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The effect of betaine on nitric oxide and cardiovascular response to exercise

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THE EFFECT OF BETAINE ON NITRIC OXIDE AND CARDIOVASCULAR RESPONSE TO EXERCISE

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by

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CERTIFICATE OF APPROVAL

MASTER OF SCIENCE THESIS

This is to certify that the Thesis of
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submitted in partial fulfillment of the requirements for the degree of
Master of Science in the School of Health Sciences and Human Performance
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ABSTRACT

Objective: The purpose of this study was to investigate the effects of acute betaine (BT) supplementation, and exercise, on plasma nitric oxide (NO) levels and the related cardiovascular response (CVR). **Background:** BT is a nutrient found in foods such as wheat germ, bran, spinach, and seafood. It is thought that BT supplementation will improve athletic performance by, among other pathways, increasing endothelial NO production. **Methods:** This study followed a placebo controlled, double-blind, repeated measures design. Placebo and BT trials were administered in a cross-over, partially randomized, and counterbalanced fashion. Subjects consumed either a 250 ml placebo (carbohydrate-electrolyte beverage, CHO) or 250 ml CHO + 2.5 g BT. Subjects sat for 45 min then cycled for 30 min at 60 rpm with a resistance of 2.5% body weight (kg). Blood was drawn before and 45 min after BT supplementation, and immediately post exercise, to assess plasma NO. HR and BP were also measured. **Statistical Analysis:** A 2 x 3 repeated measures ANOVA across treatments (CHO + BT and CHO) and times (-45, 0 and 30 min) assessed differences in NO. Another 2 x 5 repeated measures ANOVA across the same treatments and times (-45, -15, 0, 15, and 30 min) assessed differences in HR, BP, and MAP. Significant results were further analyzed using multiple pairwise comparisons with Bonferroni adjustments and alpha was set at 0.05. **Results:** No significant interactions or differences between groups were found for plasma NO levels or CVR variables with acute BT supplementation. A significant time effect ($P \leq 0.013$) for all CVR variables was found and expected due to the effect of exercise. **Conclusions:** Acute BT supplementation did not increase plasma NO levels or alter CVR at rest or during light to moderate cycling.

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Chapter 1

INTRODUCTION

Betaine (BT) is a naturally occurring compound found in many foods such as wheat germ, bran, spinach, beets, shrimp and other seafoods (Sakamoto, Nishimura, Ono, & Sakura, 2002; Zeisel, Mar, Howe, & Holden, 2003). BT supplementation improves athletic performance by enhancing metabolism when consumed with a carbohydrate-electrolyte fluid replacement drink. For example, BT supplementation improved selected anaerobic power performances (Czapla, Pryor, Swensen, & Craig, unpublished data, 2009; Hoffman et al., 2009; Maresh el al., 2007). The ergogenic effect of BT is likely a product of an ability to donate methyl groups thereby upregulating creatine synthesis in skeletal muscle (Borsook & Dubnoff, 1940; Craig, 2004). BT is also an osmolyte, which plays an important role in fluid homeostasis, possibly protecting cells against dehydration when electrolyte levels or temperatures are extreme (Craig; Sizeland, Chambers, Lever, Bason, & Robson, 1993). Even though it is an osmolyte, current research on BT enhancing aerobic performance in hot conditions is inconclusive (Armstrong et al., 2008; Klasing, Adler, Remus, & Calvert, 2002; Millard-Stafford, 2005).

In addition to these potential roles, BT has also been theorized to upregulate gene expression of nitric oxide synthase (NOS) resulting in increased nitric oxide (NO) availability (Jallel-Messadek, 2007). NO is a key factor in maintaining proper flow-mediated vascular tone. In addition to causing vasodilation, NO also inhibits vasoconstriction and platelet and leukocyte aggregation to vascular endothelium. Further, NO mediates precursors of inflammation, neurotransmission, muscle contraction,

glucose uptake and metabolic regulation (Detopoulou, Panagiotakos, Antonopoulou, Pitsavos, & Stefanadis, 2008; Kingwell, 2000; Mellion et al., 1981). These functions and properties make NO a potentially important factor in cardiovascular health. Research has demonstrated a positive linear relationship between exercise intensity and NO production (Pogliagi, Krasney, & Pendergast, 1997; St. Croix, Wetter, Pegelow, Meyer, & Dempsey, 1999). The cardiovascular response (heart rate, blood pressure, and blood flow distribution) to exercise is directly proportional to intensity (Laughlin, 1999). Although currently debated in the literature, elevated NO could contribute to greater hyperaemia in working muscles, thereby potentially improving exercise performance (Clifford & Hellsten, 2004; Kingwell, 2000; Tschakovsky & Joyner, 2008).

While the exact pathway of increasing NO via BT supplementation is yet to be fully elucidated, research shows that BT alters plasma NO. For example, 6 $g \cdot d^{-1}$ of BT increases resting levels of plasma NO from 28.8 ± 3.4 μM to 82.3 ± 13.2 μM after one week of supplementation (Iqbal et al., 2006). Increased NO availability through BT supplementation may be advantageous from both a health (e.g., anti-inflammatory, cardiovascular benefits, immune and male sexual function) and exercise performance standpoint (e.g., vasodilation promoting exercise hyperemia results in improved clearance of muscle metabolites and substrate delivery). However, little is known about the combined effects of acute BT supplementation and exercise on plasma NO levels.

It is possible that exercise and NO may work synergistically to elevate plasma NO. Accordingly, BT supplementation could benefit both exercise performance and cardiovascular health through association with NO. The aim of this investigation was to determine if acute BT supplementation alters resting plasma NO. An additional aim was

to examine the effects of acute BT supplementation combined with exercise on plasma NO. The final purpose of the study was to determine if acute BT administration (with potential NO effects) alters the cardiovascular response to exercise.

Statement of Purpose

The purpose of this study was to investigate the effects of acute BT supplementation, and exercise, on plasma NO levels and the related cardiovascular response.

Hypothesis

The hypotheses of this study were:

1. Acute supplementation of BT (2.5 g in 250 ml CHO solution) increases resting plasma NO levels more than a 250 ml CHO solution alone after 45 min.
2. Acute supplementation of BT (2.5 g in 250 ml CHO solution) plus exercise increases exercise plasma NO levels more than CHO plus exercise.
3. Acute supplementation of BT (2.5 g in 250 ml CHO solution) causes a different resting cardiovascular response (i.e., HR, SBP, DBP and mean arterial blood pressure (MAP)) than CHO solution alone after 45 min.
4. Acute supplementation of BT (2.5 g in 250 ml CHO solution) plus exercise will cause a different cardiovascular response (i.e., HR, SBP, DBP and MAP) to exercise than CHO plus exercise.

Assumptions of The Study

1. BT increased endogenous production of NO by methylating NOS.

2. The 45 min resting period after BT supplementation allowed for peak circulating BT.
3. The colorimetric assay measuring nitrate plasma levels were representative of total NO production.

Delimitations of The Study

1. Exercise consisted of 30 min of cycling at 60 rpm with 2.5% body weight (kg) pedal resistance.
2. The subjects were healthy, recreationally active college-aged males.
3. A colorimetric nitrate assay was used to determine NO levels in the plasma.
4. Supplementation was a single dose of BT (2.5 $g \cdot d^{-1}$) in a 250 ml aqueous CHO solution given 45 min before exercise.
5. Plasma NO was measured 45 min prior to, immediately before (0 min), and immediately after the 30 min of exercise.
6. Cardiovascular measures of HR, SBP, DBP and MAP were made 45 and 15 min prior to the exercise bout and at 0, 15 and 30 min during the exercise bout.

Limitations of The Study

1. Results may not be generalizable to exercise other than cycling at 60 rpm with 2.5% body weight (kg) pedal resistance.
2. Results may not be generalizable to populations other than healthy, recreationally active college-aged males.
3. Colorimetric nitrate assay techniques may not measure total plasma NO levels.

4. BT doses, other than 2.5 $g \cdot d^{-1}$ in a 250 ml CHO solution, may produce different NO levels and cardiovascular response to exercise.
5. NO was measured 45 min prior to, immediately before (0 min), and immediately after the 30 min exercise which may be generalizable to NO production at other times.
6. Results may not be generalizable to cardiovascular responses other than HR, SBP, DBP and MAP (e.g., blood vessel diameter and stroke volume) or to measurements taken at other times.

Definition of Terms

1. Acute BT supplementation- a single dose of BT (2.5 g) plus CHO in a 250 ml aqueous solution.
2. Healthy college-aged- free from any orthopedic, metabolic, viral, bacterial or genetic disease and between the ages of 18 and 26 years.
3. Recreationally active- participating in light to moderate exercise ≥ 3 per week and not part of, or engaged in, a collegiate sport or periodized endurance or resistance training program.
4. Cardiovascular response- the HR, BP and MAP reaction to the stress of physical activity.
5. Ergogenic aid- any substance used with the intention of, or may result in, improving exercise performance.

Summary

Although not fully elucidated, the extant literature on BT implies several ergogenic properties. These may include its role as an osmoprotectant, methyl group

donor, and putatively upregulating NOS gene expression. Numerous studies show physical activity increases NO production. If BT further increases NO availability during exercise (i.e., a cumulative or synergistic effect), both exercise performance and vascular health may benefit. The combined effect of acute BT supplementation plus exercise NO warrants study.

