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EXERCISE INDUCED OXIDATIVE STRESS: EXAMINING THE ANTIOXIDANT CAPABILITIES OF BETA-ALANINE SUPPLEMENTATION

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A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

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Real knowledge is to know the extent of one's ignorance. – Confucius

Throughout my graduate studies I continue to realize that there is so much knowledge left to be unlocked, yet I complete my PhD with a renewed desire to seek out the vast areas of research. I am humbled daily by my graduate colleagues and astounding professors that share their unique knowledge and ideas and hope to share that passion with future students.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS…… iv.

TABLES…….. viii.

FIGURES……. ix.

ABSTRACT…… x.

CHAPTER

I. INTRODUCTION…….. 1.

Hypothesis…….. 6.

Definition of Terms……. 7.

Abbreviations…….. 8.

Delimitations……... 8.

Assumptions…… 9.

Limitations…….. 10.

II. REVIEW OF LITERATURE……. 11.

Oxidative stress markers and timing of sampling…….. 11.
Khassaf, Child, McArdle, Brodie, et al…….. 11.
Michalidis, Jamurtas, Nikolaidis, et al…… 12.

Effects of acute exercise on oxidative stress…….. 14.
Schneider, Barp, Ribeiro, et al……. 14.
Alessio, Hagerman, Fulkerson et al…… 16.
Lovlin, Cottle, Pyke, Kavanagh, Belcastro…… 18.

The influence of exogenous antioxidants on oxidative stress…… 19.
Bloomer, Goldfarb, Mckenzie……. 19.
Goldfarb, McKenzie, Bloomer…… 21.
Watson, Callister, Taylor, Sibbritt, et al…….. 23.

Beta-alanine and its role in increasing muscle carnosine levels……. 25.
Baguet, Reyngoudt, Pottier, Everaert, et al…… 25.
Dunnett, Harris, Dunnett, Harris……. 26.

Dunnett, Harris…… 27.
Harris, Tallon, Dunnett, Boobis, Coakley, et al…….. 28.
Suzuki, Nakao, Maemura, Sato, Kamahara, et al…… 30.

The effects of beta-alanine supplementation on performance…… 31.
Suzuki, Ito, Mukai, Takahashi, et al…… 31.
Stout, Cramer, Zoeller, Torok, Costa, et al……. 32.
Hill, Harris, Kim, Harris, Sale et al……. 33.
Van Thienen, Van Proeyen, Eynde, Puype et al……. 34.

Carnosine as an antioxidant……. 35.
Babizhayev, Sequin, Gueyne, Evstigneeva, et al…… 35.
Boldyrev, Stvolinksy, Tyulina, Koshelev, et al…… 37.
Boldyrev, Song, Lawrence, Carpenter, et al…… 38.
Chan, Decker, Chow, Boissonneault…… 39.
Decker, Crum, Calvert…….. 41.
Decker, Ivanov, Zhu, Frei……. 42.
Kohen, Yamamoto, Cundy, Ames……... 43.
Egorov, Kurella, Boldyrev, Krasnovsky…….. 45.
Salim-Hanna, Lissi, Vid…… 46.

III. METHODS…… 48.
Participants…… 48.
Research Design…… 49.
Instrumentation…… 50.
Maximal Oxygen Consumption……... 51.
Oxidative Stress Run…… 51.
Blood Collection……... 51.
Statistical Analyses…….. 54.

IV. RESULTS……. 56.
Aerobic Performance…… 56.
Forty-minute Run-HR, RPE……. 57.
Oxidative Stress Biomarkers…… 59.
Nutrition and Exercise Status…… 62.

V. DISCUSSION…… 64.
Effects on aerobic performance…… 64.
Forty-minute Run…… 67.
Oxidative stress…… 68.
Conclusion…… 75.

REFERENCES…… 78.

APPENDIX…….. 87.
Experimental Protocol……. 87.

Figures……. 88.
Tables…….. 100.
Figure Legends…… 105.
Informed Consent…… 109.

LIST OF TABLES

Table 1. Demographic characteristics for men and women.

Table 2. Magnitude inferences for aerobic performance.

Table 3. Magnitude inferences for heart rate and perceived exertion.

Table 4. Magnitude inferences for oxidative stress.

LIST OF FIGURES

Figure 1A-D. Change in aerobic performance.

Figure 2A-F. Heart rate and perceived exertion responses.

Figure 3A-C. Total Antioxidant Capacity

Figure 4A-C. Superoxide Dismutase

Figure 5A-C. 8-Isoprostane

Figure 6A-C. Glutathione

ABSTRACT

EXERCISE INDUCED OXIDATIVE STRESS: EXAMINING THE ANTIOXIDANT CAPABILITIES OF BETA-ALANINE SUPPLEMENTATION

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PURPOSE: The primary purpose of this study was to evaluate the effects of twenty-eight days of beta-alanine supplementation on markers of oxidative stress in men and women. METHODS: Twenty-five men (Age: 22.0 ± 3.3 yrs; maximal oxygen consumption (VO_2max): 3.9 ± 0.5 $l \cdot min^{-1}$) and twenty-four women (Age: 21.7 ± 2.1 yrs; VO_2max: 2.6 ± 0.3 $l \cdot min^{-1}$) volunteered to participate in this double-blind, placebo-controlled study and were randomly assigned to a beta-alanine (BA, 2 x 800 mg tablets, 3 x per day; CarnoSyn®, Natural Alternatives Inc, San Marcos, CA; n=) or placebo (PL, 2x 800 mg maltodextrin tablets, 3 x per day; n=) group for twenty-eight days of supplementation. A maximal graded oxygen consumption test (VO_2max) on a treadmill was performed to evaluate VO_2max, time to exahustion (VO_2TTE), ventilatory threshold (VT) and to establish peak velocity (PV). Baseline blood draws were taken before, immediately post (IP), 2h post and 4h following a 40 min treadmill run at 70%PV to evaluate total antioxidant capacity (TAC), superoxide dismutase (SOD), 8-isoprostane (8ISO) and reduced gluthathione (GSH). Heart rate and ratings of percieved exertion were recorded

during the 40 min run. Separate four- [4 × 2 × 2 × 2; acute (base vs. IP vs. 2h vs. 4h) × chronic (pre- vs. post-) × treatment (placebo vs. beta-alanine) × sex (male vs. female)] and two- [2 × 2; time (pre-supplement vs. post-supplement) × treatment (placebo vs. beta-alanine)] way ANOVAS were used to identify and group by time interactions for oxidative stress markers and aerobic performance, respectively. RESULTS: Both groups demonstrated significant improvements ($p<0.05$) in VO_2max. Percent change scores revealed a significant increase in VT for the male BA group. Heart rate and ratings of perceived exertion values were significantly improved from pre- to post-supplementation for the BA group, while the PL group demonstrated non-significant negative changes. There was a significant ($p<0.05$) exercise induced oxidative effect on all markers. The chronic effects of BA supplementation suggest there is an antioxidant effect on 8-isoprostane and GSH, with no gender differences ($p<0.05$). CONCLUSIONS: The current findings support previous performance research, demonstrated the ergogenic benefit of BA on VT. Additionally, an exercise-induced oxidative stress response, from a 40 minute treadmill run, may be slightly attenuated following twenty-eight days of beta-alanine supplementation for markers of lipid peroxidation and reduced glutathione, for both men and women.

CHAPTER I

INTRODUCTION

The cause of exercise-induced oxidative stress is not completely understood. While moderate exercise is important for health and quality of life, intense, high volume physical activity can often times lead to injury, soreness and chronic fatigue. Although these are multifaceted effects, an accumulation of free radicals may have some influence. As chemical species produced in all living cells, free radicals have the potential to react with several chemical species and play a role in several biological functions. The majority of *in vivo* free radicals are capable of oxidizing a range of biological molecules including carbohydrates, amino acids, fatty acids and nucleotides [44]. Due to production and accumulation of free radicals within the body several antioxidant defenses have evolved, utilizing antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase and vitamins C and E to prevent deleterious effects and protect from substantial tissue damage. SOD catalyzes the dismutation of superoxide to O_2 and hydrogen peroxide (H_2O_2). Additionally, GPX and reduced glutathione (GSH) reduce H_2O_2 to form oxidized glutathione (GSSG) and water [54]. In some circumstances, such as exercise, an overproduction of free radicals or a suppression of antioxidant defenses causes an imbalance, defined as oxidative stress [78, 94]. Various mechanisms support an exercise induced increase in free radicals, which in turn confers a strong relationship between exercise and oxidative stress [2, 38]. Among free radicals, reactive oxygen species (ROS) are a species derived from oxygen and metabolism, an essential aspect of exercise [38]. Previous research has suggested the mitochondria as the major source of ROS [31, 76, 97], with the oxidation of hemoglobin

[24, 73] and increases in central temperature, catecholamines and lactic acid all leading to an influx of ROS [23, 24]. There is some evidence that ROS accumulation may have positive effects on the force of muscle contraction [85, 86], however, ROS are potentially harmful provoking inflammation and altered cell function.

Of primary interest are the oxidative ramifications of ROS on lipid and protein oxidation and implications in muscular fatigue. Lipid peroxidation is initiated by ROS altering membrane fluidity, permeability and increasing inflammation [84]. Oxidation of proteins can lead to structural and enzymatic alterations of amino acids increasing protein carbonyl groups [88] and free amino acids [65, 109]. More so, muscle-induced increases in ROS are heavily associated with fatigue during and immediately following exercise. Damage to the mitochondrial respiratory chain due to excess ROS, down regulates electron transfer and ATP formation, rendering aerobic pathways less efficient. This phenomenon leads to a greater reliance on anaerobic metabolism resulting in a large amount of metabolic by-products (inorganic phosphate (P_i), and lactate) [87]. Furthermore, intracellular calcium increases, inactivation enzymes decrease and the potassium influx system are all hindered by excess ROS, resulting in muscular fatigue and altering muscle contraction [21, 67].

The endogenous and nutritional antioxidant system plays an important role in managing the balance of antioxidant enzymes to the production of free radicals in order to minimize free radical damage and reduce oxidative stress. Several exogenous antioxidants have been studied in relation to oxidative stress. One particular supplement has gained recent popularity among researchers and athletic community as an agent to delay fatigue, and has surfaced as a possible antioxidant. Carnosine (β-alanyl-L-histidine)

is a cytoplasmic dipeptide found in high concentrations in both vertebrate and non-vertebrate skeletal muscle. Carnosine is synthesized in skeletal muscle from l-histidine and beta-alanine amino acids in a reaction catalyzed by carnosine synthetase. Additionally, although skeletal muscle is able to synthesize carnosine, these cells cannot uptake carnosine from the bloodstream [9]. More so, skeletal muscle is unable to produce either L-histidine (an essential amino acid) or beta-alanine, whose production is confined to the liver [70]. Therefore, it is recognized that endogenous synthesis of carnosine is primarily dependent upon the uptake of beta-alanine and L-histidine by the muscle. Furthermore, plasma concentration and the affinity of carnosine synthetase (Km ~16.8 μM) of L-histidine is greater than plasma concentrations and synthetase affinity of beta-alanine (~1-2.3 μM). Consequently, the rate-limiting point of endogenous synthesis of carnosine in humans is the availability of beta-alanine within the muscle. Additionally, beta-alanine supplementation has been shown to significantly increase the intramuscular carnosine content [46, 47].

