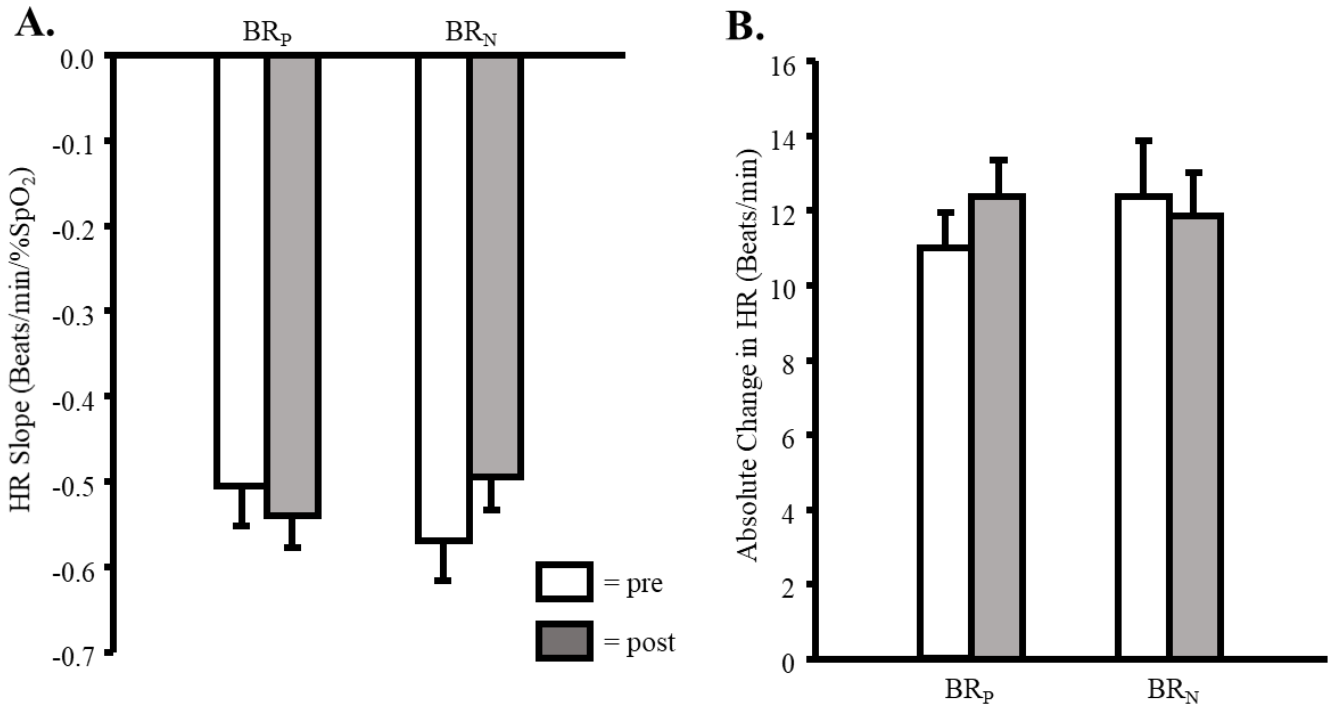

Figure 4. Heart rate (HR) response before and after supplementation of the placebo beetroot supplement lacking nitrate and nitrite (BR_P) and the beetroot containing inorganic nitrate and nitrite (BR_N) in A) HR regression against oxygen saturation and B) absolute change in HR from baseline to end hypoxia. Data are means ± SE. Pre and post refer to initial baseline hypoxia and the second bout of hypoxia two hours after supplementation.

Ventilatory Response to Hypoxia: The ventilatory responses to hypoxia are shown in Figure 5. There was no condition x time interaction for the Bl-T linear regression (p=0.08, Figure 5A), the Bl-T absolute change (p=0.11, Figure 5B), or the Bl-P linear regression (p=0.33, Figure 5C). However, the Bl-P absolute change increased in the BR_P condition (p=0.03, Figure 5D) but did not change in the BR_N condition (p=0.43, Figure 5D).

