DIETARY NITRATE SUPPLEMENTATION AUGMENTS NITRIC OXIDE SYNTHASE-MEDIATED CUTANEOUS VASODILATION DURING LOCAL HEATING IN HEALTHY HUMANS

by

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Abstract

Nitrate supplementation in the form of beetroot juice (BRJ) has been shown to increase nitric oxide (NO), where nitrate can be reduced to nitrite and NO through both nitric oxide synthase (NOS) independent and dependent pathways. We tested the hypothesis that BRJ would augment the NO component of cutaneous thermal hyperemia. Dietary intervention consisted of one shot of BRJ for three days. Six subjects were equipped with two microdialysis fibers on the ventral forearm and randomly assigned to lactated Ringer's (control) or continuous infusion of 20mM L-NAME (NOS inhibitor). The control site was subsequently perfused with L-NAME once a plateau in the local heating response was achieved to quantify NOS-dependent cutaneous vasodilation. Skin blood flow via laser-Doppler flowmetry (LDF) and mean arterial pressure (MAP) were measured; cutaneous vascular conductance (CVC) was calculated as LDF/MAP and normalized to $\%CVC_{max}$. Maximal vasodilation was achieved via local heating to 43°C and 54 mM sodium nitroprusside infusion. There was a significant decrease in DBP after BRJ (Pre-BRJ: 74 ± 1 mmHg vs. Post-BRJ: 61 ± 2 mmHg; $p < 0.05$**)** and significant reduction in MAP after BRJ (Pre-BRJ: 90 ± 1 mmHg vs. Post-BRJ: 80 ± 2 mmHg; $p < 0.05$**)**. The initial peak and secondary plateau phase of cutaneous thermal hyperemia were attenuated at sites with continuous L-NAME; however, there was no effect of BRJ on either the initial peak at control sites (Pre-BRJ: 76 ± 3%CVC_{max} vs. Post-BRJ: 75 ± 4%CVC_{max}) or L-NAME sites (Pre-BRJ: 60 ± 4%CVC_{max} vs. Post-BRJ: 59 ± 5%CVC_{max}) or the secondary plateau phaseat control sites (Pre-BRJ: 88 ± 4%CVC_{max} vs. Post-BRJ: 90 ± 4%CVC_{max}) or L-NAME sites (Pre-BRJ: 45 ± 5%CVC_{max} vs. Post-BRJ: 51 ± 3%CVC_{max}). The decrease in $\%CVC_{max}$ to L-NAME infusion during the plateau of local heating (i.e. post-L-NAME drop) was greater after BRJ (Pre-BRJ: 36 ± 2%CVC_{max} vs. Post-BRJ: 28 ± 1%CVC_{max}; $p < 0.05$). This resulted in a greater contribution of NOS to the

plateau phase of local heating (Pre-BRJ: 57±3%CVC_{max} vs. Post-BRJ: 64±2%CVC_{max}; $p < 0.05$). These data suggest BRJ modestly improves NOS-dependent vasodilation to local heating in the cutaneous vasculature of healthy humans.

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Dedication

To my family, friends, and colleagues; your support over the years has made this dream a reality.

Chapter 1 - Introduction

Cutaneous thermal hyperemia in response to rapid, non-painful local heating exhibits a biphasic response in skin blood flow. An initial transient increase in skin blood flow punctuated by a brief nadir, is followed by a prolonged secondary plateau phase mediated predominantly (~60%) by NO (Kellogg *et al*. 1999; Minson *et al.* 2001). This secondary plateau phase becomes insensitive to nitric oxide synthase (NOS) inhibition if local heating results in pain, suggesting different mechanisms facilitate a non-painful versus painful heating stimulus (Kellogg *et al.* 1999). The initial peak phase in response to local heating is predominantly mediated by a sensory nerve axon reflex, possibly through neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) (Brain *et al.* 1986). It has also been shown that adenosine receptors (Fieger *et al.* 2010), H_1 - histamine receptors (Wong *et al.* 2006), transient receptor potential vanilloid (TRPV) channels (Wong *et al.* 2010), neurokinin-1 receptors (Wong *et al.* 2011), noradrenaline and neuropeptide Y (Houghton *et al.* 2006; Hodges *et al.* 2008) and NO (Kellogg *et al.* 1999; Minson *et al.* 2001) contribute to one or more phases of cutaneous thermal hyperemia.

Although nitric oxide has been shown to modestly contribute to the initial peak and nadir response to local heating, independent studies have demonstrated inhibition of NOS significantly reduces the secondary plateau phase of cutaneous thermal hyperemia by ~60-70% (Kellogg *et al.* 1999; Minson *et al.* 2001), providing evidence that NO plays a substantial role in the plateau phase of the cutaneous thermal hyperemic response; however, NO is not the sole local vasodilator inasmuch as NOS inhibition does not abolish the response. Although the exact mechanisms underlying the increase in NO are not entirely understood, Kellogg *et al.* (2008) have recently provided evidence to suggest that most of the NO-component relies on functional endothelial nitric oxide synthase (eNOS).

Quantification of the NO-dependent vasodilation to local heating of the skin is frequently used to assess *in vivo* microvascular function in various populations, such as healthy aging, hypertension, and hypercholesterolemia (Holowatz *et al*. 2007; Minson *et al*. 2010). In these populations, NO-dependent vasodilation has been shown to be attenuated compared to healthy control subjects and reduced NO-dependent vasodilation has been shown to be correlated with atherosclerosis (Vallance *et al*. 2001). Thus, interventions that improve bioavailable NO and improve NO-dependent vasodilation in these patient populations are critical.