Carnosine and its related dipeptides can be hydrolyzed by at least two types of dipeptidases. Serum carnosinase is a secreted enzyme and is highly present and active in human plasma [6]. With a large concentration in the plasma, carnosine is virtual absent in the post-absorptive state due to its rapid and complete hydrolysis [6]. The absence of carnosinase from skeletal muscle tissue and other types of tissues was demonstrated by Severin et al. [95]. If carnosinases are absent from human muscle, then carnosine is hypothesized to remain stable within skeletal muscle and display a slow washout profile. In support, it has been shown that carnosine levels are typically highest in type II muscle fibers but still found in type I slow twitch fibers [45, 46]. Carnosine is available from

two sources; it can be readily extracted from natural sources or synthesized chemically by incorporating beta-alanine and histidine in the presence of carnosine synthase. The natural histidine-containing dipeptides, carnosine and anserine, are powerful antioxidants that are known to protect cell membranes and other cell structures. The antioxidant effect of carnosine has been demonstrated at both the cell and tissue levels with the ability to suppress peroxidation induced both enzymatically and non-enzymatically, and eliminating the products of free radical reactions [19, 20]. A modest body of literature supports the role of carnosine as an agent to delay fatigue [47, 100, 102, 106, 112]. A number of explanations have been proposed for such an effect, such as an increase in the efficiency of the electromechanical coupling [17], stimulation of ATPase activity of contractile proteins and activation of ATP producing enzymes [15, 82] and may also be related to its proposed antioxidant capacity. In general, this compound is reported to possess antioxidant, buffering, immune-enhancing and neurotransmitter actions. Carnosine is also known to be an antioxidant that is capable of preventing the accumulation of oxidized products derived from lipid components of biological membranes [27, 28]. Furthermore, carnosine has been shown to possess superoxide dismutase activity and appears to regulate lipoxygenase activity [16, 27]. The combination of histidine-containing compounds, such as carnosine, at near physiological concentrations, have resulted in synergistic antioxidant activity [63]. Several models to examine the antioxidant activity of histidine containing dipeptides have been devised consisting of determination of the accumulation of reactive oxygen species (ROS), lipid peroxidation, protein oxidation, and total antioxidant capacity via various antioxidant enzymes. Although there is no evidence that carnosine alters neuronal excitability of

modulation of synaptic transmission, as least one function of carnosine may be to prevent damage to the cell from production of reactive oxygen species [16, 20].

Boldyrev and colleagues were the first to ascertain the membrane-protecting properties of carnosine [17, 18]. Specifically, in a system containing a combination of carnosine and anserine with sarcoplasmic reticulum membranes, demonstrating that the dipeptides decreased membrane lipid oxidation rates as determined by TBARS [18]. The hydrophilic structure of carnosine plays a large role in its protective characteristic within the cytosol, where a majority of lipid oxidation catalysts and free radicals are found. Carnosine has been shown to exhibit 53% protection against lipid oxidation at a concentration of 10 mM [91]. The antioxidant mechanism of carnosine has been postulated to be due to metal chelation or free radical scavenging [40]. Furthermore, recent evidence from electronic paramagnetic resonance studies has shown that carnosine may inactivate hydroxyl radicals generated by iron and H_2O_2 [22]. Carbon-centered radicals of carnosine and homocarnosine were detected in the presence of hydroxyl radicals, demonstrating that histidine-containing dipeptides are capable of scavenging hydroxyl radicals. Furthermore, the interaction of carnosine and anserine with singlet oxygen was investigated by a direct luminescent method and both compounds were found to efficiently quench singlet oxygen at rate constants ranging from 2-4 x 10^7 M/s [35]. Ergov et al. [35] suggested that the quenching of the singlet oxygen was primarily attributed to the imidazole group of carnosine and anserine. Carcinine and carnosine have also been shown to be good scavengers of hydroxyl radicals, as detected by iron-dependent radical damage to the sugar deoxyribose suggesting that carnosine and carcinine are able to scavenge free radicals or donate hydrogen ions [7]. Boldyrev and

colleagues (1997) have suggested that the antioxidant protective effects of carnosine can be attributed to its binding affinity to reactive species, such as hydroxyl radicals and ROS and therefore preventing compounded damage [20].

While carnosine has demonstrated positive effects on oxidative stress biomarkers, all of the current studies have been conducted in vitro and in animal models. Additionally, research suggests that carnosine is not stable within the blood. Therefore, further research is warranted on the ability of carnosine to sequester free radicals in vivo and elicit the effect of its constituent, beta-alanine.

Purpose

1. The primary purpose of this study was to evaluate the effects of twenty-eight days of beta-alanine supplementation on markers of oxidative stress.
2. A secondary purpose was to investigate the effects of beta-alanine supplementation on measures of aerobic performance, maximal aerobic consumptions (VO_2max), time to exhaustion during VO_2max (VO_2TTE), and ventilatory threshold (VT).
3. A third purpose was to evaluate the effects of a 40 minute run, at 70%VO_2max, as a method to induce oxidative stress.
4. Finally, a fourth purpose of this study was to evaluate the sex differences in response to an acute 40-minute running bout of induced oxidative stress.

Hypotheses

1. It was hypothesized that an acute bout of aerobic exercise would induce oxidative stress. Oxidative stress markers would be altered from rest to immediately post (IP), up to two hours post (2h), and returning baseline by four hours (4h).

Specifically, superoxide dismutase (SOD) would be reduced immediately post exercise and increase above baseline values during recovery. Glutathione (GSH) would be reduced immediately post exercise, reaching its lowest values at 2h post. Lipid peroxidation, measured by 8-isoprostane, would demonstrate and increase IP and return to baseline by 2h. Total antioxidant capacity would increase IP.

2. It was hypothesized that twenty-eight days of beta-alanine supplementation would reduce oxidative stress markers. The reduction in SOD and GSH would be blunted compared to baseline levels. Both markers would increase during recovery to a lesser degree than during baseline evaluations. The increases in 8-isoprostane, total antioxidant capacity would be blunted with supplementation.
3. It was hypothesized that the twenty-eight days of beta-alanine supplementation would have no influence on VO_2max, while there would be an ergogenic effect on VT and VO_2TTE.
4. It was hypothesized that a 40-minute run at 70%VO_2max would be sufficient to induce an oxidative stress response.
5. It was hypothesized that the acute and chronic oxidative stress responses would not be different between genders.

Operational Definitions

Maximal Oxygen Consumption – (VO_2max) The maximum rate at which oxygen can be taken up and used by the body during exercise.

Supplement Loading Phase – The loading phase consistend of supplementing with 1600 mg beta-alanine three times daily (4.8 g) for twenty-eight days.

Oxidative Stress-Is a condition in which the balance between pro-oxidant (free radicals) production and their amelioration via the antioxidant defense system becomes skewed in favor of free radical expression leading to lipid and protein peroxidation and DNA damage.

Abbreviations

VO_2max- maximal oxygen consumption

SOD- superoxide dismutase

GSH- reduced glutathione

8-Isoprostane- measure of lipid peroxidation from the breakdown of arachidonic acid.

TAC- total antioxidant capacity

Delimitations

Twenty-six men and twenty-eight women between the ages of 18 and 35 years were recruited for this study. All participants completed a health history questionnaire and a written statement of informed consent prior to any testing. To be eligible for inclusion in this study, participants were determined to be moderately physically active, which was defined by 3-7 days per week of structured and/or recreational exercise, but they could not be untrained (1-2 hours of exercise per week) or competitive athletes. Volunteers from this study were free from any current musculoskeletal injuries and did not sustain any injuries within the four weeks prior to baseline testing. The participants were encouraged to remain involved in their current exercise training regime, but they were unable to begin any new endurance or resistance training program. Subjects could not have taken (within three months) or begin taking any nutrition supplements. A multi-vitamin was acceptable, but had to be recorded ahead of time and maintained throughout the duration of the study. A list of such supplements included, but were not limited to the

following: creatine, beta-alanine, branched chain amino acids, vitamin E, vitamin C, and weight loss pills. Volunteers were instructed to refrain from caffeine, alcohol, analegesics (asprin, acetaminophen, non-steroidal anti-inflammatories, and glucoceroticoids) and cigarettes during pre- and post-testing weeks.

Assumptions

Theoretical Assumptions

1. Subjects accurately answered the health history questionnaire.
2. All subjects gave maximal effort when performing VO_2max tests.
3. Participants maintained their current training routine throughout the duration of the study.
4. Absention from caffeine, alcohol and analegesics (asprin, acetaminophen, non-steroidal anti-inflammatories, and glucoceroticoids) on all testing days during pre- and post-trials.
5. Participants consumed a similar diet prior to the oxidative stress run for the pre- and post-testing sessions.
6. Subjects were compliant with the supplementation protocol.
7. Twenty-eight days of beta-alanine supplementation significantly augmented muscle carnosine levels.
8. The subject's knowledge of their assigned supplementation group did not influence the outcomes of the post-testing VO_2max assessments.

Statistical Assumptions

1. The population from which the samples are drawn was normally distributed.

2. The sample was randomly selected and the treatment order was randomly placed.

3. The data met the assumption of sphericity. Sphericity requires that the repeated measures data demonstrate both homogeneity of variance and homogeneity of covariance.

Limitations

1. Subjects were recruited as students from several departmental courses and responded to advertisements located within and around the Huston Huffman Center; therefore, the process of subject selection may not have been truly random. In addition, the sample was made up of volunteers, therefore not meeting the underlying assumption of random selection.

2. Due to time constraints of the supplementation protocol, repeated blood draws, and intensity of the testing session, participant withdrawl was inevitable.

3. Daily dietary fluctuations in endogenous antioxidant consumption may have occured, influencing the oxidative stress responses. A decrease in immune function and/or cold symptoms may have also influenced antioxidant response.

CHAPTER II

REVIEW OF LITERATURE

Oxidative stress markers and timing of sampling

Khassaf, Child, McArdle, Brodie, Esanu, Malcolm, Jackson, 2001 [59]
Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise.

The primary aim of this study was to evaluate the time course response of common antioxidant enzymes in human skeletal muscle after a period of exhaustive, nondamaging aerobic exercise. Seven male participants completed an incremental test to exhaustion on a cycle-ergometer (Monark 864), using a single leg, increasing 35 W every four minutes. Subsequently, participants performed a single-leg constant-load ride at 70% of VO_2peak for 45-min using the opposite leg from the previous test. Muscle biopsies using the Bergstrom-needle were taken from the vastus lateralis of the exercised leg at seven days prior to the exercise protocol, and at one, two, three and six days after. Muscle samples were homogenized in phosphate buffer, pH 7.0, and analyzed for catalase activity by the kinetic decomposition of hydrogen peroxide spectrophotmetrically. Total superoxide dismutase (SOD) activity was measured using a SOD-525 kit (R & D Systems Europe). HSPs were also analyzed using monoclonal antibodies. Peripheral blood samples were also obtained from the subjects before the muscle biopsy at seven days before exercise and at one and three days postexercise and analyzed for total creatine kinase activity. Muscle superoxide dismutase activity increased to a peak at three days post-exercise, with no change in muscle catalase activities. Muscle content of HSP60 and HSP70 increased by 190% and 3,100% of preexercise values, respectively. Ultimately, these

results indicate that human skeletal muscle responds to a single bout of nondamaging exercise by increasing superoxide dismutase activity and suggests an upregulation of HSP content of human muscle after a submaximal exercise bout.