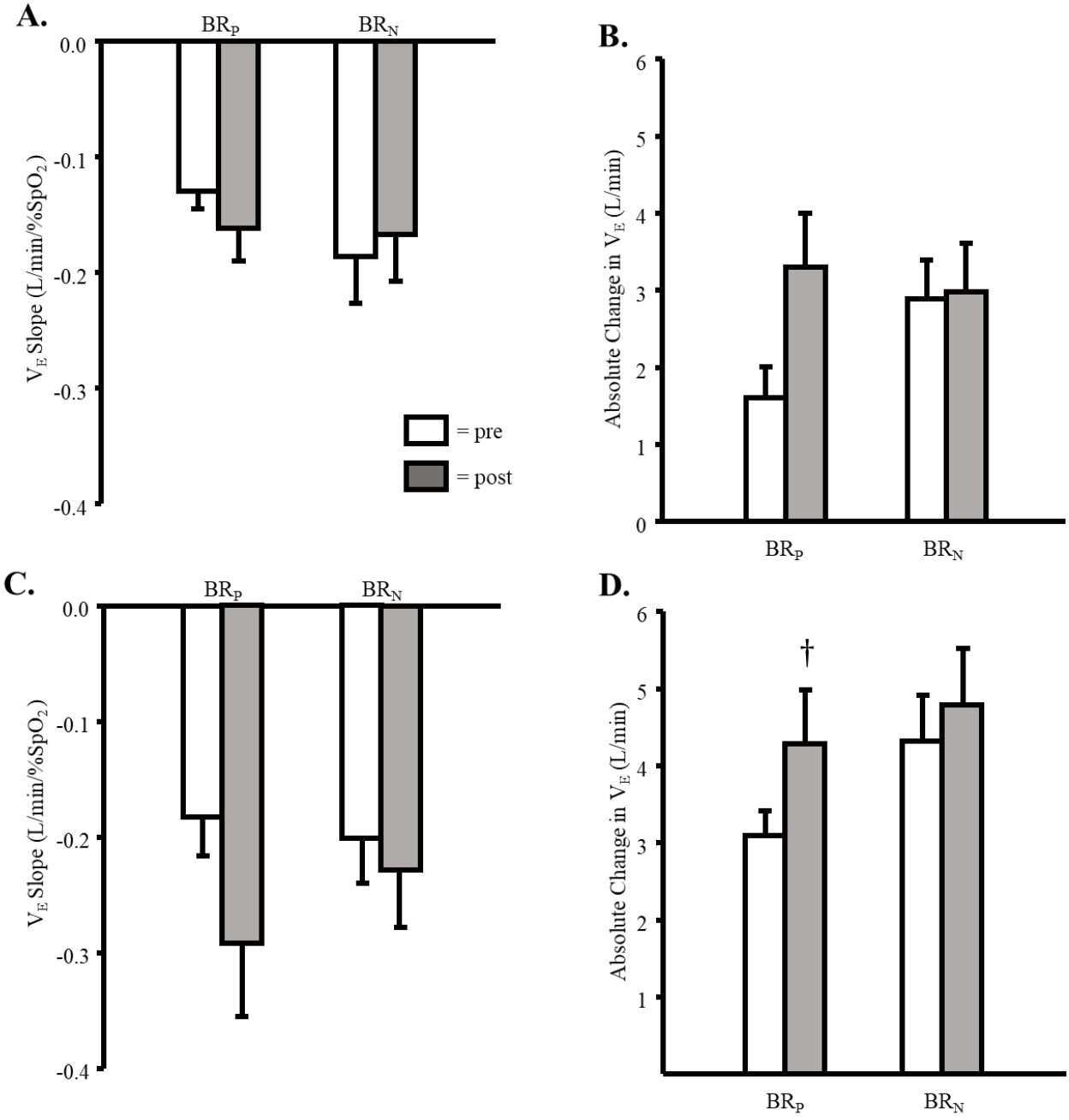

Figure 5. The ventilatory responses to hypoxia expressed as A) V_E regression against oxygen saturation (SpO_2) from baseline to target hypoxia (Bl-T), B) absolute change in V_E for Bl-T, C) the V_E regression against SpO_2 from baseline to peak response (Bl-P), and D) the absolute change in V_E for Bl-P before and after supplementation of the placebo beetroot supplement lacking nitrate and nitrite (BR_P) and the beetroot containing inorganic nitrate and nitrite (BR_N). Data are means ± SE. Pre and post refer to initial baseline hypoxia and the second bout of hypoxia two hours after supplementation. † $p<0.05$ vs pre.