Dietary nitrate supplementation via beetroot juice (BRJ) has been shown to have several positive health benefits, including reduced blood pressure (Kapil *et al.* 2010) and platelet aggregation (Webb *et al.* 2008), increased blood flow to contracting skeletal muscle (Ferguson *et al.* 2013), and enhanced peripheral vasodilation and endothelium-derived NO (Kenjale *et al.* 2011). These improved physiological markers have been shown to occur in both healthy humans and in disease populations, suggesting BRJ supplementation has important and powerful health benefits. The increase in NO bioavailability from nitrate supplementation has been shown to occur through both NOS independent (Lundberg *et al.* 2004) and dependent (Vanin *et al.* 2006) pathways. Many studies have demonstrated that reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and, subsequently, to NO can provide a valuable pathway in which the body can utilize NO through a NOS-independent system unlike the traditional L-arginine pathway that is reliant on the NOS enzyme and cofactors (Ca^{2+} and BH_4). Through this NOS-independent pathway, NO_3^- can be reduced to NO_2^- and NO through several different mechanisms including reductase enzymes in bacteria in the mouth and acidic environment of the stomach (Benjamin *et* al. 1994), various enzymes and proteins including deoxymyoglobin and xanthine oxidoreductase (Cosby *et al*. 2003; Shiva *et al.* 2007; Lundberg *et al*. 2004), and in hypoxic conditions (Vanhatalo *et al.*2011).

In the presence of a reduced partial pressure of oxygen (PO_2), eNOS has been shown to be able to utilize NO_3^- to produce NO (Gautier *et al*. 2006). Thus, nitrate supplementation may provide a means by which to improve NO-dependent vasodilation through both NOS-independent and NOS-dependent mechanisms. As stated above, NO is an essential component to the skin blood flow response to local heat stress. Therefore, it is possible BRJ supplementation and the associated increase in bioavailable NO levels may provide beneficial effects in the skin, particularly in pathological conditions that have impaired NO function such as obesity or aging (Minson *et al.* 2002).

Augmenting NO bioavailability in the systemic circulation has been suggested to improve endothelial function in various patient populations (Gautier *et al*. 2006). Microvascular and endothelial function in the cutaneous circulation has been shown to have a high correlation with responses observed in the systemic circulation; therefore, a minimally invasive model (i.e. laser-Doppler flowmetry combined with intradermal microdialysis) to assess microvascular function specifically in the skin without influence from underlying muscle blood flow can be used as a comparative method between these two vascular beds (Holowatz *et al.* 2007; Saumet *et al.* 1988, Minson *et al.* 2010). Local heating of the skin is used as a clinical tool to assess microvascular and endothelial function in various patient populations including diabetes, obesity, Raynaud's phenomenon, and aging, (Cracowski *et al.* 2006; Minson *et al*. 2002). Because the underlying mechanisms of the cutaneous thermal hyperemic response remain unclear, it is important to understand these mechanisms in healthy humans. Inasmuch as NO_3^- supplementation with BRJ has been shown to have positive health benefits in healthy humans and animal models, the purpose of this study was to investigate the role of dietary NO_3^- supplementation via BRJ in the skin blood flow response to local heating in healthy humans. We

tested the hypothesis that nitrate supplementation would augment the NOS-dependent component and contribution to cutaneous thermal hyperemia in healthy humans.

Chapter 2 - Methods

Ethical Approval

The Institutional Review Board at Kansas State University approved all protocols of this study. A written informed consent was reviewed and signed by each subject prior to participation. All procedures and protocols were performed in conjunction with the standards set forth by the *Declaration of Helsinki.*

Subjects

Six subjects (6 men: age 24 ± 1 yr, height 177 ± 6 cm, mass 80 ± 13 kg, body mass index 26 ± 3 kg/m^2), participated in this study. All were healthy, non-smokers, taking no medications and were free of cardiovascular, respiratory, and metabolic disease as determined by a medical history questionnaire. Subjects were asked to refrain from exercise, consumption of abnormal amounts (>300g/day) of leafy green vegetables (e.g. spinach), and the use of mouthwash products (e.g. Listerine) during the BRJ supplementation period as shown by previous experiments (Govoni *et al.* 2008; Bailey *et al.* 2009). A study by Lundberg *et al.* (2004) demonstrated that if subjects used antiseptic mouthwash and/or did not swallow after nitrate ingestion, there was a reduction in circulating plasma NO_2^- levels (Lundberg *et al*. 2004, Govoni *et al.* 2008). This suggests that bacteria in the mouth and saliva play an important first step in the reduction process of NO_3^- to NO_2^- to NO. Subjects were asked to refrain from caffeine and alcohol at least 12 hours prior to the study. Testing was administered in a thermoneutral environment with a room temperature of ~23°C.

Dietary Intervention

Finger-stick blood draws following an overnight fast of ~8-12 hours for the determination of blood glucose, C-reactive protein, and blood lipid variables were collected prior to the local

heating protocol for both pre- and post-BRJ. Upon completion of pre-intervention testing, subjects received three days of nitrate rich BRJ (4.7-5.5 mM/day; 0.4g of nitrates) (Beet It, James White Drinks, Ipswich, UK). Subjects were instructed to drink the supplement throughout the day and take the last shot approximately 1.5-2 hours prior to reporting to the laboratory for the local heating protocol. This time frame has been previously shown to elicit peak circulating NO_2^- levels (Bailey *et al.* 2009, Vanhatalo *et al*. 2010) and peak circulating NO_2^- levels have been shown to be correlated with reductions in systolic and diastolic blood pressure (Vanhatalo *et al*. 2010).