Michalidis, Jamurtas, Nikolaidis, Fatouros, Koutedakis, Papassotiriou, Kouretas, 2007 [72]

Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress

The purpose of this study was to thoroughly investigate the time-course changes of several commonly used markers of oxidative stress by performing serial measurements during a 24-h period after an acute bout of cardiovascular exercise. A batter of oxidative stress markers were used due to the lack of research on just one single biomarker to reliably describe oxidative damage. Eleven untrained men (age, 23 ± 6 yr, ht: 175 ± 3 cm, wt: 75 ± 5 kg; %BF: 14 ± 3%, VO2max: 47 ± 6 mL/kg/min) performed two trials in a random, counterbalanced design. An initial VO2max measurement was conducted to assess the experimental intensities. Seven to 14 days following the VO_2 test, subjects either rested (control trial) or exercised on a treadmill at 70-75% of their VO_2max for 45 minutes (experimental trial). During the experimental trial, the intensity was increased to 90% VO_2max after 45 minutes and exercise was terminated at exhaustion. Water was given ad libitum, heart rate, RPE were measured throughout and food consumption was kept constant with a banana given during the first hour of the post-exercise, followed by standard meals at lunch and dinner. Blood samples were drawn via catheter at rest, immediately after exercise and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, and 24h post. Directly after taking the blood samples, 5% trichloroacetic acid (TCA) was added to whole blood collected in EDTA tubes for reduced glutathione (GSH) analysis. For oxidized glutathione (GSSG) analysis, 5% TCA and 2-vinyl pyridine were added to whole blood

collected in EDTA tubes. The whole-blood samples were centrifuged at 4000g for 10 min at 4ºC. Two hundred µL of the supernatant were dispensed in tubes and mixed with 60 µL of 5% TCA and centrifuged again at 28,000g for 5 min at 4ºC, and the clear supernatants were collected and stored at -30ºC until analysis. Another portion of the blood was collected in plain tubes, left on ice for 20 min to clot, and centrifuged at 1500g for 10 min at 4ºC for serum separation. Serum was transferred in Eppendorf tubes and used for the determination of thiobarbituric acid reactive substances (TBARS), protein carbonyls, catalase and total antioxidant capacity (TAC). Samples were stored in aliquots and -30ºC and were thawed before analysis. For GSH, 20 µL of whole blood was treated and incubated in the dark for 45 min and the absorbance was read at 412 nm. GSSG was assayed by treating 260 µL of whole blood with TCA, NaOH, 2-vinyl pyridine and incubated for 2h at room temp, followed by the addition of TCA, sodium phosphate, NADPH, DTNB, glutathione reductase and then read for absorbance at 412 nm for 3 min. For TBARS, 100 µL of serum was mixed with TCA and Tris-HCL, incubated for 10 minutes, followed by the addition of Na2SO4 and 55mM thiobarbitruic acid solution and incubated at 95ºC for 45 min. The samples were collected on ice for 5 min, centrifuged at 15,000g for 3 min and the absorbance of the supernatant was read at 539 nm. To measure protein carbonyls, 50 µL of TCA was added to 50 µL of serum and incubated in an ice bath for 15 min and centrifuged at 15,000g for 5 min at 4ºC. The supernatant was discarded and 500 µL of 2,4-dinitrophenylhydrazine was added to the pellet and incubated in the dark for 1h with intermittent vortexing every 15 min, followed by a washing step repeated 3 times, and then read at an absorbance of 375 nm. For TAC, 480 µL of 10 mM sodium potassium phosphate and 500 µL of 0.1 mM 2,2-diphenyl-1-

picrylhydrazyl free radical were added to 20 µL of serum, and the samples were incubated in the dark for 30 min at room temp. Samples were centrifuged for 3 min at 20,000g and the absorbance was read at 520 nm. Macronutrient and antioxidant intake was controlled by having participants consume the same diet before both trials. The time to lowest concentration after exercise was 1.7 ± 0.7h for GSH/GSSG, and the time to highest concentration after exercise was 1.2 ± 0.6h for TBARS, 4.4 ± 0.5h for protein carbonyls, 0.5 ± 0.4h for catalase, and 2.2 ± 0.9 for TAC. The greatest change after exercise was -74 ± 9% for GSH/GSSG, 129 ± 29% for TBARS, 135 ± 53% for protein carbonyls, 51 ± 16% for catalase, and 24 ± 10% for TAC. In conclusion, there is no best time point applying to all markers for collecting blood samples after aerobic exercise. The optimum postexercise time points for blood collection in untrained individuals are immediately after exercise for catalase, 1h for TBARS, 2h for TAC, GSH, and GSSG, and 4h after exercise for protein carbonyls.

Effects of acute exercise on oxidative stress.

Schneider, Barp, Ribeiro, Bello-Klein, Oliveira, 2005 [92]

Oxidative stress after three different intensities of running

The purpose of study was to examine the effect of three exercise intensitites based on ventilator thresholds on blood enzyme activities (superoxide dismutase, catalase, glutathione peroxidase), antioxidant capacity (TRAP), and lipid peroxidation (LPO). Participants were assigned to groups based on training status with triathletes (T; n=8) and physical education students (UT; n=9). Each subject underwent four exercise test on a treadmill with the first test being an incremental test to exhaustion to determine VO_2max, aerobic threshold (AeT) and anaerobic threshold (AnT) starting at 5 $km \cdot h^{-1}$ and

increasing by 0.5 5 $km \cdot h^{-1}$ every 20 s (T) or every 30 s (UT) . The subsequent three exercise tests were performed in random order and were designed so that the subjects could maintain a steady state VO_2 corresponding to 10% below AeT (VT1- low intensity), 10% below AnT (VT2-intermediate intensity) and the average between AnT and VO_2max (AnM-high intensity) for 30 minutes, respectively. Blood samples were taken within 15 minutes before exercise and immediately after exercise by venous puncture. Blood samples were drawn into a tube with 100 µL EDTA 10% for hemoglobin (Hb), lipid peroxidation, TRAP, SOD, catalase (CAT), and CPx analysis. After collection, samples were centrifuged for 5 min at 1,000g. Plasma was stored at -70 ºC for further analysis of TRAP. Erythrocytes were washed and centrifuged three times with the same volume of saline solution. Then 75 µL of erythrocytes were diluted in 500 µL of saline solution for LPO and Hb analysis. Remaining erythrocytes were prepared and stored at -70 ºC for protein and antioxidant enzyme analysis. SOD activity was determined by the inhibition rate of pyrogallol auto-oxidation at 420 nm from a standard curve of commercially available SOD kit. Glutathione peroxidase activity was measured by following NADPH oxidation at 340 nm. Catalase activity was measured by following the decrease in absorption at 240 nm. Lipid peroxidation was measured in erhthyrocytes using 400 $mmol \cdot L^{-1}$ tert butyl hydroperoxide initiated chemoluminescence and then light emission was measured using a tritium channel. Total antioxidant capacity was measured in plasma using 320 $\mu mol \cdot L^{-1}$ trolox as standard antioxidant. In plasma, LPO decreased from 3589 ± 193 to 3274 ± 223 $cps \cdot mg\ Hb^{-1}$ and TRAP increased from 340 ± 45 to 384 ± 57 $\mu mol \cdot L^{-1}$ trolox after high intensity exercise in the Trained group. GPx activity increased in the T group only after exercise at moderate and high intensities. Superoxide

dismutase activity increased after exercise at low (8.35 ± 0.85 to 9.23 ± 1.03 U SOD·mg protein^{-1}) and moderate (8.89 ± 0.98 to 10.44 ± 0.86 U SOD·mg protein^{-1}) intensity in UT. There were no changes in catalase activity. In summary, the data indicate that total antioxidant capacity was increased after exercise, and that GPx activity was higher in triathletes than in untrained subjects. CAT activity showed no changes, indicated that the ROS involved in this model were scavenged by GPs. The authors also speculate that these models of exercise could raise plasma uric acid, vitamins, and other anti-oxidants, and therefore increasing serum antioxidant capacity and reduce exercise-induced oxidative stress in T individuals.

Alessio, Hagerman, Fulkerson, Ambrose, Rice, Wiley, 2000 [3]

Generation of reactive oxygen species after exhaustive aerobic and isometric exercise

The purpose of this study was to compare biomarkers of oxidative stress: lipid peroxidation, protein oxidation, and total antioxidants in blood after exhaustive aerobic (AE) and nonaerobic isometric exercise (IE). Twelve men and women completed an initial 2-day diet recall followed by a maximal AE test to exhaustion on a treadmill. Immediately pre, immediately post, and 1 h post exercise, a blood sample was drawn from an antecubital vein into an evacuated tube, and centrifuged immediately at 4ºC. Serum was separated and immediately frozen at -80ºC. Blood levels of malondialdehyde equivalents (MDA), lipid hydroperoxides (LH), protein oxidation, antioxidant activity, and lactate were measured. One week later, subjects performed a maximum nonaerobic (IE) test using a hand grip dynamometer, by performing and MVC with the dominant hand while in the sitting position. Blood samples were drawn in the same fashion to the AE test. Venous blood was collected. Samples for lactate concentration (0.050 mL)

were immediately placed in 6% perchloric acid. Blood was centrifuged at 3000 rpm at 4ºC, serum was separated from red blood cells and stored. LH were measured with chemicals purchased from Cayman Chemical. Absorbance was measured at 500 nm. Lipid peroxidation by-products (MDA) were measured using chemicals from Calbiochem. Absorbance was measured at 586 nm. Protein carbonyls were precipitated from 50 µL aliquots of plasma with trichloroacetic acid and reacted for 60 min at room temp. The derivitized proteins were isolated by precipitation with tricholoacetic acid and washed extensively. Absorbances were recorded at 364 nm. Antioxidant activity was indicated by the oxygen radical absorbance capacity (ORAC) in plasma. The ORAC assay is an inhibition method in which a sample of serum is added to a free radical-generating system, using 10 µL of plasma. VO2 increased 14-fold with AE compared with 2-fold with IE. Protein carbonyls increased 67% pre- to immediately and 1 h post-AE, and 12% pre- to immediately post-IE and returned to baseline 1 h post-IE. TBARS did no increase significantly with either treatment. LH increased 36% above rest during IE compared with 24% during AE. ORAC increased 25% pre- to post-AE, compared with 9% pre- to post-IE. In the current study, lipids were more likely to undergo peroxidation during exhaustive IE and proteins were more likely to be oxidized and form carbonyls during exhaustive AE. Compared with IE, AE was associated with an approximately 2.5-fold increase in peroxyl radical quenching activity. The stimulus for pro-oxidant and antioxidant activity must differ between AE and IE due to the different metabolic demands, although a mechanistic basis for this distinction is not clear.