Chapter 2

REVIEW OF LITERATURE

Introduction

Research on the potential ergogenic effects of BT is at its genesis. Among the putative ergogenic effects, BT may upregulate NOS gene expression resulting in increased NO levels, yielding a potential health and exercise performance boon. NO is a key mediator in vasodilation and vascular tone. Vasodilation and corresponding hyperaemia to working muscles is essential for exercise performance. The chemical and metabolic properties of BT and NO will be explored in this chapter as well as the relationship between BT and NO. The specific topics for this review are: (1) betaine, (2) ergogenic effects of betaine supplementation, (3) nitric oxide, (4) exercise and the nitric oxide response, and (5) betaine and nitric oxide.

Betaine

BT is a naturally occurring compound found in many foods such as wheat germ, bran, spinach, beets, shrimp and other seafoods (Sakamoto et al., 2002; Zeisel et al., 2003). BT is also endogenously synthesized from choline containing compounds via oxidation pathways (Craig, 2004). Otherwise known as a trimethyl glycine, BT is a derivative of the amino acid glycine and a quaternary ammonium compound with zwitterionic properties (net charge of zero and pH 7.0). As a methylamine, BT contains three chemically reactive methyl groups which allows for methyl group donation (Craig; Yancey, Clark, Hand, Bowlus, & Somero, 1982). BT provides transmethylation in the methionine cycle decreasing the concentration of homocysteine in the body, a role reserved for only BT or folate.

Craig (2004) estimated that the average dietary intake of BT in humans is 1 to 2.5 $g \cdot d^{-1}$, with the higher end of this range seen in diets high in seafood and whole wheat. BT is absorbed from the duodenum and peaks in the serum roughly one hour postprandial, typically reaching 20-70 $\mu mol \cdot L^{-1}$ in humans depending on the meal (Craig; Lever et al., 2002). BT is metabolized fairly quickly in the liver in both single (0.43 $mmol \cdot kg^{-1}$ dose has an elimination half-life of 14.4 ± 7.2 h) and multiple dosages (0.43 $mmol \cdot kg^{-1}$ dose every 12 h over a 5 d period has an elimination half-life of 41.2 ± 13.5 h) (Schwahn et al., 2003).

Betaine Metabolism

In the liver, BT is catabolized as a substrate by the enzyme betaine-homocysteine methyltransferase (BHMT, EC 2.1.1.5) producing the byproduct dimethylglycine (DMG). BHMT catalyzes the remethylation of homocysteine to methionine thereby increasing methionine, which is required for protein synthesis; as a result, this process decreases homocysteine concentrations. Studies show that BT provides the necessary methyl group for methionine to methylate glycocyamine (guanidoacetic acid) in the production of creatine in skeletal muscle (Borsook & Dubnoff, 1940; Vigneaud, Simonds, Chandler, & Cohn, 1946). This methylization process posits BT as an ergogenic aid by increasing the production of creatine in skeletal muscle (Borsook & Borsook, 1951; Craig, 2004; Vigneaud et al., 1946).

When not metabolized, BT acts as an organic osmolyte maintaining osmotic homeostasis (Alfieri et al., 2006; Caldas, Demont-Caulet, Ghazi, & Richarme, 1999; Olsen, Ramlov, & Westh, 2007; Sizeland, Chambers, Lever, Bason, & Robson, 1993). In this capacity, BT plays an integral role in sustaining water balance, cell volume and

structure *in vivo*. These osmoprotectant properties suggest prevention of dehydration and protein denaturation, which could theoretically improve aerobic, anaerobic, strength, and power performance (Craig, 2004).

Betaine and Health Promotion

Betaine has been extensively researched in human and animal clinical studies for health promoting potential. As discussed in a review by Eklund, Bauer, Wamatu, and Mosenthin (2005), BT improved carcass composition (lean mass, fat mass ratio) in porcines, rats, and poultry to name a few (Fernández-Fígares et al., 2002; Hayes, Pronczuk, Cook, & Robbins, 2003; Wray-Cahen, Fernández-Fígares, Virtanen, Steele, & Caperna, 2004). BT also improved exercise recovery time in untrained horses (Warren, Lawrence, & Thompson, 1999). These effects in animals generated interest in exploring possible health and performance enhancements from BT in humans.

To that end, BT has been used for the treatment of non-alcohol steatohepatitis, hepatic ethanol induced seatosis, and decreasing risk factors to heart health (Abdelmalek, Angulo, Jorgensen, Sylvestre, & Lindor, 2001; Barak, Beckenhauer, Junnila, & Tuma, 1993; Borsook & Borsook, 1951; Graybiel & Patterson, 1951; Mukherjee et al., 2005). Recently, Xu et al. (2009) analyzed data from the Long Island Breast Cancer Study Project and suggested high intake of BT and choline may be a prophylactic strategy to avoid breast cancer. Conversely, Olthof, van Vilet, Verhoef, Zock, and Katan (2005) theorized BT may increase low density lipoprotein (i.e. bad cholesterol) concentrations, however, as reviewed by Zeisel (2006), these results are preliminary and require further investigation. Wilcken, Wilcken, Dudman, and Tyrrell (1983) was the first to show that BT lowers serum homocysteine in subjects with homocystinuria. Interestingly,

inadequate amounts of BT increase the risk of distal colorectal adenoma in females and exacerbate metabolic syndrome and cardiovascular disease risk (Cho et al., 2007; Konstantinova et al., 2008). It is clear from these studies that BT may be involved in a number of pathways integral to maintaining optimal health.

Ergogenic Effects of Betaine Supplementation

The ergogenic potential of BT is derived from the ability to function as an osmolyte, methyl donor, and upregulator of endothelial nitric oxide synthase (eNOS). Putative performance enhancements, therefore, include prevention of dehydration, improved cardiorespiratory functions, increased skeletal muscle creatine concentration, and increased vasodilation in active muscles via stimulation of eNOS gene expression. In addition, an emerging use of BT is for the ability to reduce inflammation. In 2008, Detopoulou et al. (2008) demonstrated the potential role of BT (>360 $mg \cdot d^{-1}$) supplementation to attenuate (10 - 19% decrease) inflammatory markers in healthy adults compared to lower doses of BT supplementation (<260 $mg \cdot d^{-1}$ and 260 - 350 $mg \cdot d^{-1}$). Reduction of inflammatory compounds would benefit cardiovascular health. Furthermore, sports performance may be enhanced by attenuating recovery periods and potentially increasing playing time due to reduced inflammation.

Osmoprotectant Role and Effects on Cardiovascular Exercise

BT is an osmolyte that defends osmotic pressure by raising pressure in the cytoplasm and stabilizing proteins and cell membranes when dehydration, electrolyte levels, or temperatures are extreme. In particular, during a dehydration state, osmolytic compounds such as BT attenuate water loss against larger osmotic gradients (Klasing et al., 2002). In animals, BT can protect fish as they swim through waters with varying

degrees of salinity (Virtanen, 1995). In humans, during an exercise bout, a salinity oscillation effect may occur secondary to hydration levels. Armstrong et al. (2008) investigated the effects of BT on strenuous running and sprinting to exhaustion in a heated environment (31.1° C). They hypothesized the osmoprotectant and cardiovascular benefits of BT would improve endurance training in the heat. After a dehydration period, ten male runners were rehydrated with either: 1 L water (W), W + BT (5 $g \cdot L^{-1}$), an electrolyte carbohydrate containing fluid (CHO), or CHO + BT (5 $g \cdot L^{-1}$). Subjects ran for 75 minutes at 65% VO_2max followed by a timed performance run at 84% VO_2max to volitional exhaustion. The CHO + BT time to exhaustion was greater than CHO by 32 s (16%) and W + BT time to exhaustion was greater than W by 38 s (21%). The CHO + BT solution also increased oxygen consumption 4-5% more than CHO at the same workloads, thereby effectively increasing energy production via aerobic metabolism. Interestingly, there was greater plasma volume loss in subjects who consumed W + BT versus W, even though BT is suggested to attenuate and even protect against plasma volume loss and dehydration. Investigators speculate, however, that chronic BT supplementation may elicit different results than acute ingestion.

In another study, Millard-Stafford (2005) found cycling performance increased 10% with a 6% CHO solution and 14% with a 7% CHO + BT solution compared to W. Subjects cycled for 120 min varying between 60 - 75% VO_2max followed by a 15 min time trial. The cycling protocol is representative of typical race-day experiences and supplementation with CHO + BT was more effective than CHO or W alone at improving cycling time trials. Future animal and human studies are needed to elucidate the role of BT as an osmoprotectant and proponent of cardiovascular exercise.

Other Potential Effects on Aerobic Activity

Human and animal studies show trends that BT improves metabolic and chemical indices important to cardiovascular activity. The beneficial mechanisms of BT may involve affecting plasma lactate metabolism, fatty acid metabolism, protection of citrate synthases from thermo-denaturation, and decreasing homócysteine (Craig, 2004). Researchers ran trained and untrained horses to volitional fatigue then measured blood lactate post exercise. Supplementation with BT for 14 d reduced plasma lactate at 60 min of recovery in the untrained horses (Warren et al., 1999). These data suggest BT may be beneficial for lactate metabolism in the untrained; however, BT was not effective in trained horses under the same conditions. Although improved lactate recovery in untrained horses elucidates another possible ergogenic property of BT, the generalization of results to human performance may be limited.