Subject Instrumentation

Testing was conducted with the subject resting in the supine position with the experimental arm at heart level. To avoid the use of anesthetics, ice was used to numb the desired location prior to placement of microdialysis fibers (Hodges *et al.* 2009). Fibers were placed approximately 3-5 cm apart. Microdialysis fibers 10 mm in length with a 55-kDa molecular mass cutoff (CMA 31 Linear Probe; CMA Microdialysis, Kista, Sweden) were placed by first threading a 23-gauge needle through the intradermal layer of the skin on the ventral aspect of the left forearm. A fiber was then threaded through the lumen of the needle, and the needle was then removed, leaving the membrane in place. Approximately 45-90 minutes were allowed for resolution of the trauma response following microdialysis fiber placement before the continuation of the protocol. During this time, all fibers were perfused with lactated Ringer's solution at a rate of 2 µl/min.

Mean arterial pressure was monitored beat-by-beat via photoplethysmography (NexfinHD; BMEYE, Amsterdam, The Netherlands) and verified every five minutes via

automated brachial auscultation (S/5 Light Monitor; Datex-Ohmeda, GE Healthcare; Madison, WI, USA).

Red blood cell (RBC) flux was used as an index of skin blood flow via laser-Doppler flowmetry (LDF) (PeriFlux 5010 laser-Doppler perfusion monitor; Perimed; Jarfalla, Sweden). Laser-Doppler flowmetry provides a minimally invasive, highly reproducible technique without influence from underlying skeletal muscle blood flow (Saumet *et al.* 1988). Local heating units (PF5020 local heating units and PeriFlux 5020 Temperature Unit; Perimed; Jarfalla, Sweden) were placed on the skin directly over each microdialysis membrane, and an integrated laser-Doppler probe (Probe 413; Perimed; Jarfalla, Sweden) was placed in the center of each local heating unit to measure RBC flux directly over each microdialysis site.

Drugs Administered

Lactated Ringer's solution was administered at the control site. A 20 mM concentration of the L-arginine analog N^G *–nitro-L-arginine* methyl ester (L-NAME) has been previously shown to non-selectively inhibit all isoforms of NOS in human cutaneous microvasculature (Kellogg *et al*. 1999). At the end of the local heating protocol, a 54 mM concentration of sodium nitroprusside (SNP) (i.e. NO donor) was administered while simultaneously locally heating the skin to 43°C to elicit maximal vasodilation (Kellogg *et al*. 2008). All drugs were infused through the microdialysis fibers at a rate of 2 µl/min via microinfusion pumps (Bee Hive controller and Baby Bee Syringe Pumps; Bioanalytical Systems, West Lafayette, IN, USA) to each randomly assigned site.

Experimental Local Heating Protocol

Baseline skin blood flow, blood pressure, and mean arterial pressure were collected for approximately 5-10 minutes after the trauma resolution period. Subsequently, one of two

treatments were infused at random to each microdialysis site: 1) lactated Ringer's solution (control site) or 2) 20 mM L-NAME (non-selective NOS inhibitor). After a period of at least 45 minutes of drug infusion, temperature of the local heating units were increased from 33°C to 42°C at a rate of 1°C/10sec (i.e. rapid local heating) until a steady-state was achieved for approximately 20-30 min. Once a plateau was established and maintained, the control site was then infused with 20 mM L-NAME in order to quantify NOS-dependent vasodilation during local heating. Once a 5-10 minute plateau to L-NAME infusion at the control site was established (i.e. post-L-NAME drop), each site was infused with SNP and heated to 43°C to achieve maximal vasodilation.

Data Collection and Analysis

Data was digitized and stored at 100 Hz on a personal computer. Data were analyzed offline using signal-processing software (Windaq; Data Instruments, Akron, OH, USA). Skin blood flow measurements were normalized to mean arterial pressure and expressed as cutaneous vascular conductance (CVC), calculated as the ratio of red blood cell flux to mean arterial pressure (i.e. RBC flux/MAP). Cutaneous vascular conductance values were normalized as a percentage of maximal vasodilation (%CVC_{max}) via SNP infusion and local heating to 43°C.

All data were analyzed via Sigma Stat 3.5 (Systat Software; Point Richmond, CA, USA). All values are presented as mean ± SEM, and P-values < 0.05 were considered to be significant. A two-way ANOVA with repeated measures was used to compare pre- and post-BRJ supplementation CVC values at control and L-NAME sites for: 1) the initial peak phase of cutaneous thermal hyperemia; 2) the plateau phase of cutaneous thermal hyperemia; and 3) absolute maximal CVC values. The percent NOS-dependent vasodilation and all blood pressure variables for pre- and post-BRJ supplementation were compared using a paired t-test. Percent

NOS contribution was calculated at the control sites as ((LH Plateau - Post L-NAME Drop/LH Plateau)*100).

Chapter 3 - Results

Blood pressure data pre- and post-BRJ (nitrate supplementation) are summarized in Table 1. There was no effect of nitrate supplementation on systolic blood pressure pre-BRJ (122 ± 2 mmHg) to post-BRJ (118 ± 2 mmHg). Diastolic blood pressure was significantly reduced from pre-BRJ (74 ± 1 mmHg) to post-BRJ (61 ± 2 mmHg; $p<0.05$). Mean arterial pressure was significantly reduced from pre-BRJ (90 ± 1 mmHg) to post-BRJ (80 ± 2 mmHg; $p<0.05$). Blood lipid variables including total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, blood glucose, and C-reactive protein for pre- and post-BRJ are shown in Table 2. Only triglycerides were significantly reduced from pre-BRJ (95 ± 12 mg/dL) to post-BRJ (72 ± 6 mg/dL; $p<0.05$).