DISCUSSION

This study sought to examine the effects of inorganic nitrate supplementation on the sensitivity of the peripheral chemoreflex in individuals with OSA. Acute inorganic nitrate supplementation substantially increased bioavailable NO in subjects with mild to moderate OSA measured by plasma increases in plasma nitrate (1,023%) and nitrite (198%) concentrations (Figures 2A and B, respectively). SBP under BR_P increased, a change not observed, and possibly mitigated in the BR_N condition (Figure 3A). DBP did not change in response to either supplementation (Figure 3B). Generally, the peripheral chemoreflex response to hypoxia, did not vary according to supplementation as observed in HR and V_E measures (Figures 4 and 5 respectively). Taken, together, the hypothesized blunting of the reflex with inorganic nitrate was not supported.

Significant literature suggests that inorganic nitrate supplementation can reduce BP [26]. This trend is the largest when examining the decreases in context of SBP (2.3 to 8.9), however it has also been observed in reference to DBP (0.2 to 5 mmHg) [26]. While the BP results of our study did not follow the documented BP decrease following inorganic nitrate, it does express a blunting of a rise in BP due to potential diurnal changes. The temporal fluctuations in BP, or diurnal variation, exhibit a strong morning increase and peak associated with wakening and increased activity [27]. Other factors such as stress, diet, and smoking can impact the magnitude of this phenomena [28]. Study days, all scheduled in the early morning would have observed subjects during this diurnal rise, and thus may explain the SBP increase under BR_P (Figure 3A). The absence of an equivocal rise under BR_N hints that nitrate supplementation may play a role in mitigating this BP increase. Furthermore, the greater inorganic nitrate blunting of SBP in the literature, when compared to DBP, may explain why the mitigation of diurnal rise was only

observed in SBP. It must be noted that this rise is blunted when individuals remain supine following waking [28]. Over the course of our study days, subjects spent a significant amount of time in a low activity state, at times lying supine. Further compelling this area of study, individuals with OSA commonly show irregularities in BP especially in pertinence to the diurnal BP cycle. In particular, hypoxic arousals drive an increased nightly BP and may be responsible for nondipping nocturnal BP trends. Characterized by a lack of the regular decrease in BP during sleep, nondipping BP places individuals at increased risk of CVD and hypertension [29]. Pathophysiological prevention of the dipping seen in OSA could be offset by a nitrate induced blunting of the morning BP increase in an effort to mitigate overall hypertension.

An increase in HR is a key component of the peripheral chemoreflex in response to hypoxia. For individuals with OSA, however, this response to hypoxia is exaggerated. Compared to age and BMI matched healthy individuals, Narkiewicz et al. [11] reported that OSA individuals exhibit a greater HR response to hypoxia. In the current study, this increased response was not mitigated by increasing NO bioavailability by means of inorganic nitrate supplementation (Figure 4). This absence of NO blunting on HR translates to the same observation by Bock et al. [24], which utilized a longer-term (4-week) inorganic nitrate study in healthy older adults. However, as no healthy controls were enrolled in the present study, analyzing the degree of HR response augmentation in OSA individuals was not possible. In retrospect, a true control group would have allowed us to quantify if the OSA subjects in the current study did indeed have an augmented HR response to hypoxia.

While significant research has attempted to examine the effects of nitrate on high altitude/hypoxic exercise, little work has been done to study its effects on the ventilatory response. As mentioned, one long term supplementation study showed a nitrate associated decrease in ventilatory response to acute hypoxia for older adults [24]. The current study assessed ventilatory response utilizing two analysis techniques, linear regression and absolute change, over two temporal criteria, Bl-T and Bl-P. The linear regression was used to quantify the rate at which V_E changed according to SpO_2 while the absolute change simply considered the response magnitude. While the regression allowed for a numeric rate analysis, it assumed a linear response. However, several subject's response seemed to progress in a nonlinear fashion (e.g., their greatest ventilatory response was not observed at the lowest %SpO2). Additionally, in reference to the temporal organization, Bl-P was utilized to avoid underreporting ventilatory responsiveness, another consequence of a possible nonlinear relationship. Our results showed no impact of either inorganic nitrate or a placebo on V_E response except in the BR_P Bl-P absolute change criteria. However, since no difference was observed in the other three V_E analyses criteria, it is likely that the low p-value in BR_P Bl-P was simply a result of repeated testing. Thus holistically, our study saw no decrease in the ventilatory response to hypoxia following inorganic nitrate supplementation. As this trend was not seen in this study, two elements must be considered: duration of supplementation and effects of OSA morbidity. Unlike Bock et al.'s [24] study which followed a daily inorganic nitrate beetroot dosage over four weeks, this study utilized an acute supplementation taken during both study days. Thus, reproducing the blunting effect in OSA may require a more tonic supplementation approach. Additionally, OSA's physiological consequences may disrupt Bock et al.'s [24] trend which was produced in healthy individuals.