Other research by Penry & Manore (2008) suggests the ergogenicity of BT supplementation may improve aerobic performance by decreasing homocysteine, a byproduct of choline interaction. High levels of homocysteine are produced during long endurance exercise, these high homocysteine levels might increase BT mediated methyl metabolism. Further human studies are needed to confirm or refute the role of BT in endurance exercise performance.

Regardless of any ergogenic potential of BT, Craig (2004) suggested recovery rehydration with BT would promote blood volume changes, lower LDL and homocysteine levels, and thereby begin preparation for future training sessions. Restoring lost fluids, improving cholesterol profiles, and maintaining safe plasma homocysteine levels along with exercise should also be advantageous to cardiac health.

Betaine and Anaerobic Performance

Little is known about the exact mechanism(s) by which BT improves "anaerobic" performance. Studies suggest that in the mitochondria of the liver and kidney, a series of enzymatic reaction occur by which BT provides an essential methyl groups in the production of creatine via the methionine cycle (Borsook & Dubnoff, 1940; Vigneaud et al., 1946). Although this has not been established, increases in muscle creatine and protein syntheses would provide additional energy and protein synthesis for "anaerobic" activity theoretically benefiting power, strength, and force output.

Millard-Stafford (2005) found CHO + BT supplementation attenuated loss of force in isometric knee extension compared to W and CHO after 120 min cycling and 15 min time trial. W lost 16.8-20.8% force, CHO lost 14.3 - 18.3% force, and CHO + BT lost 11.1 - 14.5% force compared to baseline measures. In other studies using the same subject population characteristics (recreationally active, college-aged students), Maresh et al. (2007) and Hoffman et al. (2009) explored the relationship between BT supplementation and "anaerobic" performance and found diverging results. Hoffman et al. recruited 24 males that underwent 15 d of BT supplementation (1.25 g in 240 ml solution twice per day). Subjects were tested on muscle endurance, power, and rate of fatigue indices, but no improvements in power measures (Wingate anaerobic test, vertical jump, and bench press throw) or upper body exercises were observed. The researchers did find BT improved muscle endurance and quality of repetitions at 90% of the subject's one repetition maximum during a squat exercise. Interestingly, these benefits were apparent within only seven days of BT supplementation.

Maresh et al. (2007) had 12 male subjects, with at least a three month history of resistance training which included the squat exercise, consume 1.25 g BT with 240 ml Gatorade twice per day for 14 d. Before and after supplementation, the subjects performed a series of typical power and strength tests. BT significantly increased isometric squat force by 10.4%, isometric bench press force by 19.8%, and bench press throw by 13.7%. BT supplementation did not alter muscular endurance (repetitions), vertical jump, or jump squat power. Unlike Hoffman et al. (2009), the results from Marsh et al. clearly indicated that BT improves strength and power performance. It remains unclear, however, why BT had a selective effect rather than a more constant and positive influence on such measures. Perhaps improvements may be attributed to the practice effect if the measures (e.g. bench press or squats) are part of the subjects training protocol.

Recently, Czapla et al. (2009, unpublished data) had 16 untrained subjects perform a series of four cycling sprints (12 s each) after one week of BT supplementation (1.25 g BT per 295 ml of CHO beverage twice a day). Peak power average, peak power maximum, mean power average, and mean power maximum increased by 6.4%, 5.7%, 5.4%, and 4.4%, respectively, compared to baseline. There were no changes in the placebo group, which consumed 295 ml of CHO beverage twice a day. In summary, BT shows promising results for improving "anaerobic" performance. The exact mechanism that underlies this effect needs to be further explored.

Nitric Oxide

Furchgott & Zawadski (1984), in their seminal study, discovered endothelial cells released many "endothelial derived relaxing factors" or EDRF. Subsequent research showed EDRF to be the highly reactive, short-lived gaseous compound NO. NOS catalyzes the synthesis of NO from the precursor L-Arginine resulting in the byproduct L-citrulline. Co-substrates to this reaction include O_2 and nicotinamide adenine dinucleotide phosphate (NADPH). Co-factors to this reaction include: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin, heme, and calcium dependent calmodulin (Reid, 1998).

There are three NOS isozymes: type I, type II, and type III. Type I or neural NOS, is equated with muscular function and metabolism and is located in the sarcolemma, but also expressed in the brain, peripheral nerves, spinal cord, and sympathetic ganglia. Type II or inducible NOS, is equated with immune function involving cytokine stimulation. Type III or eNOS, is expressed by vascular endothelial cells, smooth and cardiac muscle, and the brain. Neural and eNOS are constitutively expressed, low output isozymes, however, once synthesized by these isozymes, NO reacts rapidly as a signaling mechanism for both coronary and peripheral hemodynamic regulation (Kingwell, 2000; See Reid, 1998, for a complete review).

NO has a variety of important biological functions, among them is a capacity to cause vasodilation, inhibit platelet and leukocyte aggregation to vascular endothelium, inhibit vasoconstriction, and improve immune function (Kingwell, 2000; Mellion et al., 1981). It is evident from both human and animal studies th at NO specifically induces vasodilation of the arteriole smooth musculature.

Production of Nitric Oxide

The following is an outline of purported mechanisms in NO release: (1) basal release from the endothelial cells, (2) the shear force stress on endothelial vascular cell membranes caused by friction of blood circulation (viscosity), and (3) metabolites and other substances (e.g., hydrogen ions, carbon dioxide, oxygen, potassium, ATP, phosphate and magnesium). Metabolites and substances from muscle or neural structures increase NO release, which dilate surrounding vessels, thereby increasing blood flow and consequently further enhance endothelial shear force release of NO. Skeletal muscle also contains both neural and eNOS isozymes allowing for tissue release of NO (Burnett & Lowenstein, 1992; Joyner & Tschakovsky 2003; Kobzik, Stringer, Balligand, Reid, & Stambler, 1995; Martin, Beltran-Del-Rio, Albrecht, Lorenz, & Joyner, 1996; Toda, 1995). NO releasing mechanisms demonstrate situation specific activation, further, the abundance of NO producing modes indicate its importance in maintaining appropriate blood vessel tone.

Specific NOS isozymes are upregulated under certain conditions. For example, the constitutive upregulation of inducible NOS requires gene transcription, which delays NO production from this isozyme several hours after exposure to stimuli. eNOS is also a constitutive form of NOS, active in immediate production of NO. In the vascular system, for example, eNOS is the primary isoenzyme responsible for NO production secondary to shear stress and other metabolites such as PO_2, PCO_2, hydrogen ions, potassium, phosphate, and magnesium. Other purported conditions and pathways that elicit NO from eNOS include: potassium activation by blood flow as a result from deformation of cytoskeletal elements, calcium influx in endothelial cells, ATP release from erythrocytes,

bradykinin release, and phosphorylation of a serine residue altering eNOS affinity to intercellular calcium (Maiorana, O'Driscoll, Taylor, & Green, 2003). Kleinbongard et al. (2006) discovered a functional eNOS within human red blood cells. This suggests that erythrocytes have the ability to contribute to NO production during intense exercise that results in local hypoxia within active skeletal muscle mass. Hemoglobin in these areas act as a sensor to oxygen levels and consequently induce NO bioactivity (Isbell et al., 2008; Suhr et al., 2009). The discovery of yet another pathway highlights the key role of NO in vasodilation, while reinforcing the idea that exercise training, even in hypoxic states (i.e., anaerobically), promotes NO synthesis.

Pharmacokinetics of Nitric Oxide Mediated Vasodilation

The pharmacokinetics of NO-mediated vasodilation was first discovered by Gruetter, Kadowitz, and Ignarro (1981) in bovine coronary artery studies. NO activates soluble guanylate cyclase in smooth muscle cells by generating a NO-heme complex (heme moiety) that binds to it causing a conformational change facilitating the conversation of 3', 5'-cyclic guanosine triphosphate (GTP) to 3'-5'-cyclic guanosine monophosphate (cGMP). Elevated intracellular cGMP causes free calcium depletion from the smooth muscle cytosolic space, thereby decreasing calmodulin activation of myosin light chain kinase. Limiting the phosporylation of myosin light chains decreases smooth muscle tone. Cyclic GMP also activates protein kinase G and phosphorylates heat shock protein 20, both of which mediate force production by inhibiting cross-bridge formation and cycling (Brophy et al., 2002; Rembold, Foster, Strauss, Wingard, & Eyk, 2000). Archer et al. (1994) has also shown cGMP-dependent protein kinase-dependant potassium channel activation causes smooth muscle relaxation.