Absolute maximal CVC values are shown in Table 3 for both pre-and post-BRJ. There was no significant difference in absolute maximal CVC values among control sites (Pre-BRJ: 1.81 ± 0.21 vs. Post-BRJ: 1.61 ± 0.14) or L-NAME sites (Pre-BRJ: 1.93 ± 0.17 vs. Post-BRJ: 1.70 ± 0.18).

Figure 1 shows the group mean data for the initial peak of the thermal hyperemic response to local heating for pre-and post-BRJ at control (solid bars) and L-NAME (gray bars) sites. The initial peak at L-NAME sites was significantly attenuated ($p<0.001$) compared to control sites prior to BRJ (control: 76 ± 3%CVC_{max} vs. L-NAME: 60 ± 4%CVC_{max}) and post-BRJ (control: 75 ± 4%CVC_{max} vs. L-NAME: 59 ± 5%CVC_{max}); however, three days of BRJ had no effect on the initial peak at either control sites (Pre-BRJ: 76 ± 3%CVC_{max} vs. Post-BRJ: 75 ± 4%CVC_{max}) or L-NAME sites (Pre-BRJ: 60 ± 4%CVC_{max} vs. Post-BRJ: 59 ± 5%CVC_{max}).

Figure 2 shows the group mean data for the secondary plateau phase of the thermal hyperemic response to local heating for pre-and post-BRJ at control (solid bars) and L-NAME

(gray bars) sites. The secondary plateau was attenuated ($p < 0.001$) at L-NAME sites compared to control sites prior to BRJ (control: 88 ± 4%CVC_{max} vs. L-NAME: 45 ± 5%CVC_{max}) and post-BRJ (control: 90 ± 4%CVC_{max} vs. L-NAME: 51 ± 3%CVC_{max}). However, following three days of BRJ, there was no significant difference in the secondary plateau at either control sites (Pre-BRJ: 88 ± 4%CVC_{max} vs. Post-BRJ: 90 ± 4%CVC_{max}) or L-NAME sites (Pre-BRJ: 45 ± 5%CVC_{max} vs. Post-BRJ: 51 ± 3%CVC_{max}).

Figure 3 shows the group mean data for the post-L-NAME drop. The decrease in CVC to L-NAME infusion during the plateau phase at control sites was significantlygreater after three days of BRJ supplementation (Pre-BRJ: 36 ± 2%CVC_{max} vs. Post-BRJ: 28 ± 1%CVC_{max}, $p<0.05$). Figure 4 shows theindividual responses (thin, dashed lines) and group mean (± SEM) data (thick, solid line) depicting contribution of %NOS-dependent vasodilation following three days of BRJto the plateau phase (i.e. NO component) of the cutaneous thermal hyperemic response to local heating. Following three days of BRJ, there was a significant increase in the NO contribution to the plateau of cutaneous thermal hyperemia (Pre-BRJ: 57 ± 3%CVC_{max} vs. Post-BRJ: 64 ± 2%CVC_{max}, $p<0.05$). The modest increase in NOS dependent vasodilation was due entirely from a greater decrease in %CVC_{max} to L-NAME infusion during the plateau phase at control sites (Figure 3). As can be seen in Figure 4, most subjects showed a slight increase in NOS-dependent vasodilation from pre- to post-BRJ with one subject showing a robust (~20%) increase. Taken together, these data suggest BRJ supplementation augments the NOS-dependent component of the cutaneous thermal hyperemic response to local heating in healthy humans.

Chapter 4 - Discussion

To our knowledge, this study is the first to investigate the effects of nitrate supplementation via BRJ on cutaneous thermal hyperemia during local heating in healthy humans. The primary finding of this study suggests nitrate supplementation modestly increases NOS-dependent vasodilation. Although we observed no significant effect of nitrate supplementation on the initial peak or secondary plateau phase of cutaneous thermal hyperemia with three days of BRJ, we did find a greater decrease in CVC in response to L-NAME infusion during the plateau at control sites. This suggests nitrate supplementation provides modest improvements to the NOS-dependent component of cutaneous thermal hyperemia and the augmented post L-NAME drop accounts for the entirety of the increase in the NOS-dependent contribution (Figure 3). Inasmuch as the secondary plateau did not differ from pre-BRJ to post-BRJ, we conclude the increase in NOS-dependent vasodilation was due to further reductions in the post-LAME drop.

Kellogg *et al.* (2008, 2009) effectively demonstrated that endothelial nitric oxide synthase (eNOS) was the primary NOS isoform responsible for NO generation during local heating in the skin. Although we used a non-specific NOS inhibitor (L-NAME) in this study, data from Bruning *et al.* (2012) confirmed the observations of Kellogg *et al.* (2008, 2009), which suggests our data are indicative of an increase in eNOS-dependent vasodilation. In the context of eNOS and dietary nitrate supplementation, studies have demonstrated that eNOS can reduce NO_2^- to NO during anoxia/hypoxia (i.e. low O_2 levels) (Gautier *et al*. 2006; Vanin *et al*. 2006). Although local heating of the skin is not expected to reduce PO_2, it is possible changes in blood temperature and/or skin temperature may affect the ability of eNOS to produce nitric oxide and nitrate supplementation may augment the ability of eNOS to produce NO. The NO-dependent

vasodilation during local heating of the skin in healthy humans is robust and, as such, changes in the ability of eNOS to produce NO during local heating would appear to be minimal. However, our present data demonstrating a modest improvement in NOS-dependent vasodilation suggests nitrate supplementation may improve eNOS function. More research is needed to determine how changes in temperature (blood, skin, etc.) and nitrates interact with eNOS.