Lovlin, Cottle, Pyke, Kavanagh, Belcastro, 1987 [66]

Are indices of free radical damage related to exercise intensity

The stress of exercise, by causing substrate depletion and modification of the NADH/NADPH ratio by effecting lactate production, uptake and removal, may also directly affect free radical generation and lipid peroxidation. Therefore the aim of the present study was to evaluate a possible relationship between exercise intensity, lactate metabolism and lipid peroxidation. Six male subjects completed five exercise sessions. Two initial maximal oxygen consumption tests were conducted on a Monark ergometer, beginning with a 5 min warm-up at 88 W, then increased by 30 W each minute until exhaustion. In the remaining three exercise tests, the subjects first rested for 15 minutes on the ergometer, with a blood sample drawn after 7 min. The subjects then exercised at 40% VO_2max for 5 min and rested for 5 min. This was immediately followed by 5 min of exercise at 70% VO_2max and 5 min of rest. The subjects then completed the intermittent exercise regime by pedaling to voluntary exhaustion at an increase load (30 W each minute). Blood samples were drawn into 7 ml heparinized vacutainers at the midpoint of the rest periods and after voluntary exhaustion. The samples were immediately placed on ice and help until completion of the exercise test when they were centrifuged to separate plasma. The plasma was stored in the refrigerator and analyzed the next day. Three plasma aliquots (300 µL) were analyzed for evidence of lipid peroxidation by adding the plasma to distilled water before addition of HCL TBA reagent solution. The samples were put in a hot bath at 95 ºC for 8 min, centrifuged for 10 min and the optical density was measured at 535 nm. Lactate concentrations were determined enzymatically by converting lactate to pyruvate in the presence of lactate dehydrogenase (LDH) and NAD^+. All samples were analyzed in triplicate after adding 0.1 ml of the deproteinized samples to 2.9 ml of the reagent solution and incubated. Samples were

read at 340 nm. The same procedure was used for determination of MDA, based on MDA standards. Maximal exercise to exhaustion resulted in a 26% increase in plasma lipid peroxides. Short periods of intermittent exercise, with varied intensities, indicated a correlation between lactate and MDA. Blood lactate concentrations increased throughout this exercise regimen. A significant decrease (10.3%) in plasma MDA occurred at 40% VO_2max. At 70% VO_2max plasma MDA was still below resting values. At exhaustion, plasma MDA and lactate were significantly greater then at rest. Lovin and colleagues suggest, based on their findings, that exhaustive maximal exercise induces free radical generation while short periods of submaximal exercise (<70% VO_2) may inhibit free radical generation and lipid peroxidation.

The influence of exogenous antioxidants on oxidative stress

Bloomer, Goldfarb, Mckenzie, 2006 [12]

Oxidative stress response to aerobic exercise: comparison of antioxidant supplements

The primary aim of this investigation was to compare the effects of vitamin C + E (the most examined antioxidants studied in relation to exercise) and an ecapsulated fruit and vegetable juice powder concentrate on the oxidative stress response to acute aerobic exercise. Aerobically trained men (n=25) and women (n=23) were assigned to one of three treatments: 400 IU of vitamin E + 1g of vitamin C (V; n=15), a fruit and vegetable juice powder concentrate (FV; n=16) or a placebo (P; n=17). All participants completed a maximal graded exercise test at 0% grade at a self-selected pace. The grade was increased every 2 min while the speed was kept constant, with subjects reaching exhaustion by 8-12 minutes. The VO_2max data were used to calculate the workload for the submaximal exercise bouts and to categorize fitness levels. Subjects ran for 30 min at

80% VO_2max before, after two weeks of supplementation, and after a one-week washout period. Plasma proteins, protein carbonyls, malondialdehyde, and vitamin E were determined from blood collected via vacutainer and immediately centrifuged at 3000 rpm for 15 min at 4ºC and then stored at -80ºC until analyzed. An additional 5 mL of blood was collected into serum collection vacutainers for analysis of 8-OHdG. Blood was allowed to clot at room tem and then separated for serum and stored for later analysis. All assay procedures were performed in duplicate. Plasma protein was determined comparing samples against known standards and plasma samples were also used for the measurement of malondialdehyde. Measurement of 8-OHdG was performed using an ELISA from Genox corp. Vitamin C analysis was performed immediately on plasma by adding it to 5% TCA, 85% orthophosphoric acid and α,α' dipyridyl and were read at 525 nm and compared against a standard curve. Plasma vitamin E was determined by HPLC at 290-nm wavelength. The V treatment increased plasma vitamin C and E after two weeks of supplementation, with no change in the FV or P. Postexercise PC values were elevated for all treatments after all exercise bouts. Both V and FV attendated the exercise-induced increase in PC two weeks of supplementation (V=21%, FV=17%), and after the 1-week washout (V=13%, FV=6%) compared with P, and no differences between V and FV. MDA was unaffected by exercise and treatment. 8-OHdG was significantly lower for V than for FV and P. The authors suggest that V and FV supplementation for 2wk can attenuate the rise in protein carbonyls after 30 min of aerobic exercise, even after a 1-wk washout, with no impact on plasma MDA or 8-OHdG.

Goldfarb, McKenzie, Bloomer, 2007 [41]

Gender comparisons of exercise-induced oxidative stress: influence of antioxidant supplementation

The purposes of this investigation were (i) to ascertain if gender has an influence on exercise-induced oxidative stress using a moderate-intensity exercise with equal relative exercise intensity, and (ii) to ascertain if gender has an effect on antioxidant supplementation on blood indices of oxidative stress under controlled conditions. Twenty-five men and 23 women underwent a graded VO_2max test on a treadmill. After a five minute warm up, subjects ran at a self-selected pace at 0% grade for two minutes with an increase in grade of 2% every two minutes. Subjects were randomly assigned to one of three treatments: placebo (P), antioxidant (A: 400 IU vitamin E + 1 g vitamin C), or a fruit and vegetable powder (FV). All subjects completed three exercise bouts at 80% VO_2max for 30 minutes, before and after two weeks of treatment and again after a one-week washout period and were allowed to consume water ad libitum during the exercise. Blood samples were obtained before and immediately after each exercise bout form an antecubital vein. About 10 mL of blood was obtained by vacutainer at all collections. Samples were immediately processed for glutathione and vitamin C. Plasma was obtained and later analyzed for protein carbonyls (PC), vitamine E, and malondialdehyde (MDA). Serum was obtained and stored in separate microtubes and analyzed for 8-hydroxydeoxyguanosine (8-OHdG). Whole blood was immediately processed for glutathione. Blood was immediately treated with 10% 5-sulfosalicylic acid containing 1 mmol/L bathophenanthrolinedisulfonic acid mixed and centrifuged at10,000 r/min for 15 min at 4ºC. The supernatants were stored at -80ºC and later determined for total glutathione (TGSH) and oxidized glutathione (GSSG). TGSH and GSSG were analyzed

using 5,5'-dithiobis-2-nitrobenzoic acid and measured at 412 nm using a spectrophotometer (Shimadzu UV-1601). GSH was computed by subtracting 2 x the value obtained for GSSG from TGSH (GSH=TGSH-(GSSG)). Plasma protein was determined by comparison to a known standard and was run through columns and the effluent monitored spectrophotometrically. MDA was measured using a colorimetric method and 8-OHdG was performed using an enzyme-linked immunosorbent assay from Genox Corp. Before supplementation, women had higher resting reduced glutathione, total glutathione, and plasma vitamin E compared with men. With both A and FV supplementations, plasma vitamin E gender differences disappeared. Protein carbonyls, oxidized glutathione and malondialdehyde all increased similarly for both men and women in response to exercise. Both A and FV attenuated the reduced glutathione decrease and the oxidized glutathione and protein carbonyls increase compared with P, with no gender differences. 8-OHdG was lower with treatment A compared with FV and P only for men. Plasma vitamin C increased 39% (A) and 21% (FV) compared with P. In conclusion, this study indicates subtle differences in men and women at rest and prior to antioxidant supplementation with several oxidative stress markers. This study demonstrates that young men and women given similar relative intensity and duration of exercise will demonstrate comparable alterations in blood markers of oxidative stress. Despite slightly different antioxidant protection prior to supplementation, men and women given a similar antioxidant treatment for two weeks can enhance their antioxidant status at rest and demonstrate similar results in reducing exercise-induced oxidative stress.

Watson, Callister, Taylor, Sibbritt, Macdonald-wicks, Garg, 2005 [110]

Antioxidant restriction and oxidative stress in short-duration exhaustive exercise

Watson and colleagues primary purpose was to determine the effects of dietary antioxidant restriction on oxidative stress (as measured by F2-isoprostane), antioxidant defenses (as measured by TAC, uric acid, vitamins E, C and β-carotene, and serum glutathione), and exercise performance in athletes. Seventeen healthy, well-trained male endurance running athletes performed an initial VO_2max incremental test to exhaustion starting at 10 km·h^{-1} with 0% gradient. Speed was increased 2 km·h^{-1} every two minutes until the subject could not run any faster; the gradient was then increased by 2% each minute until the fatigue. Subsequently, participants underwent two separate exercise tests, running 30 min at 60% VO_2max, followed by an incremental running test to exhaustion immediately following, increasing the speed by 2 km·h^{-1} every two minutes to top running speed. Before the initial exercise tests, participants followed their habitual (high) antioxidant (H-AO) diets. Then they followed a two-week restricted-antioxidant (R-AO) diet before the second exercise test. Four 20-mL blood samples were collected at rest, after 30 minutes of submaximal exercise, immediately after high-intensity exhaustive exercise, and after 1h of recovery. Blood was collected into EDTA coated tubes and placed on ice. Plasma and red blood cell (RBC) pellets were obtained by centrifugation of whole blood at 3000g for 10 minutes at 4ºC. All blood analyses were corrected for changes in blood volume post exercise by measuring hemoglobin and hematocrit concentrations. Plasma-free F2-isoprostanes was added to 300 µL of plasma, followed by ethanol and was chilled to precipitate proteins. The samples were centrifuged through a series of washing steps. A total of 50 µL of purified samples was analyzed with an F2-isoprostane immunoassay kit (Cayman Chemical). Absorbance

values were determined using a plate reader at 405 nm. The antioxidant capacity of plasma was analyzed using the ImAnOx Colorimetric test system. A standard amount of hydrogen peroxide (H_20_2) was added to plasma and the reaction was determined colorimetrically. Plasma uric acid was analyzed using a commercial assay on a COBAS-BIO centrifugal analyzer. Absorbance was measured at 520 nm. TGSH and GSSG were analyzed using a commercial assay (OxisResearch) using the Ellman's reagent, which reacts with GSH to form a spectrophotometrically detectable complex at 412 nm. Vitamin E and β-carotene were separated by reverse phase high-performance liquid chromatography and measured using a programmable wavelength UV-visible detector. At 0.01 min, vitamin A was measured at 310 nm; at 5.5 min vitamin E was measured at 280 nm and at 9.0 min, β-carotene was measured at 450 nm. The R-AO diet induced a threefold reduction in antioxidant intake when compared with habitual antioxidant intake when compared with habitual-antioxidant (H-AO) diets. F2-isoprostane concentration was significantly higher after submaximal (38%), exhaustion (45%), and 1 h of recovery (31%) when following the R-AO diet compared with the H-AO diet. Rate of perceived exertion was increased on the R-AO diet although exercise time to exhaustion was not affected. Total antioxidant capacity and circulating antioxidant concentrations tended to be lower when following the R-AO diet. In summary, the athletes following a 2-week restricted antioxidant diet had an increased susceptibility to lipid peroxidation, even though their blood antioxidant defenses were not significantly decreased. These findings highlight the vital and adequate role of high-antioxidant foods have in defending against oxidative stress. However, according to the authors, there seems to be no valid reason to recommend antioxidant supplements to athletes participating in acute high-intensity

exercise events up to 40 min in duration except in those consuming a low-antioxidant diet.

Beta-alanine and its role in increasing muscle carnosine levels.