Auto-oxidation kinetics of NO to nitrate and nitrite in aqueous solutions are dependent upon a number of factors such as the concentration of NO, diffusibility, and other bioreactant concentrations. Hemoglobin, related hemoproteins, and methylene blue inhibit NO (Ignarro, Wood & Wolin, 1984). Other bioreactants and conditions that can affect NO concentrations are the type and amount of O_2-derived radicals, PO_2, blood pH, PCO_2, red blood cells (hemoglobin), adenosine triphosphate (ATP), concentration of transition metals, magnesium, potassium, and thiols. Consequently, the half-life of NO is not constant; it ranges from 0.05-1 s in circulating blood (Kelm, 1999). The short half-life of NO in the blood necessitates a constant release of NO during physical activity. It is evident from the short half-life of NO its actions are primarily local and conducive to rapid diffusion and inactivation.

Chronic aerobic exercise improves cardiovascular health, as it lowers blood pressure, improves cardiac output, coronary blood flow, capillary density, and cholesterol profiles. Such exercise also helps prevent atherosclerosis and coronary artery disease (Thompson, 2003). Cardiovascular risk factors such as hypertension, decreased coronary blood flow and hypercholesterolemia have been associated with impaired NO release (Kingwell et al., 1997). eNOS upregulation after a relatively short duration of training may be the link to long-term structural changes that improve cardiovascular health. Methods to increase upregulation of NOS gene expression should be explored as NO is beneficial from both a cardioprotective and an ergogenic standpoint.

Exercise and the Nitric Oxide Response

The control of blood pressure is a product of cardiac output and total peripheral resistance. There are myriad vasomotor control mechanisms throughout the human body

regulating blood vessel diameter with redundant and synergistic pathways. In relation to exercise performance, the regulation of blood flow and corresponding supply of oxygen and substrates are crucial elements to success. In response to exercise, blood flow in active muscles increases up to 100 fold (Walloe & Wesche, 1988).

The vasodilatory response to exercise follows the "ascending vasodilation" phenomena whereby dilation begins at the arterioles and microvessels and propagates proximally to larger arteries (Green et al., 1996). To adequately perform at desirable levels, oxygen and substrate supply must meet the demand of the active myofilaments. This is controlled by vascular tone, blood delivery to the area and oxygen/substrate extraction capacity of target cells. NO appears to facilitate both vascular tone and blood delivery processes in coronary and peripheral circulation (Maroun, Mehta, Turcotte, Cosio, & Hussain, 1995). Performance of physical activity can be positively modulated by NO because of its integral hemodynamic and metabolic regulatory effects (Kingwell, 2000).

Studies show a direct correlation between production of NO and intensity of exercise. In five healthy subjects, during an 11.3 minute cycling bout, Matsumoto et al. (1994) found exhaled NO levels increased three-fold from resting to maximum exercise (121 ± 53 ml·min^{-1} to 398 ± 164 ml·min^{-1}). Other studies found similar trends in exhaled NO concentration during moderate, submaximal cycling protocols. For example, Maroun et al. (1995) found moderate intensity exercise increased expiratory NO from 85 pM·kg^{1}·min^{-1} to 110 pM·kg^{1}·min^{-1} in sedentary adults and 85 pM·kg^{1}·min^{-1} to 450 pM·kg^{1}·min^{-1} in an athletic population. Persson, Wiklund, and Gustafsson (1993) showed expiratory NO increased from 69 nl·min^{-1} to 225 nl·min^{-1} during a submaximal cycling

protocol. Pogliaghi et al. (1997) found maximal cycling, using 50 watt increments every 3 min until volitional exhaustion, increased exhaled NO from 137.7 ± 57.8 ppb·min^{-1} to 544.7 ± 387.0 ppb·min^{-1}.

St. Croix et al. (1999) also demonstrated the more intense the cycling bout, the greater the amount of NO release. NO production was assessed during cycle ergometry at rest and at three intensities (30, 60, and 90% VO_2max) in nine healthy subjects and found NO levels to be 42.5 ± 14.7, 43.5 ± 15.0, 39.1 ± 14.0, and 44.4 ± 12.9 [NO_3^- + NO_2^-], μmol·L^{-1}, respectively. In sum, NO production is stimulated at the onset of physical activity and the concentration is correlated to intensity.

The wide range of NO variability in response to exercise may be attributed to researchers using different units of measurement, exercise intensity, dietary control of NO, collection procedures, and the biological substances collected to obtain NO levels (e.g., serum, plasma, urine, saliva and expiratory air). Furthermore, there is variability in the assays used to measure NO, such as measuring nitrite or nitrate levels (stable end products of NO) or both in serum assays. Additionally, in ventilator measurement methods there are numerous techniques, many of which can influence NO determination. A standardized protocol for all biological mediums for NO measurement needs to be agreed on within the research community to facilitate inter-study comparison.

Although exercise clearly increases NO production, controversy remains whether NO is responsible for hyperaemia induced by exercise. In a review by Kingwell (2000) of five studies using human subjects, the author concluded that NO either plays no role in exercise hyperaemia or has a redundant role. A more plausible explanation may be that NO is part of a synergistic pathway utilizing other metabolites such as adenosine,

prostacyclin, and K^+ ATPase channels in moderating blood flow to active muscle (Clifford & Hellsten, 2004; Tschakovsky & Joyner, 2008).

Betaine and Nitric Oxide

NOS gene expression is regulated by a litany of physiological and pathophysiological stimuli such as mechanical forces, cell growth, cytokines, lipoproteins, oxidative stress and growth factors (Searles, 2006). Recently, BT has been purported to upregulate NOS gene expression through mRNA methylation by both transcriptional and posttranscriptional events (increasing constitutive NOS protein levels). BT may also stimulate L-arginine or L-lysine biotransformation through either eNOS or neural NOS enzymatic activity (Jallel-Messadek, 2007). Numerous stimuli moderate eNOS mRNA expression *in vitro*.

Recently, Jallel-Messadek (2007) theorized BT upregulates NOS gene expression of NO resulting in increased bioavailability. This increase could lead to greater hyperaemia in working muscles, thereby improving exercise performance. As a methyl group donor, BT may actively donate its methyl group in the methylation and upregulation in NOS gene expression. BT is theorized to affect only constitutive eNOS and neural NOS isozymes and not the inducible NOS isozyme. While the exact pathway to increasing NO via BT has yet to be fully elucidated, recent research shows that it does alter plasma NO. For example, 6 $g \cdot d^{-1}$ of BT increases resting levels of plasma NO from 28.8 ± 3.4 μM to 82.3 ± 13.2 μM after one week of supplementation (Iqbal et al., 2006). This is the first study to demonstrate increased NO release via BT supplementation. Considering the ergogenic and health promoting additive effects of exercise, BT and NO, future research related to how these factors interact is merited.

Summary

BT is a promising supplement with potential ergogenic properties as an osmoprotectant and methyl group donor. In addition to these properties, BT is theorized to upregulate NOS gene expression. An increased production of NO is beneficial to both cardiovascular health and enhancement of exercise performance. NO is an integral signaling molecule in maintaining myriad cardiovascular functions and vascular homeostasis. NO is a key mediator in flow-mediated vasodilation and vascular tone. The vasodilation and corresponding hyperaemia to working muscles is essential for exercise performance. Based upon the literature reviewed, research is needed to assess the role of BT plus exercise in NOS gene expression and its potential for promoting cardiovascular health and sport performance.

Chapter 3

METHODS

An overview of the study procedures will be discussed in this chapter. The aim of this study was to assess the effect of BT supplementation and exercise on plasma NO levels and cardiovascular response. Sections in this chapter include: (1) subjects, (2) experimental design, (3) procedures, (4) physiological measures, and (5) statistics.

Subjects

Ten healthy college aged males volunteered to participate in the study. All subjects were recreationally active, participating in light to moderate exercise ≥ 3 per week and not part of, or engaged in, a collegiate sport or periodized endurance or resistance training program. Subjects were excluded if they reported high blood pressure, history of diabetes, renal disease, current orthopedic injuries that would prevent cycling, allergies, illness or any other medical reason that may endanger them or affect the quality of the study. Subjects who chose to participate were made aware of risks, benefits, and protocols of the study while providing informed consent (Appendix A). A brief medical history form (Appendix B) and a 24-hour history form (Appendix C) were filled out by the subject to ensure subject compliance with exclusion criteria. Subjects did not consume any caffeine, alcohol, or any other drugs within 24 h of testing sessions and maintained current physical activity and dietary habits throughout the course of the study. Subjects completed an overnight fast and did not eat breakfast on the day of testing. To ensure hydration, subjects drank 12 oz of water prior to sleeping the night before and again on the morning of data collection. The methods of this study were approved by the Ithaca College Human Subjects Review Board.

Experimental Design

This study utilized a placebo controlled, double-blind, repeated measures design. To limit the influence of diet and relative cycling workload on BT and NO levels, a crossover design was employed so each participant was his own control. Placebo and BT (BetaPower™ Danisco A/S, White Plains, NY) trials were administered in a partially randomized, counterbalanced fashion to reduce order effects. For the two trials, the subjects consumed either a 250 ml placebo (carbohydrate-electrolyte beverage, CHO) or a 250 ml carbohydrate-electrolyte beverage with 2.5 g BT (CHO + BT). To ensure beverage anonymity, all subjects received uniform opaque sport drink bottles with cap ties broken.