It has been proposed the local increase in temperature may increase bioavailable NO through the stimulation of endothelial cells in turn augmenting NO generation by increasing eNOS activity (Kellogg *et al*. 2008). It is possible that local heat application may increase the activity of heat shock protein 90 (HSP90), a signaling protein that can stimulate eNOS activity (Shah *et al.* 1999). Shastry *et al.* (2002) have shown the HSP90 inhibitor geldanamycin attenuates the secondary plateau by ~20% suggesting that HSP90 contributes to cutaneous thermal hyperemia and may enhance eNOS function and NO generation. To our knowledge, there has been no studies investigating a potential interaction between HSPs and nitrate supplementation; however, it is possible in the present study that dietary nitrates enhance HSP90 activity and, in turn, aid in the function of eNOS to produce nitric oxide. Future studies examining a potential interaction between eNOS and HSP90 while supplementing with nitrates is warranted in order to support this concept.

Although we observed a modest increase in NOS-dependent vasodilation, it is possible we did not observe a greater response due to a 'ceiling effect'. In the present study, young healthy subjects had plateau values reaching ~90%CVC_{max} and higher suggesting minimal room for improvement. Minson *et al*. (2002) with healthy aging, have demonstrated, that the secondary plateau phase and contribution of NO is attenuated compared with healthy young subjects. Minson and colleagues therefore concluded that healthy aging diminishes cutaneous

microvascular function by reducing NO responsiveness and/or the production of NO itself. It is possible greater increases in NOS-dependent and independent vasodilation may be observed in populations with reduced responses, such as aging.

Previous studies have shown the ingestion of nitrates can result in an increase in circulating nitrite levels (Lundberg *et al.* 2004). Further reductions of nitriteto NO can occur through various pathways and reduction mechanisms and can be introduced to the systemic circulation where NO can promote vascular smooth muscle relaxation and improve blood flow (Cosby *et al.* 2003). Nitrate supplementation has also been showed to improve exercise tolerance in peripheral artery disease (PAD) patients (Kenjale *et al*. 2011), lower the oxygen cost of exercise (Bailey *et al.* 2009; Larsen *et al*. 2007), and improve skeletal muscle blood flow (Ferguson *et al.* 2013). It is important to note the aforementioned studies demonstrated the effects of nitrate supplementation in the systemic circulation, whereas the present study and its' primary outcomes are directed predominantly to the microvasculature of the cutaneous circulation. Although we observed a minimal increase in microvascular blood flow, we did observe significant reductions in DBP, MAP, and triglycerides, which is consistent with previous data (Kapil *et al.* 2010, Webb *et al.* 2008, and Zand *et al.* 2011), and suggests our dosing of BRJ/nitrates was effective.

Experimental Considerations

There are at least two limitations that need to be addressed in the present study. First, this study specifically incorporated male subjects in order to reduce skin blood flow variability due to changes in sex hormone levels across menstrual cycle and oral contraceptive phase in female subjects (Charkoudian *et al.* 2010). It is possible that reproductive hormones could affect NO bioavailability. To our knowledge there have been no studies investigating the relationship

between reproductive hormones and dietary nitrates. Second, the oral dosage or concentration of BRJ (0.4g of nitrates; ~5mM/day) supplemented could be insufficient in providing systemic effects and could possibly explain why we observed no change in the initial peak and secondary plateau phase of cutaneous thermal hyperemia during local heating. This seems unlikely inasmuch as three days of BRJ supplementation resulted in an approximately 10mmHg decrease in DBP and MAP and a decrease in triglycerides of ~25mg/dL in the blood. These findings suggest the dietary intervention was effective in providing an adequate amount of nitrate demonstrated by systemic and blood lipid profile improvements. However, we cannot rule out the possibility of the dietary supplement being diluted once it reached the cutaneous vasculature. For example, Wong *et al*. (2004) demonstrated that intradermal microdialysis infusion of an H_1 histamine antagonist attenuated the skin blood flow response to passive heat stress whereas an oral dose of antihistamine had no effect on skin blood flow during heat stress. This suggests doses of drugs administered orally may not reach the skin in sufficient concentration to have an effect.

Conclusion

This is the first mechanistic study to investigate the effects of nitrate supplementation via BRJ on cutaneous thermal hyperemia during local heating in healthy subjects. Our findings suggest that nitrate supplementation provides a modest improvement of the NOS-dependent contribution of cutaneous thermal hyperemia. Future research is needed to better understand the effects of dietary nitrate supplementation on the skin blood flow response during local heating in not only healthy humans, but also patient populations where nitrate supplementation may have more marked effects in NO-mediated vasodilation.

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Table 1.

Blood Pressure Data

	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)
Pre-BRJ	122 ± 2	74 ± 1	90 ± 1
Post-BRJ	118 ± 2	61 ± 2*	80 ± 2*

Values are means ± SEM; n = 6. Hemodynamic variables are presented Pre-BRJ and Post-BRJ supplementation. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; * P<0.05 vs. Pre-BRJ

Table 2.

Blood Lipid Profiles & CRP

	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	TG (mg/dL)	Glucose (mg/dL)	CRP (mg/dL)
Pre-BRJ	154 ± 13	78 ± 12	81 ± 20	95 ± 12	95 ± 4	0.46 ± .07
Post-BRJ	156 ± 11	62 ± 10	87 ± 13	72 ± 6*	102 ± 7	0.51 ± .07

Values are means ± SEM; n = 6. Blood lipid profiles were taken prior to and after BRJ supplementation. Total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG), C-reactive protein (CRP) * P<0.05 vs. Pre-BRJ.