Baguet, Reyngoudt, Pottier, Everaert, Callens, Achten, Derave, 2009 [6]

Carnosine Loading and washout in human skeletal muscles

The aim of the present study was to investigate the magnitude of supplementation-induced amplitude of carnosine synthesis and the time course of subsequent elimination on cessation of supplementation in different human skeletal muscle types, including the soleus, anterior tibialis, and medial gastrocnemius. In a double-blind, randomized fashion fifteen male subjects supplemented with either placebo (maltodextrin) or beta-alanine (Carnosyn, National Alternatives International) for 5-6 weeks. Supplements were provided in capsules of 400 mg and were administered each day as six divided doses, with at least 2 h in between ingestions. Daily doses consisted of 2.4 g/day during the first two days, 3.6 g/day during the subsequent two days, and from then on 4.8 g/day until the end of the supplementation. Carnosine quantification was performed in the week before the start of supplementation (Pre), during the last week of supplementation (Post), and 3 weeks and 9 weeks following the end of supplementation. Carnosine content was measured using proton magnetic resonance spectroscopy in three skeletal muscles of the lower leg (%type I/II fibers: soleus, 86/14; tibialis anterior, 73/27; and gastrocnemius, 44/56). The beta-alanine supplementation significantly increase carnosine content in the soleus by 39%, +27% in the tibialis anterior and +23% in the gastrocnemius. Following the three week washout 26.1% ($p=0.171$) of the increase in the soleus, 20.1% ($p=0.50$) of the increase in the tibialis and 44.7% ($p=0.166$) of the increase in the gastrocnemius had disappeared. Nine weeks after cessation of supplementation, the carnosine content in the

soleus was not different from the initial carnosine content. The carnosine concentration of the tibialis anterior and the gastroc also returned to their initial levels, representing a washout rate of 2.5%-3.5%/week. When stratified for high responders and low responders, washout periods may range from 15 to 6 weeks, respectively.

Dunnett, Harris, Dunnett, Harris, 2002 [33]

Plasma carnosine concentration: diurnal variation and effects of age, exercise and muscle damage

The aims of this study were to 1) measure the plasma carnosine concentration range in Thoroughbred horses of differing ages and genders; 2) study the 24 h variability in plasma carnosine in fed and fasted horses, and 3) investigate possible concentration changes associated with strenuous exercise and muscle damage. Plasma carnosine concentrations were determined by high-performance liquid chromatography, and plasma aspartate transaminase and creatine kinase activities were determined by kinetic methods using a Kone Specific Autoanalyser. Four separate studies were conducted within this investigation. In study A, blood samples from Thoroughbred horses in training (26 males, 30 females, 25 geldings), yearlings (13 males, 13 females) and foals (2 males, 3 females) were drawn to determine plasma carnosine concentration and the influence of age and gender. Study B evaluated the variations in plasma carnosine concentrations over 24 hours in a fed and fasted stated, separated by two days. Plasma carnosine concentrations as a result of four-weeks of high-intensity exercise were measured before, 5 min, 30 min, 2 hr and 24 h following a stepwise maximal treadmill test. The final investigation measured the changes in plasma carnosine concentration following the onset of equine exertional rhabdomyolysis syndrome from three horses. These investigations determined that carnosine is present in equine plasma, in contrast to man,

with levels consistent with the absence of carnosinase. More so, there was a significant effect of age on plasma carnosine concentrations in resting Thoroughbred horses. Values in horses age three and older were 11.3-14.1 µmol/l, compared to young (foals/yearlings) were 3.9-8.7 µmol/l and therefore may reflect lower skeletal muscle carnosine concentrations. When looking at within-day variation of plasma carnosine concentrations, there were no differences between fed and fasted levels. Intense exercise resulted in a small significant increase in plasma carnosine concentration (pre-exercise: 10.3 ± 1.0 µmol/l; post-exercise: 12.4 ± 4.4 µmol/l). In conclusion, elevated plasma carnosine concentrations are observed following exercise induced muscle damage. Plasma carnosine measurements could provide an alternative clinical indicator of muscle damage; and in conjunction with plasma taurine measurements may be indicative of selective type 1 or type 2 muscle fiber damage.

Dunnett, Harris, 1999 [32]

Influence of oral β-alanine and L-histidine supplementation on the carnosine content of the gluteus medius

The aim of the present study was to evaluate the hypothesis that in vivo carnosine biosynthesis may be dependent upon the availability of endogenous beta-alanine, by studying the effect of chronic dietary beta-alanine supplementation in the horse on the carnosine concentrations in type I, IIA and IIB skeletal muscle fibers. The diets of six un-trained Thoroughbred horses were supplemented three times daily, for 30 days, with beta-alanine (300 mg/kg body weight) along with a comparatively low dose of L-histidine (37.5 mg/kg body weight) to ensure an adequate supply for carnosine synthesis. On day one and day 30 of the supplementation, venous blood samples were collected via an indwelling catheter. Blood samples were also taken on days 6, 18, and 24. Muscle

samples were collected by percutaneous biopsy from the right m. gluteus medius on the day immediately before supplementation commenced (Day 0) and on the day immediately after (Day 31). Plasma beta-alanine, L-histidine and carnosine concentrations were determined by HPLC. Individual muscle fibers were dissected from freeze-dried biopsies and analyzed for carnosine and taurine. Fragments of individual muscle fibers were characterized at types I, IIA or IIB by histochemical staining for myosin ATPase. Results indicate that there was an adaptive response to sustained beta-alanine administration resulting in mean ± SD beta-alanine AUC values increasing significantly from 1130 ± 612 µmol/l (Day 1) to 2490 ± 1416 µmol/l (Day 30), suggesting increased beta-amino transport across the gastrointestinal lumen. There was no consistent increase in histidine AUC between Days 1 and 30 [757 ± 447 µmol/l (Day 1) and 1162 ± 1084 µmol/l (Day 30)]. Type IIA fiber carnonsine concentrations increased from 59.9-102.6 to 76.2-112.2 mmol/kg dry weight, with significance resulting in only two of the six horses. Type IIB fiber carnosine concentrations increased from 101.3-131.2 to 114.3-153.3 mmol/kg, with significance increases in five of six horses. In conclusion, results from this investigation clearly support the hypothesis that *in vivo* the magnitude and rate of carnosine biosynthesis is greatly influenced by the availability of beta-alanine. Chronic dietary supplementation with beta-alanine can increase muscle carnosine concentration in the horse.

Harris, Tallon, Dunnett, Boobis, Coakley, Kim, Fallowfield, Hill, Sale, and Wise 2006 [46]

The absorption of orally supplied β-alanine and its effect on muscle carnosine synthesis in human vastus lateralis

The aim of this study was to investigate the intramuscular availability of β-alanine supplied orally and the effect of supplementation on muscle carnosine synthesis. A series

of 3 studies were utilized to investigate the bioavailability of β-alanine. *Study 1* used a crossover design in which six male subjects (Age: 33.5 ± 9.9 yrs; Wt: 80.2 ± 17.1 kg) underwent four treatments, out of a possible five. All subjects ingested 8 $ml \cdot kg^{-1}$ bwt of chicken broth containing a total of 40 $mg \cdot kg^{-1}$ bwt β-alanine in the form of anserine and carnosine (Treatment A). For treatments B, C, D and E, 3 of the possible 4 treatments were randomly ordered within and between the six subjects. Each subject ingested 3 $ml \cdot kg^{-1}$ bwt of a drink containing B) 0 (control), C) 10, D) 20, or E) 40 $mg \cdot kg^{-1}$ bwt β-alanine, followed by ingestion of 5 $ml \cdot kg^{-1}$ bwt of water. Venous blood and urine samples were collected at 10 min intervals for the first 90 min up to 360 min and for 9 hours, respectively. *Study 2:* Six male subjects (Age 28.3 ± 2.7 yrs; Wt 83.2 ± 14.3 kg) were catheterized after a 12 hr fast. Each subject was given three doses of 10 $mg \cdot kg^{-1}$ bwt β-alanine at 0 (9 am), 3 and 6 hours. Blood samples were taken up to 9 hours after supplementation. Subjects continued to take the supplement 3 times per day for 15 days, on the 15^{th} day, a second blood draw was performed. *Study 3:* The purpose of this segment of the study was to evaluate the effect of 4 weeks of dietary β-alanine supplementation or carnosine on muscle carnosine content. The main study was preceded by a 15-day investigation where sixteen male subjects (Age 19.4 ± 1.6 yrs; Wt 79.5 ± 9.3 kg) were randomly assigned to either β-alanine (n = 8; 800 mg) or matching placebo (n = 8) and instructed to take two, 400 mg capsules at 9 am, 12 pm, 3 pm, and 6 pm. Following the 15 day pre-study investigation, 21 male subjects (Age 26.1 ± 5.6 yrs, Wt 79.5 ± 10.5 kg) ingested β-alanine using one of two protocols; (I and II, n = 5 each); I: 800 mg of β-alanine was given 4 times per day (~3.2 g); II: 800 mg using a more frequent dosing strategy, on average consuming ~ 4 grams per day; or treatments III and

IV. III: Subjects ingested L-carnosine in the same dosing pattern as treatments II and IV: control subjects consumed a maltodextrin capsule to match groups II and III. *Study 1:* β-alanine increased in plasma following ingestion of all treatments, excluding 0 $mg \cdot kg^{-1}$, and was ~ ½ of the ingested solution. Carnosine was not detected in the plasma. Time to peak plasma concentration of all concentrations was between 30-40 minutes. In addition, a 6-8 fold increase was seen with 10 to 20 $mg \cdot kg^{-1}$ ingestion. Taurine concentration was also increased. Study 2 showed similar increases in plasma β-alanine concentrations and time to peak as in study 1. In addition, the time between dosing allowed plasma levels to return to baseline and avoided the flushing sensation. Study 3 showed no significant changes by the end of 4 weeks supplementation with 4 x 800 mg β-alanine, whereas there was a significant increase in carnosine levels with L-carnosine supplementation.

Suzuki, Nakao, Maemura, Sato, Kamahara, Morimatsu, and Takamatsu, 2006 [104]
Carnosine and anserine ingestion enhances contribution of nonbicarbonate buffering

The purpose of this study was to examine the influence of oral supplementation with chicken breast extract (CBEX), which is a rich source of carnosine and anserine, on acid-base balance and performance during intense intermittent exercise. Eight moderately trained men (age: 19.8 ± 0.5 yrs, Ht: 171.8 ± 2.3 cm, Wt: 64.7 ± 2.7 kg) performed intense intermittent exercise that consisted of ten, 5-s maximal sprints on a cycle ergometer, with 25-s recovery. Each subject ingested 190 g of either CBEX or placebo in a soup consistency, 30 minutes before beginning the exercise. Arterial blood samples were collected at rest, 1 minute before the start of the test, after each set, and immediately after the last set. Hydrogen-ion concentration (pH), carbon dioxide partial pressure (pCO2), bicarbonate ion concentration (HCO_3^-), blood lactate (La), and the

concentrations of carnosine, anserine, and the other constitutive amino acids were measured. Concentrations of anserine significantly increased 30 minutes after CBEX supplementation. Carnosine however, did not improve significantly. Two other constituents, β-alanine (27.2 ± 5.9 to 33.6 ± 6.8 μmol·L-1) and histidine (93.3 ± 12.5 to 100.9 ± 11.3 μmol·L-1) were significantly increased from pre- to post-, respectively. In addition, following CBEX supplementation and the cessation of exercise, the pH and HCO_3^- values were elevated significantly. After both the CBEX and placebo treatments, the La concentration increased throughout and after the exercise, and pCO2 increased throughout the exercise, but decreased following cessation of exercise. With regard to the mean power output for each interval set, there were no significant differences observed between the CBEX and placebo group. Furthermore, there were no significant differences between either group, for mean power for all 10 sets (9.35 ± 0.18 $W \cdot kg^{-1}$ (CBEX), 9.50 ± 0.19 $W \cdot kg^{-1}$(Pl)).