Procedures

Upon subject arrival, 3.5 ml of blood was drawn to assess baseline plasma NO levels (-45 min). Subjects then consumed either the CHO or CHO + BT beverage and sat for 45 min. Based upon human digestion pharmacokinetics of BT, 45 min allows BT to reach peak levels in the plasma (0.65 - 1.3 hrs post-digestion) (Schwahn et al., 2003). During this time, subjects were free to read, study, or surf the internet. After this 45 min period, another resting blood sample was taken (0 min). On a Monark (Series 818e, Varberg, Sweden) cycle ergometer with seat height adjusted to 10-15° knee flexion, subjects completed a 30 min cycling bout at 60 rpm with resistance set at 2.5% body weight (kg). The calculated workload was analogous to light to moderate intensity exercise. Blood was drawn immediately after the 30 min cycling bout and the subjects then cooled down for 5 min against light resistance. Subjects completed a second trial in the alternate condition at the same time of day after waiting 6 to 8 d to allow for adequate

BT wash out. The same procedures were used for both data collection sessions. All testing occurred in a temperature controlled laboratory (72° F).

Physiological Measures

In the morning and upon subject arrival, euhydration ($U_{sg} \leq 1.025$ μG) was verified using a digital refractometer (KS-0050; Kernco Instruments, El Paso, TX). The subject was then fitted with a Polar HR monitor (Model S610i; Polar Electro, Kemplele, Finland), and HR data were measured during rest and cycling (i.e., -45, -15, 0, 15 and 30 min). In addition, blood pressure data were collected at the same time intervals as HR data using auscultation and sphygmomanometer.

Plasma Collection and Nitric Oxide Assay

Venous blood from an anticubital vein was drawn at rest (-45 min, 0 min) and immediately following cycling (30 min). Blood was collected in citrate containing vials and immediately centrifuged at 3500 rpm for 10 min at 4° C. Plasma was collected, aliquoted and stored at -80° C until analysis. The high reactivity and short half-life of NO make it difficult to measure directly. Therefore, the concentration of inorganic nitrate (NO_3^-), a stable end product of NO oxidation, was assayed by a colorimetric method using the Greiss method in a total NO Nitrate/ Nitrite Assay Kit (Assay Designs Inc., Ann Arbor, MI). The mean minimum detectable dose for this assay kit was 0.625 $\mu M \cdot L^{-1}$. Samples were ultra-filtrated with 10,000 MWCO filter.

Statistics

A 2 x 3 repeated measures ANOVA across treatments (CHO + BT and CHO) and times (-45, 0 and 30 min) assessed differences in plasma NO. A 2 x 5 repeated measures ANOVA across the treatments and times (-45, -15, 0, 15 and 30 min) assessed

differences in SBP, DBP, MAP and HR. Significant results were further analyzed using multiple pairwise comparisons with Bonferroni adjustments. When assumed sphericity was violated, a Greenhouse-Geisser analysis was performed. All statistics were performed on PASW (v. 17.0) with alpha set at 0.05.

Chapter 4

RESULTS

The following chapter describes the results from examining the effects of BT, and exercise, on plasma NO levels and cardiovascular responses at rest and during exercise. Raw data are found in Appendix E. Statistical analyses of data are detailed in the following subsections: (1) subjects, (2) plasma nitric oxide, and (3) cardiovascular responses.

Subjects

Ten recreationally active, healthy college-aged males volunteered to participate in this study. Means ± SD for pertinent anthropometric data were in the expected range (height = 170.0 ± 12.4 cm, weight = 78.7 ± 11.0 kg, and age = 20.2 ± 3.6 y). Subjects completed both trials for this study within 6 to 8 d and both trials were scheduled at the same time of day for each subject.

Plasma Nitric Oxide

The 2 x 3 ANOVA (Group x Time) with repeated measures on the time variable (i.e., -45 min, 0 min, and 30 min) showed no significant interactions or differences between groups or time (Table 1). Examining descriptive data (means ± SD) in Table 2, it can be seen that NO was at the lowest point 45 min after BT supplementation. NO also returned back to pre-BT supplementation levels after 30 min of exercise.

Table 1.

Plasma Nitric Oxide ANOVA Summary Table

	SS	DF	MS	F	*p**
Time	674.9	1.6	427.4	2.3	0.13
Time * Group	449.9	1.6	285.0	1.5	0.23
Error	4963.3	26.8	184.9		
Group	350.2	1.0	350.2	0.3	0.31
Error	5395.1	17.0	317.4		

Note. * Sphericity was not met, Greenhouse-Geisser corrections were applied to all *p* - values

Table 2.

Plasma Nitric Oxide

	Time (min)	Group	NO ($\mu M \cdot L^{-1}$)	Average ($\mu M \cdot L^{-1}$)
Rest	-45	Control	55.1 ± 14.0	53.5 ± 12.8
		Betaine	51.9 ± 11.6	
	0	Control	46.3 ± 11.7	46.8 ± 12.0
		Betaine	47.2 ± 12.2	
Exercise	30	Control	60.8 ± 21.1	54.5 ± 16.8
		Betaine	48.2 ± 11.5*	

Note. Values are mean ± SD, * n = 10 except this data point where n = 9

Cardiovascular Responses

Systolic Blood Pressure

The 2 x 5 ANOVA (Group x Time) with repeated measures on the time variable (i.e., -45 min, -15 min, 0 min, 15 min, and 30 min) showed no significant interactions or differences between groups for SBP (Table 3). There was, however, a significant main effect for time (F (4, 72) = 137.2, p = 0.00). Descriptive data (means ± SD) are illustrated in Table 4. Multiple pairwise comparisons using Bonferroni adjustments found SBP at 15 and 30 min was statistically greater than -45 min ($p \leq 0.013$), -15 min ($p \leq 0.013$), and 0 min ($p \leq 0.013$). SBP at 30 min was also significantly greater than SBP at 15 min ($p \leq 0.013$). As expected, the 30 min cycling bout beginning at 0 min was responsible for the main effect of time on SBP.

Diastolic Blood Pressure

A 2 x 5 ANOVA (Group x Time) with repeated measures on the time variable (i.e., -45 min, -15 min, 0 min, 15 min, and 30 min) showed no significant interactions or differences between groups for DBP (Table 5). There was, however, a significant main effect for time (F (1.7, 30.3) = 6.8, p = 0.01). Descriptive data (means ± SD) are illustrated in Table 6. Multiple pairwise comparisons using Bonferroni adjustments found DBP at 30 min was statistically less than -45 min ($p \leq 0.013$), -15 min ($p \leq 0.013$), and 0 min ($p \leq 0.013$). Again, the 30 min cycling bout beginning at 0 min was likely responsible for the main effect of time on DBP. From 0 to 15 min a 6.4 mm Hg decrease occurred and a further 8.4 mm Hg drop occurred from 15 to 30 min totaling a 14.8 mm Hg decrease from resting DBP during exercise which was not expected.

Table 3.

Systolic Blood Pressure ANOVA Summary Table

	SS	DF	MS	F	*p*
Time	49942.3	4	12485.6	137.2	0.00*
Time * Group	149.7	4	37.4	0.8	0.74
Error	4939.0	72	91.0		
Group	25.0	1	25.0	0.0	0.87
Error	13186.1	18	732.6		

Note. * $p \leq 0.013$

Table 4.

Systolic Blood Pressure

	Time (min)	Group	SBP (mm Hg)	Average (mm Hg)
Rest	-45	Control	123.5 ± 12.1	123.9 ± 11.8
		Betaine	124.4 ± 11.5	
	-15	Control	119.0 ± 8.6	120.9 ± 9.0
		Betaine	122.9 ± 10.4	
	0	Control	118.1 ± 10.9	119.9 ± 10.5
		Betaine	121.7 ± 12.0	
Exercise	15	Control	163.9 ± 21.7	162.9 ± 18.9*
		Betaine	161.9 ± 16.1	
	30	Control	171.5 ± 19.7	170.8 ± 19.2*#
		Betaine	170.1 ± 18.7	

Note. Values are mean ± SD, * Greater than other times (-45, -15, and 0; $p \leq 0.013$),

Greater than 15 min ($p \leq 0.013$)

Table 5.

Diastolic Blood Pressure ANOVA Summary Table

	SS	DF	MS	F	*p**
Time	3320.0	1.7	1960.7	6.8	0.01**
Time * Group	367.3	1.7	217.0	0.8	0.46
Error	8837.4	30.3	290.0		
Group	249.6	1.0	249.6	1.1	0.32
Error	4250.6	18.0	236.3		

Note. * Sphericity was not met, Greenhouse-Geisser corrections were applied to all *p - values*, ** $p \leq 0.013$

Table 6.

Diastolic Blood Pressure

	Time (min)	Group	DBP (mm Hg)	Average (mm Hg)
Rest	-45	Control	82.9 ± 9.5	81.5 ± 8.1
		Betaine	83.4 ± 6.7	
	-15	Control	82.0 ± 7.7	81.8 ± 8.5
		Betaine	81.6 ± 9.4	
	0	Control	82.4 ± 5.7	82.7 ± 6.4
		Betaine	82.9 ± 7.1	
Exercise	15	Control	73.4 ± 16.7	76.3 ± 18.0*
		Betaine	79.1 ± 19.2	
	30	Control	63.2 ± 11.7	67.9 ± 14.6*
		Betaine	72.7 ± 17.5	

Note. Values are mean ± SD, * Greater than other times (-45, -15, and 0; $p \leq 0.013$)

Mean Arterial Blood Pressure

A 2 x 5 ANOVA (Group x Time) with repeated measures on the time variable (i.e., -45 min, -15 min, 0 min, 15 min, and 30 min) showed no significant interactions or differences between groups for MAP (Table 7). There was, however, a significant main effect for time (F (4, 72) = 6.5, p = 0.00). Descriptive data (means ± SD) are illustrated in Table 8. Multiple pairwise comparisons using Bonferroni adjustments found MAP at 15 min significantly greater than -15 min ($p \leq 0.013$).