Table 3.

Absolute Maximal CVC Values	
Treatment Site	**Maximal CVC**
Control Pre-BRJ	1.81 ± 0.21
L-NAME Pre-BRJ	1.93 ± 0.17
Control Post-BRJ	1.61 ± 0.14
L-NAME Post-BRJ	1.70 ± 0.18

Values are mean ± SEM. There was no statistical difference between maximal CVC Pre-BRJ or Post-BRJ supplementation in either control or L-NAME sites.

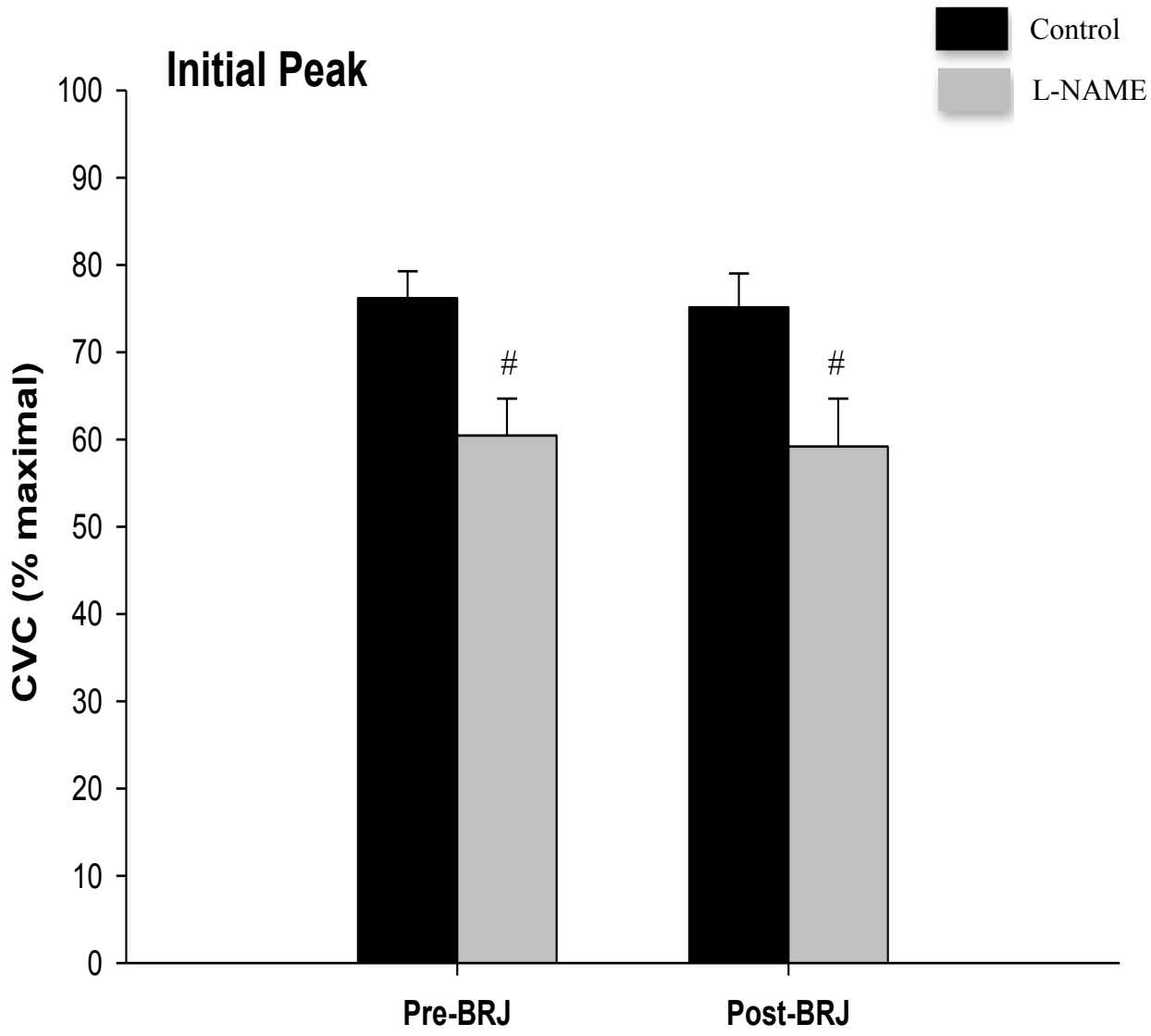

Figure 1. Group mean (± SEM) CVC data depicting initial peak following three days of BRJ
# P<0.001 vs. Control