The effects of beta-alanine supplementation on performance.

Suzuki, Ito, Mukai, Takahashi, and Takamatsu, 2002 [103]

High level of skeletal muscle carnosine contributes to the latter half of exercise performance during during 30s maximal cycle ergometer sprinting

The purpose of this study was to examine the relationship between the carnosine concentration in skeletal muscle, fiber-type distribution, and high-intensity exercise performance. Eleven recreationally active men (age: 21.7 ± 1.4 years, Ht: 171.8 ± 6.1 cm, Wt: 60.5 ± 3.9 kg, VO_2max: 48.2 ± 2.3 $ml \cdot kg^{-1} \cdot min^{-1}$) performed an initial 30-s maximal cycle ergometer sprint at 7.5% of their body weight. To determine the intramuscular concentration of carnosine, muscle biopsies were taken from the vastus lateralis, 6-7 days before the sprint cycle bout, and further analyzed with an amino acid

auto analyzer. Fiber-type distribution was also analyzed. The carnosine concentration was significantly correlated with mean power (r = 0.785), and demonstrated a positive, but non-significant correlation with peak power (r = 0.598). The correlations observed between the carnosine concentration and the mean power of the final 2 phases of the 30-s sprint, were the most highly correlated (21-25s: r = 0.694; 26-30s: r = 0.660). A significant, positive correlation between % Type II_x fiber type content and carnosine concentration was reported. In addition, % Type II_x fiber area was significantly correlated with mean power (r = 0.809) and peak power (r = 0.732).

Stout, Cramer, Zoeller, Torok, Costa, Hoffman, and Harris, 2007 [101]

Effects of beta-alanine supplementation on the onset of neuromuscular fatigue and ventilator threshold in women

The purpose of this study was to examine the effects of 28 days of β-alanine supplementation on PWC_{FT}, VT, VO_2max and TTE in women. This double-blind, randomized, placebo controlled study, included 22 women (age: 27.4 ± 6.1 yrs) that were placed into a β-alanine (n = 11) or placebo (n = 11) group. Each subject went through a series of tests to measure PWC_{FT}, VO_2max, VT, and TTE, before and after the supplementation period. All data was collected and values were determined from one initial trial of the PWC_{FT}. Subjects performed a graded cycle ergometer test, beginning at 40 W and increasing 20 W every 3minutes until the individual could no longer maintain a speed of 70 rpm. During the PWC_{FT} test, oxygen consumption measurements were collected, using open circuit spirometry and used to calculate VO_{2MAX} and VT. In addition, bipolar surface electrodes were placed over the vastus lateralis of the right thigh to collect muscle activity. Following initial testing, subjects began a 28 day supplementation period in which β-alanine was taken at 4 different times each day.

During days 1-7, 3.2 $g \cdot d^{-1}$ were consumed, and 6.4 $g \cdot d^{-1}$ were taken for the remaining duration (days 8-28). There were 13.9, 12.6 and 2.5% significant increases in VT, PWC_{FT}, and TTE, respectively, for the β-alanine group, but no changes observed in the placebo group. However, there were no changes in VO_2max, for either group. In addition, there was no significant difference between groups for their compliance rate (89.5% for the β-alanine group; 91.7% for the Placebo group), which represents a 5.0 $g \cdot d^{-1}$ intake.

Hill, Harris, Kim, Harris, Sale, Boobis, Kim, and Wise, 2006 [47]

Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity

The aims of this study were to examine the effects of a 10 week β-alanine supplementation protocol on muscle carnosine accumulation to determine if changes were specific to Type I or Type II muscle fibers, and to determine the effects of β-alanine on performance during a high-intensity, endurance cycling capacity test. Twenty-five physically active males were randomly assigned to either a β-alanine (β-ala, n = 13) or a placebo group (P, n = 12) (age: β-ala: 25.4±2.1, P: 29.2 ± 6.9 yrs, Ht: β-ala: 184.1±7.5, P: 182.1±7.9 cm). Subjects performed a high-intensity cycling test in which the power output began at 100 or 150 W, depending on fitness, with an increasing intensity of 12.5 W every 30 s until volitional exhaustion. Maximum power output was calculated and used at a 110%, to complete two cycle capacity tests (CCT110%). Subjects exercised at power outputs corresponding to 80% of VO_2max (Wmax) for the first 15 s, 95% Wmax for the second 15 s and then 110% Wmax until volitional exhaustion. The time and average power output were recorded and used to calculate total work done (TWD: average power (W) x time (seconds). To evaluate the changes in intramuscular carnosine

concentration, muscle biopsies were taken and analyzed by HPLC from the mid section of the vastus lateralis, 1-2 days after the pre-supplementation cycle test, and 1-2 days after the 4 and 10 week tests. Muscle carnosine concentrations were significantly increased by 58.8% and 80.1% after 4 and 10 weeks of β-alanine supplementation, respectively. The initial carnosine concentration was 1.71 times higher in type IIa fibers than in Type I fibers, but increased equally in both Type I and IIa fibers, but, there were no increases observed in the placebo group. TWD was significantly increased following 4 and 10 weeks of β-alanine supplementation (7.3 ± 1.3 and 8.6 ± 3.1 kJ), compared with no change in the placebo group. In addition, TWD and increased muscle carnosine concentration were correlated, but not significant.

Van Thienen, Van Proeyen, Eynde, Puype, Lefere, Hespel, 2009 [106]
β-Alanine improves sprint performance in endurance cycling

Van Thienen and colleagues investigated the effect of short-term β-alanine (βALA) administration in a simulated cycling race. In a double-blind, placebo-controlled approach, 17 male cyclists supplemented for eight weeks with βALA or placebo (PL), interspersed with two experimental sessions (pretesting vs. posttesting). βALA was administered in 500-mg capsules to be ingested at regular intervals throughout the day. Dosage was gradually increased from 2 $g \cdot d^{-1}$ (weeks 1 and 2) to 3 $g \cdot d^{-1}$ (weeks 3 and 4) and eventually 4 g daily from week 5 to the end of the study. PL capsules contained maltodextrin. Subjects completed an initial incremental exercise test starting at 100 W and increasing 40 W per 8-minute stages until volitional exhaustion. One week later (and following 8 weeks of supplementation), the subjects participated in a simulated road race, receiving a standardized dinner the evening before. The exercise protocol started with a 110-min intermittent endurance exercise bout during which exercise intensity was varied

between 50% and 90% (10-min stages) of their previously estimated max. Immediately at the end of the intermittent exercise bout, a 10-min time trial was started at 100% max workload (~89% VO_2max), with the subjects voluntarily increasing/decreasing the workload at 1-min intervals according to their perception of fatigue. At the end of the time trial, subjects were allowed to recover for 5 min at a workload corresponding to 50% of their max. Finally, they performed a 30-s all-out sprint in an isokinetic mode. Mean power output during the time trial was approximately 300 W and was similar between PL and βALA during both the pretesting and the posttesting. However, compared with PL, during the final sprint after the time trial, βALA increased by 11.4% (95% confidence interval = +7.8 to + 14.9%), on average, whereas mean power output increased by 5.0% (95% confidence interval = +2.0 to +8.1%). Blood lactate and pH values were similar between group at all times points. In conclusion, oral βALA supplementation can significantly enhance sprint performance at the end of an exhaustive endurance exercise bout.

Carnosine as an antioxidant

Babizhayev, Seguin, Gueyne, Evstigneeva, Ageyeva, Zheltukhina, 1994 [5]
L-carnosine (β-alanyl-L-histidine) and carcinine (β-alanylhistamine) act as natural antioxidants with hydroxyl-radical scavenging and lipid-peroxidase activities

The purpose of this study was to elucidate the molecular basis of the protective effect of carnosine in biological systems by testing the anti- or pro-oxidant activities of carnosine and other imidazole-containing compounds (i.e. carcinine, histamine, and histidine) physiologically linked to carnosine in the carnosine-histidine-histamine metabolic pathway. Both carnosine and carcinine (10-25 mM) are capable of inhibiting the catalysis of linoleic acid and phophatidylcholine liposomal peroxidation (LPO) by the

$O2^{-\cdot}$ dependent ion-ascorbate and lipid-peroxyl-radical generating linoleic acid 13-monohydroperoxide (LOOH)-activated haemoglobin systems, as measured by thiobarbituric-acid-reactive substance. Linoleic hydroperoxide was quantified using an iodometric technique. Peroxidation of the phosphatidylcholine liposomes or the emulsion of linoleic acid was initiated, and the concentration of LPO products in the oxidized lipid substrates were measured by reaction with thiobarbituric acid. Carcinine and carnosine are good scavengers of $OH^{\cdot}$ radicals, as detected by iron-dependent radical damage to the sugar deoxyribose suggesting that carnosine and carcinine are able to scavenge free radicals or donate hydrogen ions. The iodometric, conjugated diene and t.l.c. assessments of lipid hydroperoxides (13-monohydroperoxide linoleic acid and phosphatidylcholine hydroperoxide) showed their efficient reduction and deactivation by carnosine and carcinine in the liberated and bound-to-artificial-bilayer states. Different constituents and imidazole-containing compounds were compared for their ability to inhibit the iron-ascorbate and LOOH/Hb-dependent oxidation of LA and PC liposomes. The results demonstrate that carnosine and carcinine significantly inhibited the LPO reaction at 5-25 mM concentration. The constituents histidine and histamine exhibited stimulatory peroxidative effects for free LA or PC liposomes. The other tested compounds, i.e. imidazole, beta-alanine and the mixtures of beta-alanine + histidine or beta-alanine+ histamine, were inactive or showed a slight stimulatory activity to LPO suggesting that the redox potential of the whole β-alanyl-imidazole-containing molecule is essential for its antioxidant activity. Both carnosine and carcinine were effective at inhibiting the production of TBARS in the LOOH-activated Hb catalysis of LPO (which is not sensitive to chelating agents) suggesting that the antioxidant mechanism depended

on the ability to scavenge lipid peroxy radicals or donate hydrogen ions and not solely on chelation. Degradation of deoxyribose was found to be strongly inhibited by carnosine and carcinine and therefore delaying a rise in $OH^{\cdot}$ radicals. These results suggest that carnosine and carcinine appear to be physiological antioxidants able to efficiently protect the lipid phase of biological membranes and aqueous environments.

protect the lipid phase of biological membranes and aqueous environments.