Heart Rate

A 2 x 5 ANOVA (Group x Time) with repeated measures on the time variable (i.e., -45 min, -15 min, 0 min, 15 min, and 30 min) showed no significant interactions or differences between groups (Table 9). There was, however, a significant main effect for time (F (4, 72) = 325.0, p = 0.00). Descriptive data (means ± SD) are illustrated in Table 10. Multiple pairwise comparisons using Bonferroni adjustments found HR at 15 and 30 min were significantly greater than -45 min ($p \leq 0.013$), -15 min ($p \leq 0.013$), and 0 min ($p \leq 0.013$). HR was also significantly greater at 30 min than 15 min HR ($p \leq 0.013$). The exercise effect on HR and the upward drift of HR from 15 to 30 min was expected.

Summary

There were no statistically significant interactions, or group or time main effects for NO. There were no statistically significant interactions or group main effects for any cardiovascular response (SBP, DBP, MAP and HR). All cardiovascular variables significantly changed over time, specifically, from 0 min to 30 min as the body expectedly responded to the exercise bout.

Table 7.

Mean Arterial Blood Pressure ANOVA Summary Table

	SS	DF	MS	F	*p*
Time	1712.8	4	428.2	6.5	0.00*
Time * Group	91.4	4	22.9	0.4	0.84
Error	4718.8	72	65.5		
Group	148.8	1	148.8	0.5	0.47
Error	5008.1	18	278.2		

Note. * $p \leq 0.013$

Table 8.

Mean Arterial Blood Pressure

	Time (min)	Group	MAP (mm Hg)	Average (mm Hg)
Rest	-45	Control	96.4 ± 2.7	96.8 ± 2.7
		Betaine	97.1 ± 2.7	
	-15	Control	94.3 ± 2.7	94.8 ± 2.7
		Betaine	95.4 ± 2.7	
	0	Control	94.3 ± 2.4	95.1 ± 2.4
		Betaine	95.8 ± 2.4	
Exercise	15	Control	103.6 ± 4.2	105.2 ± 4.2*
		Betaine	106.7 ± 4.2	
	30	Control	99.3 ± 4.1	102.3 ± 4.1
		Betaine	105.2 ± 4.1	

Note. Values are mean ± SD, * Greater than -15 min ($p \leq 0.013$)

Table 9.

Heart Rate ANOVA Summary Table

	SS	DF	MS	F	*p*
Time	89179.1	4	22294.8	325.0	0.00*
Time * Group	134.3	4	33.6	0.5	0.74
Error	4939.0	72	68.0		
Group	466.6	1	466.6	0.8	0.40
Error	11223.6	18	623.5		

Note. * $p \leq 0.013$

Table 10.

Heart Rate

	Time (min)	Group	HR (bpm)	Average (bpm)
Rest	-45	Control	68.4 ± 8.9	68.0 ± 7.8
		Betaine	67.5 ± 7.0	
	-15	Control	71.0 ± 10.3	67.9 ± 10.3
		Betaine	65.2 ± 10.2	
	0	Control	73.8 ± 10.6	70.5 ± 10.2
		Betaine	67.1 ± 9.4	
Exercise	15	Control	126.7 ± 17.7	125.6 ± 16.9*
		Betaine	124.5 ± 16.1	
	30	Control	136.5 ± 18.4	133.5 ± 16.6*#
		Betaine	130.5 ± 18.7	

Note. Values are mean ± SD, * Greater than other times (-45, -15, 0, and 15; $p \leq 0.013$), # Greater than 15 min ($p \leq 0.013$)

Chapter 5

DISCUSSION

The purpose of this study was to investigate the effects of acute BT supplementation and exercise on plasma NO levels and cardiovascular response. Previous research has demonstrated increased resting NO levels after 6 $g \cdot d^{-1}$ for one week of BT supplementation (Iqbal et al., 2006). Many authors have shown that there is a positive linear relationship between exercise intensity and NO production (Matsumoto et al., 1994; Pogliaghi et al., 1997; St. Croix et al., 1999). However, little is known about the combined effects of acute BT supplementation and exercise on plasma NO levels, blood pressure, and heart rate. This chapter elaborates on the findings of this study in the following subsections: (1) plasma nitric oxide, (2) cardiovascular responses, and (3) practical implications.

Plasma Nitric Oxide

Iqbal et al. (2006) found chronic BT supplementation (6 $g \cdot d^{-1}$ for one week) increases plasma NO at rest. The present study found, however, acute BT supplementation (2.5 $g \cdot d^{-1}$) does not significantly increase plasma NO levels at rest. The conflicting results may be a function of supplementation dosage and time whereby the amount of BT titration, with chronic doses, may enable upregulation of NOS in the blood vessels. Future research should examine the effect of chronic BT supplementation similar to Iqbal et al. and exercise on plasma NO.

The results of this study also demonstrated plasma NO did not increase with exercise. However, many studies show a positive linear relationship with exercise intensity and NO levels during exercise (Maroun et al., 1995; Matsumoto et al., 1994;

Persson et al., 1993; Pogliaghi et al., 1997; St. Croix et al., 1999). While exercise intensity is correlated with NO release, the prescribed exercise intensity in this study may not have been of sufficient intensity to elicit NO release. Goto et al. (2003) compared the effects of training at three exercise intensities for 30 min in healthy young men and found moderate intensity (50% VO_2max) training enhanced endothelium-dependant vasodilation while low (25% VO_2max) and high (75% VO_2max) intensities did not. The added oxidative stress associated with high intensity training may reduce NO bioavailability, diminishing any vascular effect seen as a result of NO production (Maiorana et al., 2003). Additionally, low intensity exercise may not provide a vasodilatory stimulus strong enough to reach a threshold to promote NO release.

NO is difficult to measure directly considering the extremely short half life (ranging from 0.05-1 s in circulating blood; Kelm, 1999). NO is quickly oxidized to the stable end products nitrite (NO_2^-) and NO_3^- which are then typically measured. This study assayed NO_3^- levels only, the addition of a NO_2^- assay may have shown the positive linear relationship between NO and exercise.

A potential limitation of the current study was measuring plasma NO immediately after the cycling bout. Based upon the reviewed literature, it is difficult to ascertain whether this methodological factor would significantly affect measured NO levels. Studies with similar methodology have shown both significant (Node et al., 1997) and non-significant (Matsumoto et al., 1994; St. Croix et al., 1999) results. However, studies linking NO release with exercise intensity typically measured NO during exercise.

Inconsistencies between studies may be attributed to differences in methodology, specifically, exercise intensity and duration. Studies involving graded exercise or

moderate (>50% VO_2max) to near- maximal or maximal intensities clearly demonstrated increased NO release (Maroun et al., 1995; Matsumoto et al., 1994; Node et al., 1997; Persson et al., 1993; Pogliaghi et al., 1997; St. Croix et al., 1999). Studies using longer exercise duration also show increased NO release (Jungersten, Ambring, Wall, & Wennmalm, 1997). Perhaps a longer duration or higher intensity may have increased NO production post-exercise in the current study.

Exogenous sources of NO_3^- from foods with high NO_2^- or NO_3^- levels may alter resting plasma NO measures and potentially exercise NO measures. In the current study, subjects were instructed to fast 12 hrs prior to testing to limit this effect. When compared to other studies with similar methodology, resting plasma NO levels were slightly higher at rest, however, this small difference had no apparent effect on post-exercise plasma NO measures.

A final potential limitation of this study may be the venous blood sampling from a peripheral site rather than the vascular bed of the active muscle. Local (quadriceps muscle) increases in NO may not be sufficient to overcome the diluting effect when circulating throughout the body to be detected at peripheral (anticubital space) sampling sites. A similar theory was purported in a study by St. Croix et al. (1999) when blood was drawn at a similar peripheral site.

Cardiovascular Responses

In the current study, an acute dose (2.5 g) of BT, had no significant effect on resting or exercise HR. The results of this study are in agreement with other research involving BT and HR. For example, Armstrong et al. (2008) found no statistically significant differences for HR between treatments (W, W + BT, CHO, or CHO + BT)

after subjects ran in a heated environment at 65% VO_2max for 75 min followed by a timed performance run at 84% VO_2max to volitional exhaustion. In another study, no differences in HR were observed between groups (PL, CHO + BT, or CHO) when eight trained cyclist cycled for 120 min alternating between 60% and 75% VO_2max followed by a 15 min time trial (Millard-Stafford, 2005).