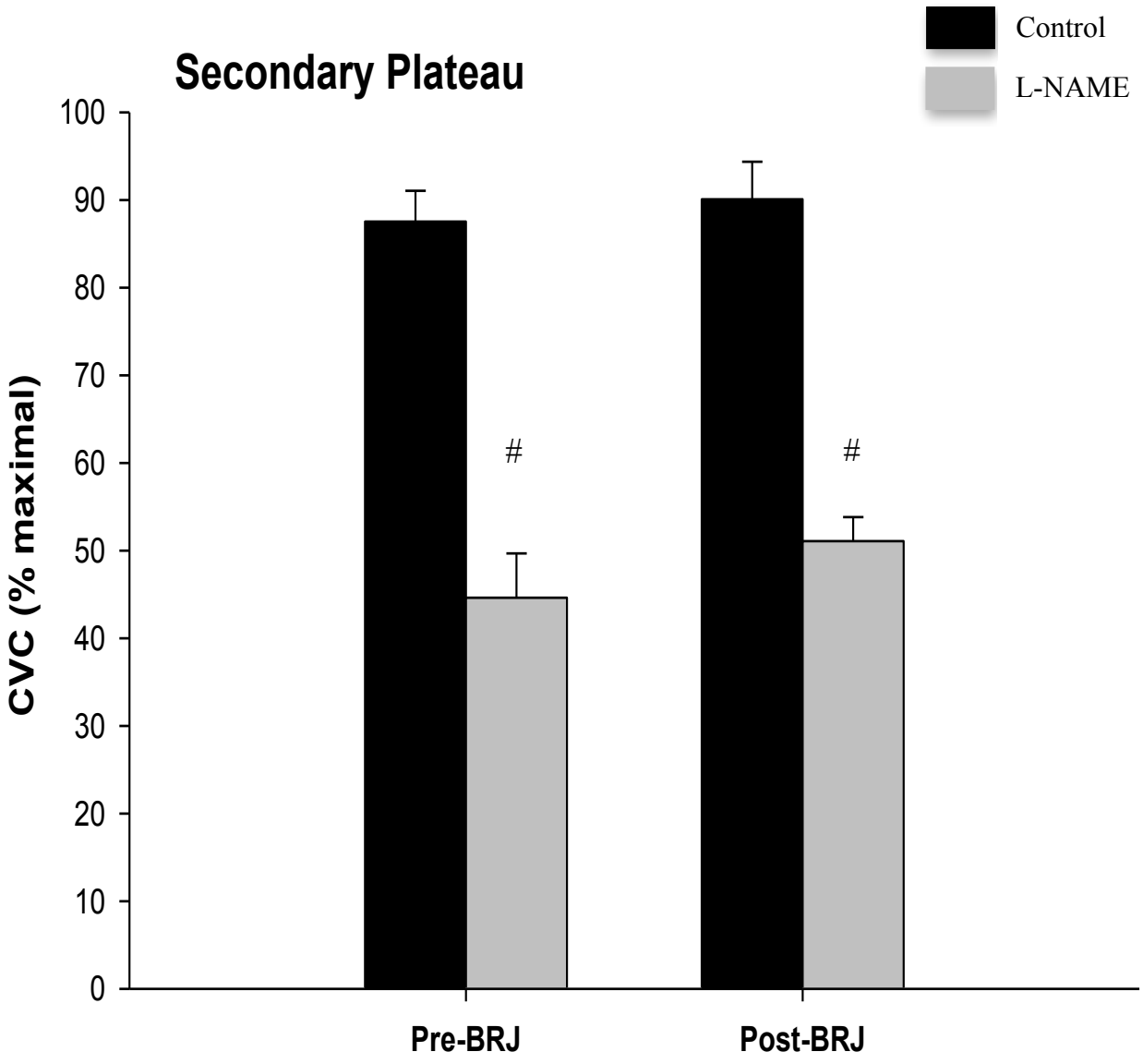

Figure 2. Group mean (± SEM) CVC data depicting secondary plateau following three days of BRJ.# $p<0.001$ vs. Control.