Boldyrev, Stvolinsky, Tyulina, Koshelev, Hori, Carpenter, 1997 [20]

Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals

The current study examined the actions of carnosine and its natural derivatives on two isolated enzyme systems, and on two physiological models of hypoxia, to test their protecting efficiency on the brain systems under injury. Na/K-ATPase was prepared from ox brain gray matter and assayed for activity by determining inorganic phosphate released. Oxidation was triggered by adding 10 mM hydrogen peroxide prior to activity determination for periods of up to 40 min, and controls, buffered with PIPES, compared with preparations of enzyme containing either carnosine of homocarnosine. Ischemia was applied to living brain slices, immersed in oxygenated Krebs-Ringers solution and then stimulated on the lateral olfactory tract. Additionally, whole-animal hypoxia experiments were performed on ten white rats in each experimental group. Each animal was injected with either carnosine (250 mg/kg) or saline in a single intraperitoneal injection 20 mi prior to the experiment. Experiments were performed in a 5-L chamber with equalized pressure corresponding to an altitude of 10,500 m above sea level. The resistance of each rat to hypoxia was judged by the time interval to loss of ability to stand, as well as the time to cessation of spontaneous breathing. After cessation of

breathing, the pressure was returned to atmospheric levels and the time needed to recover the ability to stand was recorded. Boldyrev and colleagues demonstrated that both carnosine and homocarnosine had a facilitating effect on inorganic phosphate liberations by ATPase, even at time 0. The enzyme activity in the control decayed rapidly over the 40 min experiement, as the result of destruction triggered by the hydrogen peroxide, while both carnosine and homocarnosine were shown to be protective. The ability of carnosine to function as a $OH^{\cdot}$ scavenger should serve to protect NA/K-ATPase and support functional activity of neurons. Additionally, tyrosine hydoxylase is a system for assay of oxidant actions. In the presence of carnosine (20 μM) and homocarnosine, tyrosine hydoxylase was found to be reduced to 26% and 60% of the control, respectively. With respect to evaluating whether carnosine can protect the brain from ischemic damage, Boldyrev et al fail to demonstrate direct excitation of neurons by carnosine suggesting that carnosine does not operate as a neurotransmitter, which is consistent with previous literature. More so, when relating carnosine to whole-animal hypoxia, carnosine resulted in a strong protective action delaying the time to loss of coordination and cessation of breathing and shortening the recovery. In summary, although there is no evidence that carnosine alters neuronal excitability of modulation of synaptic transmission, as least one function of carnosine may be to prevent damage to the cell from production of reactive oxygen species.

Boldyrev, Song, Lawrence, Carpenter, 1999 [16]

Carnosine protects against excitotoxic cell death independently of effects on reactive oxygen species

This investigation explored the actions of carnosine, homocarnosine and N-acetylcarnosin on acutely isolated cerebellar granule cells, studied in a flow cytometer

monitoring cell death upon application of excitatory amino acids and measuring ROS accumulation. Wistar rats (10-12 days-old weight 18-22 g) were used in all experiments. Cerebellar granule cell neurons were obtained from cerebellum slices that were incubated and filtered. Changes in ROS concentration were measured by use of 2',7'-dichlorofluorescein diacetate (DCF-DA) to determine oxidation and damage plasma membranes. In order to distinguish necrotic from apoptic cell death FITC-labeled Annexin-V was used to detect cells in early apoptosis. Using the method of blotting propidium iodide (PI) allowed the authors to follow the progression to cell death by either necrosis or apoptosis. For all of the flow cytometric measurements, carnosine, homocarnosine or N-acetylcarnosine were added to the cells at the beginning of the 1 h incubation period at a concentration of 10 mM. Boldyrev and colleagues reported that carnosine, N-acetylcarnosine and homocarnosine at physiological concentrations are all potent in suppressing fluorescence of 2',7'-dichlorofluorescein, which reacts with intracellularly generated reactive oxygen species. Only carnosine in the same concentration range was effective in preventing apoptotic neuronal cell death as demonstrated from the DNA binding dye, PI and FITC-labeled Annexin-V. These results indicate that carnosine and related compounds are effective scavengers of reactive oxygen species generated by activation of ionotropic glutamate receptors, but this action does not prevent excitotoxic cell death. Some other process, which is sensitive to carnosine, but not the related compounds is a critical factor in cell death.

Chan, Decker, Chow, Boissonneault, 1994 [22]

Effect of dietary carnosine on plasma and tissue antioxidant concentrations and on lipid oxidation in rat skeletal muscle

The objective of the present study was to determine how dietary carnosine, at a concentration equivalent to that in a diet containing 20% beef, would affect carnosine, anserine, histidine and α-tocopherol concentrations in both α-tocopherol-deficient and α-tocopherol-adequate rats. The secondary aim of this study was to determine the effect of dietary carnosine and α-tocopherol on the formation of thiobarbituric acid reactive substances (TBARS) in rat skeletal muscle homogenates. Utilizing three groups containing 1) 0.0875% carnosine, 2) α-tocopherol acetate or 3) or carnosine (0.0875%) plus α-tocopherol acetate. Carnosine, anserine and histidine concentrations in the derivatized extract were determined by HPLC using an FS 970 Spectrofluoro Monitor at 310 nm for excitation and 375 nm for emission, based on peak areas and standard curves obtained on 10-100 µg/mL of carnosine and histidine and 100-500 µg/mL anserine. Dietary supplementation with carnosine did not increase carnosine concentrations in heart, liver or skeletal muscle. Dietary supplementation with both carnosine and α-tocopherol increased carnosine concentrations in liver 1.56-, 1.51- and 1.51-fold as compared with diets lacking carnosine, α-tocopherol or both carnosine and α-tocopherol, respectively. To determine the oxidation of skeletal muscle homogenates the extent of lipid oxidation was determined at various time points by measuring TBARS. Dietary supplementation with carnosine, α-tocopherol or both carnosine and α-tocopherol was effective in decreasing the formation of TBARS with α-tocopherol and α-tocopherol plus carnosine being more effective than carnosine alone. In summary, dietary carnosine supplementation did not affect carnosine concentrations in heart, liver and skeletal muscle. Dietary supplementation with carnosine, α-tocopherol, or both carnosine and α-tocopherol effectively decreased lipid oxidation in rat skeletal muscle.

Decker, Crum, Calvert, 1992 [27]

Differences in the antioxidant mechanism of carnosine in the presence of copper and iron

The objectives of this research were to determine if the antioxidant mechanism of carnosine was different in the presence of copper or iron. Differences in antioxidant mechanism were determined by the ability of carnosine to chelate iron and copper as determined by changes in the catalytic activity of the metals and by changes in the ^{1}H NMR spectra of carnosine in the presence of iron or copper. Carnosine (1.0-25 mM) is capable of inhibiting copper- and iron-catalyzed oxidation of phosphatidylcholine liposomes. Lipid oxidation was performed in a model system containing 0.02 mg of phophatidylcholine liposomes/mL of 0.12 M KCl-5 mM histidine buffer (pH 7.0) plus ascorbate, FeCl3 or CuCl2 and various concentrations of carnosine. All reactions were run at 37˚C for 30 min. Lipid oxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) and lipid peroxides with assays containing 0.3 mM ADP, 0.3 mM NADPH, 10 µM FeCl2, 25 mM carnosine, and 0l5 mg of microsomal protein per ml of 0.12 M KCl-5 mM histidine buffer, compared to controls void of carnosine. Percent inhibition was calculated as (1-[(activity in the presence of inhibitor)/(activity in the absence of inhibitor)])x 100. ^{1}H NMR spectra of carnosine and carnosine in combination with either copper or iron were recorded on a Varian VXRS-400 spectrometer at 400 MHz. Decker et al. demonstrated that carnosine was capable of inhibiting both iron- and copper-catalyzed oxidation of phosphatidylcholine liposomes. When lipid oxidation was measured by TBARS, low carnosine concentrations (≤10 mM) more effectively inhibited lipid oxidation catalyzed by copper than by iron. At 1, 5, and 10 mM, carnosine inhibition of TBARS production was 11-, 2.6-, and 1.3-fold higher in

the presence of copper than in iron. At higher carnosine levels, antioxidant responses were similar between iron and copper. More so, the antioxidant activity of carnosine was greater in the presence of copper than of iron when measuring the oxidation of phosphatidylcholine liposomes by lipid peroxides. Neither hisitidine or beta-alanine (25 mM) was found to inhibit iron- or copper-catalyzed formation of TBARS or lipid peroxides. Low concentrations of carnosine (0.1 and 0.05 mM) slightly accelerated the catalysis of ascorbate oxidation by both metals, whereas higher concentrations had no effect. Carnosine was also capable of inhibiting ghost production of TBARS by and iron/NADPH-dependent lipid oxidation system in chicken muscle microsomes. In summary, in addition to iron and copper, carnosine can also inhibit nonmetal lipid oxidation catalysts including peroxyl radicals, lipoxidase, and photoactivated riboflavin. The ability of carnosine to inhibit iron-catalyzed lipid oxidation without chelation and the ability of carnosine to inhibit nonmetal lipid oxidation catalysts suggest that carnosine could be capable of inactivating free radicals and therefore its antioxidant mechanism may be multifunctional.

Decker, Ivanov, Zhu, Frei, 2001 [28]

Inhibiton of low-density lipoprotein oxidation by carnosine and histidine

This research investigated the ability of carnosine to inhibit the oxidation of low-density lipoprotein (LDL) in comparison to its constituent amino acid, histidine. Carnosine was obtained from Sigma Chemical Co. and LDL was prepared from blood plasma of healthy individuals. Oxidation of LDL (20 μg of protein/mL of PBS) was promoted by either Cu^{2+} or 2-amidinopropane dihydrochloride (AAPH). Measurment of TBARS was performed to assess oxidation. Comparisons of the inhibitory effects of carnosine and

hsitidine were determined within the same experiment to minimize variations. The ability of carnosine and histidine to alter the redox cycling of copper was determined by monitoring changes in Cu2+-promoted ascorbic acid oxidation rates. None of the carnosine concentrations tested (3.0-24.0 μM) inhibited Cu^{2+}-promoted tryptophan oxidation after two hours of incubation. Carnosine (3 μM) inhibited Cu^{2+}-promoted LDL oxidation at carnosine/copper ratios as low as 1:1 as determined by loss of tryptophan fluorescence and formation of conjugated dienes after three hours. Carnosine (6 μM) lost its ability to inhibit conjugated diene formation and tryptophan oxidation after two and four hours of incubation. Carnosine and histidine were able to significantly inhibit carbonyl formation in both oxidation systems at concentration ≥0.2 mM. The ability of carnosine and histidine to inhibit LDL oxidation by inactivating free radicals was determined by oxidizing LDL with peroxyl radicals generated from AAPH. At higher concentrations (250 μM) both purified carnosine and histidine have been found to inhibit the peroxyl radical with no differences between dipeptides. The current study demonstrated that histidine was more effective at inhibiting copper-promoted formation of carbonyls on bovine serum albumin than carnosine, but carnosine was more effective at inhibiting copper-induced ascorbic acid oxidation than histidine. Neither dipepetide was a strong inhibitor of AAPH-promoted oxidation of LDL, indicating that their main antioxidant mechanism is through copper chelation.