The results of this study show acute BT supplementation (2.5 g) has no significant effect on resting or exercising SBP, DBP and MAP. Similarly, Schwab et al. (2002) found no significant differences between control and BT groups after a 12 wk (6 $g \cdot d^{-1}$) hypoenergetic diet among 42 obese subjects (male = 14) for resting SBP and DBP. Conversely, Konstantinova et al. (2008) recently demonstrated BT concentration in plasma is inversely related to components of metabolic syndrome. Among those tested, SBP and DBP were identified as having such a chronic relationship (-0.73, 95% CI: [-1.03, -0.43] and -0.86, 95% CI: [-1.13, -0.59], $P < 0.0001$, respectively). However, the impact of the small yet statistically significant improvements in SBP and DBP resulting from BT supplementation may not be clinically relevant.

It has been well established that HR and SBP, and also blood flow to working muscles are linearly proportional to exercise intensity (Ehrman, deJong, Sanderson, Swain, Swank, & Womack, 2010; Laughlin, 1999). The 30 min cycling bout in the present study was responsible for a significant increase in SBP and HR. Although workload was held constant throughout the exercise, steady state was not achieved for these variables. The significant, but expected increased HR during exercise may be explained by the cardiac drift effect whereby stroke volume decreases while HR increases with time during exercise to maintain cardiac output. This effect can begin within about

10 min of exercising (Coyle & Gonzalez-Alonso, 2001). Dehydration and hypovolemia are two leading hypothesis that contribute to cardiac drift, decreased stroke volume, and increased HR during exercise (Ehrman et al., 2010). In the current study, euhydration ($U_{sg} \leq 1.025$ μG) was verified before exercise possibly suggesting dehydration did not occur. Hydration was not, however, checked again following exercise.

Progressive increases in HR due to cardiac drift during exercise may also be explained by hyperthermia and subsequent increased sympathetic nervous system activation to maintain BP (Coyle & Gonzalez-Alonso, 2001). Perhaps over the course of the 30 min cycling bout, to attenuate core body temperature increases, blood flow to the skin increased. This thermoregulatory vasodilation caused a trend towards decreased DBP, ultimately threatening MAP. Additionally, subjects may have experienced muscular fatigue towards the end of the 30 min cycling bout eliciting increased muscle fiber recruitment. In order to maintain MAP and meet this added metabolic demand, the cardiovascular control center increased HR and SBP during the cycling bout by releasing catecholamines from the adrenal medulla. In sum, the combined effects of muscular fatigue and the vasodilation due to thermoregulation may have challenged MAP maintenance but HR and SBP significantly increased to compensate and maintain MAP during the 30 min cycling bout as expected.

Practical Implications

An acute dose (2.5 g) of BT has no cardiovascular effect during rest or 30 min of light-moderate cycling. Additionally, acute BT supplementation did not increase resting or post-exercise plasma NO levels. While other ergogenic effects may occur with acute BT supplementation, benefits sought with improved NO production does not appear to

occur. Although it has not been investigated, future research should explore whether exercise increases NO levels with chronic BT supplementation. One dose may not be enough and the impact of BT on NO may require at least several days or a week as seen under resting conditions in the work of Iqbal et al. (2006). Further, other resistance and endurance training modes, intensities, and durations may exhibit different effects of BT on NO production and should be explored.

Summary

Within the limitations of this study, acute BT supplementation did not affect plasma NO levels at rest or immediately after light-moderate cycling or cardiovascular responses at rest, during, or after exercise. While not studied in this investigation, chronic BT supplementation may produce different NO levels during exercise and should be explored. The lack of effect of exercise on NO may be because of insufficient intensity and duration which appear to play a role in NO production with exercise.

Chapter 6

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This study examined the effects of a single dose of BT on resting and exercise plasma NO levels and related cardiovascular responses (SBP, DBP, MAP and HR). Ten male, recreationally active Ithaca College students volunteered to serve as subjects in this study. This study utilized a placebo controlled, double-blind, repeated measures design. In a counterbalanced fashion, subjects ingested either 250 ml placebo (CHO) or 2.5 g of BT + 250 ml CHO beverage then rested 45 min while HR, BP, and plasma NO measures were taken. After this time, subjects cycled 30 min against 2.5% body weight (kg) at 60 rpm, after which the last plasma NO measure was taken. Subjects completed a second trial in the alternate condition at the same time of day after waiting 6 to 8 d to allow for adequate BT wash out.

Plasma NO levels were indirectly measured through a stable end product of oxidized NO, NO_3^-. This was determined using the colorimetric method and Greiss technique in a total NO nitrate/nitrite assay. A HR monitor obtained HR and BP was measured by auscultation and sphygmomanometer at rest and during exercise. NO, HR, SBP, DBP and MAP data were statistically analyzed with a two-way repeated measures ANOVA for each dependant variable. Acute BT supplementation did not significantly increase plasma NO levels or alter cardiovascular responses at rest or after light-moderate cycling.

Conclusions

Results of this study support the following conclusions:

1. Acute consumption of BT (2.5 g) does not significantly increase resting or light-moderate exercise plasma NO levels as indirectly measured by plasma nitrate levels.
2. Acute ingestion of BT (2.5 g) does not significantly alter HR, SBP and DBP during rest or light-moderate cycling.

Recommendations

The following recommendations for future study are to:

1. Examine the effects of chronic BT supplementation and exercise on NO levels.
2. Include NO_2^- in the assay to determine plasma NO production or use other methods of NO detection *in vivo* (i.e., exhalation) and during exercise.

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APPENDIX A

Informed Consent

Effect of Betaine Supplementation and Exercise on Plasma Nitric Oxide

1. Purpose of the Study: The purpose of this study is to examine the effect of betaine, a natural ingredient found in food, and exercise on blood plasma nitric oxide levels. Danisco, the maker of BetaPower or betaine, will provide the betaine and other funds needed for this project.

2. Benefits: You may benefit from participating in this study because you will get first hand experience on how scientific data are collected and know your resting nitric oxide levels. Your participation will also benefit the researchers, who are learning how to conduct a scientific study. Last, it is hoped that the data, and the subsequent publications generated from it, will benefit the scientific community.

3. Your Participation Requires you to be at least 18 - 27 years old and in good health. You will be contacted via email to meet several days before data collection in the Exercise Physiology Laboratory in CHS 303. You will complete a health history questionnaire; it is possible that you may be excluded from exercising if health risks are identified in this questionnaire. Your bicycle seat height will also be determined and you will be given written instructions on how to come prepared for data collection if you are eligible to participate. To ensure hydration, you will drink 12 oz of water before going to bed the night before and 12 oz of water in the morning prior to reporting to CHS 303.

For the following two weeks, you will visit the lab in the morning following an overnight fast; your last meal can be no later than 12 am the night before. Upon arrival, I will assess hydration levels through a urine sample. Then, I will draw a 3.5 ml sample of venous blood from an anticubital vein in your arm. I will follow the procedures used by the Cayuga Medical Center. The blood sample will be used to determine your plasma nitric oxide level. Similar blood samples will also be drawn 45 minutes after ingestion of the supplement and at the end of the cycling exercise.

After the first sample is drawn, you will consume up to 250 ml aqueous betaine solution (1% betaine or 2.5 grams). After consuming the betaine, you will remain in the laboratory in a seated position for 45 minutes. You are free to read, study, or surf the internet while in the lab. You will then cycle at a cadence of 60 rpm at a relative resistance of 2.5% body weight (kg) for 30 minutes. I will draw blood samples at the aforementioned times. In total, three 3.5 ml samples or 10.5 ml in total will be drawn per session. Each visit to the lab will take approximately 1.5 hours (3 hours total time).

4. Risks of Participation: There are no known risks associated with the solutions provided to you. The risks associated with the bicycle test include skeletal muscle injury and possibly a cardiac event, which could be fatal. The chances of a cardiac event are low in your age group. You may also have sore muscles 24 to 48 hours after the tests; the forearm region that is lanced may also be tender for a few days. The study involves venipuncture, which will be done with aseptic techniques to avoid risk of infection at the puncture site. To minimize risks, you will warm-up and cool-down before and after each test. If you feel poorly during the test, you may terminate it at any time. The technicians will promptly provide standard first aid procedures in the event you are injured. Since there will be at least two research technicians present during any test, those not involved with immediate care will call 911 to seek additional assistance if warranted.

5. Compensation for Injury: If you suffer an injury that requires any treatment or hospitalization as a direct result of this study, the cost of such care is your responsibility. If you have insurance, you may bill your insurance company. Ithaca College and the investigator will not pay for any care, lost wages, or provide other compensation.

6. If you would like more information about this study at anytime prior to, during, or following the data collection, you may contact Luke Pryor at jpryor1@ithaca.edu or 814.380.4359 or Dr. Tom Swensen at tswensen@ithaca.edu or 607.274.3114.

7. Withdrawal from the study: Participation in this study is voluntary and you may withdraw at any time if you so choose. You will not be penalized for withdrawing and will still be eligible for extra credit.

8. Confidentiality: Information gathered during this study will be maintained in complete confidence. Only the researchers will have access to this information, which will be stored in a locked cabinet in room 320 in the Center for Health Sciences at Ithaca College or on password protected computer. You and your name will never be associated with this information in any future disclosures. To further insure confidentiality, all files will be number coded and data collection instruments will be kept separately from Informed Consent Forms and sign-up sheets. There will also be a separate form for signing up for extra credit, which will be kept separately from all other forms.