For this purpose, studying inorganic nitrate supplementation in CPAP competent OSA individuals might expose such a relation. CPAP, the most robust available treatment option for OSA, successfully blunts pathologically elevated blood pressure (BP), sympathetic nervous system activation, and risk of death due to CVD [2, 3, 5]. Furthermore, CPAP has been shown to restore endothelial function (observed by flow mediated dilation), increase circulating NO levels, increase levels of substrate L-arginine, and mitigate chemoreflex sensitization in patients with OSA [7, 15]. Removing some of the pathophysiological consequences of OSA through use of CPAP may allow the inorganic nitrate impact observed in Bock et al.'s [24] study. Furthermore, as CPAP is such a functionally robust treatment, inorganic nitrate serves not as a replacement option for treating OSA but rather as a possible avenue for an adjunct therapy. Future studies should consider including a CPAP competent arm in order to study this possible interaction.

In design, there are several distinct limitations to this study. Considering the wide breadth of subject demographics, AHI ranged from 5.6 to 29.9 events/hour. AHI and consequently OSA severity correlate with sympathetic activation magnitude as well as with hypertension [4, 32]. Additionally, in considering BP, one subject in the current study was hypertensive. Literature demonstrates that both OSA severity and hypertension correlate with increase peripheral chemoreflex sensitivity [3, 8, 11]. Thus, the large variability in AHI and the mixture of normotensive, prehypertensive and hypertensive individuals may have increased intersubjective variability in response to hypoxia. As the effects of inorganic nitrate on hypoxic response have never been assessed in populations with an augmented chemoreflex, it is possible that this intervention only yields a therapeutic effect in cases of severe response augmentation. If this

where the case, a lack of blunting in milder OSA could obscure significant blunting of the HR and V_E response in more severe OSA subjects. Thus, narrowing the demographic breadth to populations with greater sensitization may see the proposed therapeutic effects. Future studies should consider subpopulations such as hypertensives and/or individuals with severe OSA who demonstrate a more robust response to hypoxia [2, 11].

Additionally, because this study looked simply for an interaction between inorganic nitrate supplementation and hypoxic responsiveness, only one concentration of nitrate was utilized. Literature suggests a dose dependent relationship between inorganic nitrate and cardiovascular outcomes. Lara et al. [33] showed increased endothelial function at increased inorganic nitrate doses. Additionally, multiple studies have found that inorganic nitrate's BP lowering effect is dose dependent [21]. Accordingly, future studies should consider utilizing multiple concentrations of inorganic nitrate to asses for possible dose dependent changes.

Beyond demographics and supplementation considerations, monitoring sympathetic activation would further develop our understanding of inorganic nitrate's impact on OSA. Within the peripheral chemoreflex, sympathetic activation plays a key role in effector signaling. As discussed earlier, apneas mechanistically facilitate an increase in sympathetic outflow due to the inhibition of ventilatory stretch afferents negative regulation of the sympathetic nervous system (SNS). Furthermore, in comparison to healthy individuals, SNS activity is increased in OSA [2]. This increase is likely due to the repeated activation of the peripheral chemoreflex from nightly apneas. Tonic activation of the SNS may ultimately facilitate hypertension, arrythmias, and heart failure [2]. Furthermore hypertension, often comorbid with OSA, sees an increased sympathetic

response to apnea [3, 27]. Because of its key role in the hypoxic response, future studies analyzing the effects of inorganic nitrate supplementation on OSA should consider recording sympathetic activation.

Holistically, inorganic nitrate supplementation did not affect the HR and ventilatory response to hypoxia. These findings directly contrast several trends observed in the literature (ventilatory response blunting and BP lowering). The absence of these trends in the current study suggest a lack of a therapeutic effect of inorganic nitrate on the peripheral chemoreflex in OSA. Nonetheless, the current study provides support for further consideration of a possible interaction between inorganic nitrate and diurnal rise in BP. Further studies should be conducted to analyze these trends especially in context of CPAP, and SNS activation.

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