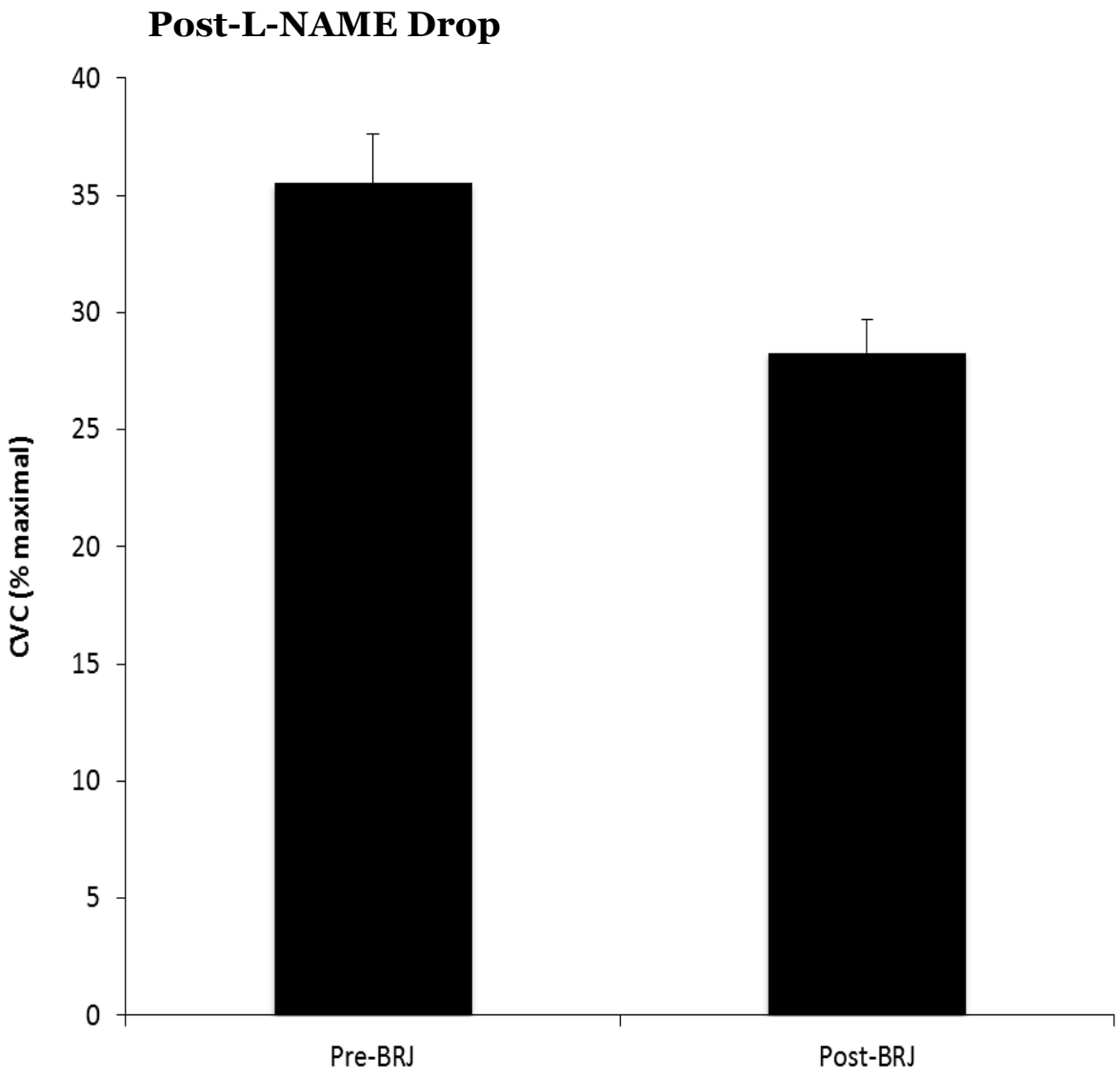

Figure 3. Group mean (± SEM) CVC data depicting post-LNAME drop following three days of BRJ.* $p<0.05$ vs. Pre-BRJ.

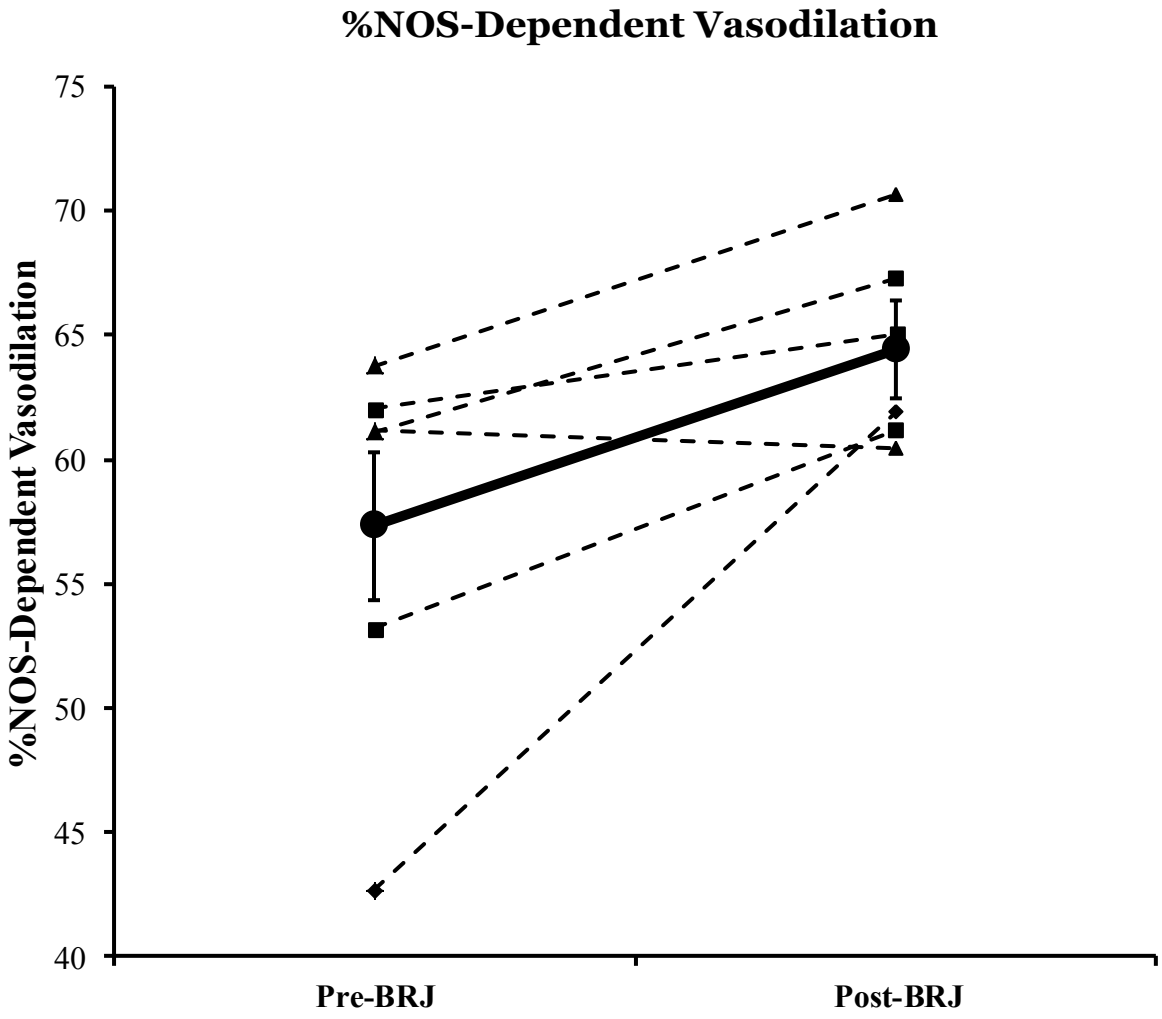

Figure 4. Individual responses (thin, dashed lines) andgroup mean (± SEM) data (thick, solid line) depicting contribution of %NOS-dependent vasodilation following three days of BRJ.
* $p<0.05$ vs. Pre-BRJ for the group data.