I have read and understood the above document. I agree to participate in this study and realize that I can withdraw at anytime. I also understand that I can and should address questions related to this study at any time to any of the researchers involved. I also verify that I am at least 18 years of age.

_____ _____

Your Name (please print) Your Signature Date

APPENDIX B

Medical History Form

Name: _____

Age: _____ Weight: _____ Sex: _____

Medical/Health History (please check all that apply)

[ ] Heart/Disease
[ ] Lung Disease
[ ] Stroke
[ ] Diabetes
[ ] Heart Murmur
[ ] Epilepsy
[ ] Skipped, rapid or irregular heart rhythms
[ ] Injuries to back, hips, knees, ankles, or feet
[ ] High Blood Pressure
[ ] Renal Disease
[ ] High Cholesterol
[ ] Allergic reactions to Latex
[ ] Rheumatic Fever
[ ] Other conditions/comments: (please explain)

Present Symptoms (please check all that have applied within the last **six months**)

[ ] Chest pain
[ ] Ankle/Leg Swelling
[ ] Shortness of Breath
[ ] Joint/muscle injury requiring medical attention
[ ] Lightheadedness
[ ] Allergies (if yes, please list)
[ ] Heart Palpitations
[ ] Loss of consciousness
[ ] Illness, surgery, or hospitalization
[ ] Other conditions (please explain)

Current medications (please list all medications presently being taken)

Are you taking any supplements that are said to increase nitric oxide levels? If so, please list.

Exercise Habits

Do you presently engage in physical activity? [ ] Yes [ ] No

If so, what type of exercise? [ ] Aerobic [ ] Strength Training [ ] Both

How hard do you exercise? [ ] Easy [ ] Moderate [ ] Hard

How many times a week do you work out on average? _____

How many times a day do you work out on average? _____

Have you ever had any discomfort, shortness of breath, or pain while exercising?

[ ] Yes [ ] No

Sleep Patterns

On average, how many hours do you sleep per night? _____

Would you say you have good sleeping patterns? [ ] Yes [ ] No (explain)

APPENDIX C

24-Hour Health History Form

Name: _____ Date: _____

Current Health Status (please check all that apply)

[ ] Nausea	[ ] Sore Throat	[ ] Headache
[ ] Body Ache	[ ] Chills	[ ] Lethargy
[ ] Nasal Drip	[ ] Cramping	[ ] Muscle Aches
[ ] Chest Pain	[ ] Shortness of Breath	[ ] Dizziness

Diet

Did you drink 12 ounces of water last night? [ ] Yes [ ] No

Did you drink 12 ounces of water this morning? [ ] Yes [ ] No

Have you consumed alcohol in the last 12 hours? [ ] Yes [ ] No

Have you used caffeine or nicotine in the last three hours? [ ] Yes [ ] No

Did you eat any food in the last twelve hours? [ ] Yes [ ] No

If so, please list:

Has your diet changed drastically since the last exercise test? [ ] Yes [ ] No

If so, please describe:

Please describe your last meal and the **time** of day:

Exercise

Have you exercised in the last 24 hours? [ ] Yes [ ] No

If so, please describe:

Has your exercise routine changed at all since the last test? [ ] Yes [ ] No

If so, please explain:

Over-the-Counter and/or Prescription Drug Use

Have you taken any over the counter drugs (e.g., cold meds) in the last 24 hours?

[ ] Yes [ ] No

Has there been any change in your use of prescription drugs? [ ] Yes [ ] No

If so, please explain:

Injury

Have you experienced any physical pain in the last 24 hours? [ ] Yes [ ] No

If so, please explain:

Is there any physical injury we should know about before you perform the test?

[ ] Yes [ ] No

If so, please explain:

Sleep Pattern:

Has your sleep pattern changed since the last exercise test? [ ] Yes [ ] No

Do you feel drowsy, tired, or run down at this time? [ ] Yes [ ] No

If so, please describe:

Has there been any change since the last exercise test that you feel could compromise your performance on today's exercise test?

[ ] Yes [ ] No

If so, explain:

Other questions/comments/concerns please state below.

APPENDIX D

Pre-Test Instructions

Test Date:_____ Test Time: _____

Your performance depends upon the adherence of these instructions:

1. Do not eat 12 hours before your lab visit.
2. Avoid over-the-counter medications for the 12 hours preceding the lab visit. (However, cancel the appointment if you are ill and treat yourself accordingly; we can always reschedule)
3. Please, sustain your same lifestyle habits (eating, exercise, medication, etc.) between tests.
4. Bring something to read or study to help keep you occupied during the lab visit.
5. Do not drink alcohol for 12 hours before the test.
6. Do not use stimulants such as caffeine (e.g., coffee) or nicotine (i.e., cigarettes) for three hours preceding the test
7. Wear comfortable clothing and appropriate shoes during the test. (i.e., shorts, t-shirt, and sneakers are recommended)
8. To ensure hydration prior to the test, drink **12 ounces** of water before going to bed the night before (Saturday) and **12 ounces** of water in the morning before reporting to CHS 303.

We thank you for your cooperation!

APPENDIX E

Raw Data

Subject	Trial	Group	Weight (kg)	Height (cm)	Age	SBP (mm Hg) -45 min	-15 min	0 min	15 min	30 min	DBP (mm Hg) -45 min	-15 min
1	1	2	73.2	173	19	142	134	138	156	186	86	80
2	1	1	64.6	170	27	110	108	104	161	178	82	76
3	1	1	103.2	138	19	130	118	118	190	196	89	86
4	1	2	80.5	172	18	120	128	112	172	176	90	92
5	1	1	85.0	175	18	118	116	110	160	176	80	82
6	1	1	86.8	183	19	110	116	120	140	160	70	72
7	1	2	73.8	166	27	138	139	138	192	196	90	98
8	1	2	69.1	172	18	132	120	122	150	155	74	75
9	1	2	72.3	172	19	118	126	128	170	188	78	76
10	1	1	78.6	181	18	122	118	120	160	182	78	85
1	2	1	-	-	-	148	138	130	198	190	95	88
2	2	2	-	-	-	110	112	104	159	165	80	77
3	2	2	-	-	-	132	132	132	175	172	95	90
4	2	1	-	-	-	116	112	108	135	132	72	78
5	2	2	-	-	-	118	116	110	160	176	80	82
6	2	2	-	-	-	126	108	114	134	135	78	66
7	2	1	-	-	-	138	124	138	190	187	100	91
8	2	1	-	-	-	124	128	125	150	154	84	92
9	2	1	-	-	-	119	112	108	155	160	79	70
10	2	2	-	-	-	108	114	119	151	152	83	80

Note: Group 1 = Placebo, 2 = Betaine

APPENDIX E (Continued)

Raw Data

DBP (mm Hg)			HR (bpm)					NO ($\mu M \cdot L^{-1}$)			
0 min	15 min	30 min	-45 min	-15 min	0 min	15 min	30 min	-45 min	0 min	30 min	U_{sg} (μG)
84	110	110	68	72	72	121	132	48.6	40.9	43.2	1.024
80	90	90	75	74	86	119	146	68.4	64.4	51.3	1.019
80	60	64	77	80	86	165	170	44.4	30.4	72.0	1.018
90	80	80	60	54	55	104	106	66.3	72.6	59.6	1.025
78	92	70	60	60	67	106	106	49.5	50.6	62.5	1.020
78	72	64	79	74	70	120	135	40.3	43.1	44.3	1.022
90	75	70	65	59	70	147	151	57.4	90.7	-	1.005
78	68	64	82	79	74	129	131	43.1	43.0	33.2	1.022
82	64	60	65	49	51	116	124	66.9	47.4	29.2	1.024
80	106	50	72	75	64	120	135	62.7	31.5	40.2	1.014
90	64	60	55	60	76	124	132	40.9	43.5	33.7	1.011
72	108	95	65	66	69	132	152	43.0	30.1	58.6	1.022
95	80	55	74	80	84	153	159	51.5	53.0	56.2	1.024
78	70	69	59	65	66	119	118	83.9	53.0	85.3	1.024
78	92	70	60	60	67	106	106	66.6	53.9	60.1	1.009
77	55	60	73	70	66	122	127	40.2	36.3	48.6	1.014
94	60	60	73	84	76	147	154	63.1	51.3	92.4	1.007
86	60	50	74	83	89	135	145	51.0	34.6	82.4	1.024
80	60	55	60	55	58	112	124	47.4	60.6	43.6	1.019
83	59	63	63	63	63	115	117	40.8	47.7	45.3	1.007

APPENDIX E (Continued)

Raw Data

MAP (mm Hg)				
-45 min	-15 min	0 min	15 min	30 min
91	93	97	99	103
93	90	93	95	94
106	112	106	114	112
100	104	97	111	112
105	98	102	125	135
90	89	83	125	118
107	104	107	112	94
93	93	89	115	105
94	80	89	81	85
91	91	95	90	93
93	96	93	124	94
83	87	92	95	96
93	93	89	115	105
103	97	93	103	108
91	87	88	114	119
113	105	103	109	103
87	89	88	92	90
113	102	109	103	102
97	104	99	90	85
92	84	89	92	90