Kohen, Yamamoto, Cundy, Ames, 1988 [63]

Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain

The aim of the current study was to explore the role of carnosine and its analogues (homocarnosine and anserine) as protectors against oxidative stress and the continued

possibility of carnosine and related compounds of serving as natural antioxidants in skeletal muscle and brain. Using Sigma materials the azo compounds 2,2'-azobis(2,4-dimethylvoleronitrile) (AMVN) and 2,2'-azobis(2-amidinopropane dihydrochloride) (AAPH) were used as free-radical initiators in both homogeneous and liposome system. The rate of peroxyl-radical formation from these initiators is constant at a given temperature and, once produced, can initiate free-radical chain oxidation. The free-radical chain oxidation products linoleic acid hydroperoxide and was determined by injection of 10 µl of the reaction solution onto a 25-cm oxtadecylsilane LC-18 column. The oxidation of soybean PtdCHo was measured by the formation of PtdCho hydroperoxide. The effects of carnosine were also measured by the formation of 8-hydroxy-deoxyguanosine (8-OHdGuo). The pH of all solutions were tested and adjusted to 7.2-7.6. Results from this study demonstrated that when carnosine was introduced into the AMVN and linoleic reaction mixture, there was a decrease in the rate of oxidation of linoleic acid to linoleic acid hydroperoxide. Carnosine at concentrations of 1, 7.5, and 10 mM reduced the rate of oxidation of the linoleic acid with rates of 14, 6.5 and 4 $\mu M \cdot min^{-1}$. The evaluation of antioxidant activity in the AAPH system were minimized, similar to the AMVN at 2.8, 1.9, 1.7 and 0.9 $\mu M \cdot min^{-1}$ for the same carnosine concentrations compared to the antioxidant control at 3.6 $\mu M \cdot min^{-1}$. Homocarnosine and anserine demonstrated similar antioxidant protective activity at 56% protection compared to 53% protection of carnosine. The structural component responsible for the antioxidant activity was evaluated demonstrating that L-Alanyl-L-histidine (which differs from carnosine only in the form of the amino acid alanine), shows a less-pronounced antioxidant activity than carnosine. The alanyl residues alone (L-alanine or β-alanine), the combination of

both (l-alanyl-l-alanine or β-alanyl-l-alanine) and γ-aminobutryric acid (GABA) (present in homocarnosine) failed to show any antioxidant activity, suggesting that the imidazole ring is responsible for the antioxidant activity. Carnosine prevented oxidative damage of deoxyguanosine induced by ascorbic acid and copper ions. Conclusions regarding carnosine, homocarnosine and anserine suggest antioxidant activity. All of these compounds showed peroxyl radical trapping activity and were electrochemically active as reducing agens. Furthermore, carnosine inhibited the oxidative hydroxylation of 8-OHdGuo induced by copper ions. Kohen and colleagues also demonstrate other roles of carnosine, such as chelation of metal ions, quenching of singlet oxygen, and binding of hydroperoxides.

Egorov, Kurella, Boldyrev, Krasnovsky, 1997 [35]

Quenching of singlet molecular oxygen by carnosine and related antioxidants. Monitoring 1270-nm phosphorescence in aqueous media

The goal of the current study was to quantitatively assess the rate constants of $^1O_2$ quenching by carnosine and its components: beta-alanine, L-histidine, and imidazole, and compare their activities with other natural metabolites of muscle tissues- anserine and taurine that are considered to contribute to tissue antioxidant systems. $^1O_2$ quenching was obtained using the most reliable method, by time-resolved monitoring of 1270-nm $^1O_2$ phosphorescence. In short, utilizing a series of pulse beams at differing wavelengths allowed for measurement of phosphorescence kinetics, quantifying the quenching activities (K_q) for taurine, L-anserine, L-carnosine, imidazole, L-histidine, ergothioneine, and beta-alanine. The rate constants of $^1O_2$ quenching (K_q) for carnosine, histidine, and imidazole were similar to each other and ranged from $2x10^7$ to $4x10^7$ $M^{-1}s^{-1}$. The activity of non-aromatic amino acids – taurine and beta-alanine, demonstrated very low

quenching activities at $<3x10^3$ $M^{-1}s^{-1}$. These results indicate that the imidazole residue in the carnosine molecule is responsible for its quenching activity and the peptide body between histidine and alanine does not influence the K_q value. Additionally, taurine did no show any measureable quenching activity in the concentration range. The results of the current study suggest that the dipeptides can be used as potent water-soluble protectors against $^1O_2$ attack whereas under natural conditions, either radical scavenging or other biochemical properties of carnosine, related histidine-containing dipeptides and taurine may be more important for other biological functions rather than interaction with singlet oxygen.

Salim-Hanna, Lissi, Vid, 1990 [91]

Free radical scavenging activity of carnosine

The aim of the current study was to assess the ability of carnosine to interfere with the free radical-mediated inactivation of horseradish peroxidase and lysozyme, and to inhibit lipid peroxidation either in a metal-depended process (oxidation of brain homogenates) or in a metal-independent system (red cell membranes supplemented with 2,2'-azobis-(2-aminopropane) (ABAP)). Thiobarbituric acid reactive substance (TBARS) of lipid peroxidation were determined in brain homogenates and erythrocyte ghost membranes before and after 1 hours incubation. Horseradish peroxidase activity was measured by recording the increase in absorbance at 420 nm produced as a consequence of the oxidation of pyrogallol. Solutions of lysozyme and/or tryptophan, with or without carnosine were incubated to evaluate lysozyme activity from the lysis rate of *micrococcus luteus*. Carnosine addition up to 17 mM did not produce any protection in either lipid peroxidation system, as assayed by the oxygen uptake rate. However, results obtained for

carnosine and beta-alanine indicate that both additive concentrations are able to afford complete protection to the HRP enzyme and lysozymal radical mediated inactivation. The mean carnosine concentrations required to inhibit the inactivation by 50% were 0.13 mM and 0.6 mM for HPR and lysozyme, respectively. Furthermore, while carnosine is not particularly reactive towards alkylperoxyl radicals, its capacity to interact with these radical might contribute to decreasing protein damage by reactive oxygen species. When evaluating lipid peroxidation, results demonstrated that carnosine did not alter the erythrocyte ghost membrane system. In conclusion, carnosine does not prevent metal ion-independent oxidation of red cell membranes, and has only a small effect upon the brain homogenate oxidation rate, when employed in high concentration. More so, the addition of L-histidine or beta-alanine up to 12 mM did not produce any noticeable effects upon the oxygen consumption rate.

CHAPTER III

METHODS

Participants

Fifty-four moderately trained men (N=26) and women (N=28) were recruited for this investigation. One male subject was lost during pre-testing due to a musculoskeletal injury. One male was excluded from the oxidative stress analyses due to scheduling. Two females dropped out due to the inability to complete the 40 minute run and a musculoskeletal injury, respectively. Therefore, pre- and post-supplementation data resulted in 25 men and 24 women (Table 1). All subjects completed a health history questionnaire containing a brief survey to quantify each participant's physical activity and supplementation status. None of the participants reported any current or ongoing musculoskeletal injury at the time of initiation. Participants were asked to refrain from caffeine, alcohol, analegesics (asprin, acetaminophen, non-steroidal anti-inflammatories, and glucoceroticoids) and cigarettes prior to testing weeks and specifically 48-hours prior to the oxidative stress run. All participants were moderately trained, engaging in 3-7 days per week of aerobic, resistance or recreational activities, but were not highly trained competitive athletes. This study was approved by the University's Institutional Review Board for Human subject and all subjects completed a written informed consent form (Appendix A). Using the procedures described by Howell et al [53] for estimating samples sizes for repeated measures designs, a minimum sample size of n=12 was required for each group to reach a statistical power (1-β) of 0.80 based on the findings of Derave et al. [30], Stout et al. [102], and Hill et al. [47]. A sample size of n=13 per group was recruited to account for subject dropout.

Research Design

A randomized, placebo controlled, mixed factorial design [acute (0 vs. IP vs. 2h vs. 4h) × chronic (pre- vs. post-supplementation) × treatment (beta-alanine vs. placebo) × sex (male vs. female)] was used to examine the effects of beta-alanine loading on markers of oxidative stress. All other performance variables were assed using separate two-way mixed factorial models (time × treatment). Each participant visited the laboratory four times to undergo pre- and post-testing, plus an additional visit to monitor supplement compliance. During week 1 of pre-testing, participants completed an initial run to establish their maximal oxygen consumption (VO_2max) and to determine the peak velocity (PV). Within two to three days, participants returned to the lab for baseline blood draws followed by a non-damaging treadmill run for 40 minutes at 70% PV [75]. Additional blood samples were drawn immediately post (IP), two hours (2h) and four hours (4h) following completion of the run. All samples were centrifuged and stored immediately. Hydration status was measured and controlled for, using handheld specific gravity (VEE GEE Refractometers, Model CLX-1), before the oxidative stress run to decrease the risk of hydration and any potential impact on performance. Each participant was randomly assigned to either a placebo (PL; 800 mg/tablet of maltodextrin; 2 tablets 3 times daily) or beta-alanine (BA; 800 mg/tablet; 2 tablets 3 times daily; CarnoSyn®, Natural Alternatives Inc, San Marcos, CA) supplementing group, following the 40 minute run, for the remainder of the study. Participants were required to visit the lab after two weeks of supplementation to report product intake, any side effects and return completed dietary recalls. Following twenty-eight days of supplementation, participants returned to the lab for post-testing consisting of the same pre-testing assessments (VO_2max, 40 min treadmill run at baseline 70% PV).

Dietary Analysis

To control for the effect of previous dietary antioxidant levels on the outcome measures of the study, and to establish similar levels of macronutrient and antioxidant intake, participants were asked to complete a 3-day dietary recall prior to pre-testing and at the beginning of post-testing, consuming the same macro and micronutrient intake before the oxidative stress testing.

Variables

The independent variables included: (a) treatment [beta-alanine vs. placebo], (b) acute [baseline vs. IP vs. 2h vs. 4h], (c) chronic [week 1 vs. week 7] and (d) sex [male vs. female]. The dependent variables measured include: (a) maximal oxygen consumption (VO_2max), (b) time to exhaustion during VO_2max (VO_2TTE), and (c) oxidative stress markers [total antioxidant capacity (TAC), superoxide dismutase (SOD), isoprostanes (8-isoprostane), and reduced glutathione (GSH).

Instrumentation

- Open circuit spirometery (True One 2400® Metabolic Measurement System, Parvo-Medics Inc., Sandy, UT) was used to determine maximal oxygen consumption (VO_2max).
- A high-speed treadmill (Woodway, Pro Series, Waukesha, WI) was used for determination of VO_2max, as well as to induce oxidative stress.
- Handheld specific gravity (VEE GEE Refractometers, Model CLX-1) was used to measure hydration.
- Wavelengths for the TAC, SOD, GSH, and 8-isoprostanes were measured using a micro plate reader (Model #680, Bio Rad, Hercules, CA).

Pre- and Post-testing Procedures

Determination of VO_2max

All participants performed a graded exercise test (GXT) to volitional exhaustion on a treadmill (Woodway, Pro Series, Waukesha, WI) to determine VO_2max. Based on the protocol of Peake et al [43], the initial GXT velocity was set at 10 $km \cdot h^{-1}$at a 0% grade and increased 2 $km \cdot h^{-1}$ every two minutes up to 16 $km \cdot h^{-1}$, followed by 1 $km \cdot h^{-1}$ increments per minute up to 18 $km \cdot h^{-1}$. The gradient then increase by 2% each minute until VO_2max was achieved. Open-circuit spirometry was used to estimate VO_2max ($l \cdot min^{-1}$) with a metabolic cart (True One 2400® Metabolic Measurement System, Parvo-Medics Inc., Sandy, UT) by sampling and analyzing the breath-by-breath expired gases. The metabolic cart software calculates VO_2 and determines the VO_2max value for each GXT. The highest velocity achieved was recorded as peak velocity (PV).

Exercise Test (Oxidative Stress Run)

During the experimental protocol, each subject ran the treadmill at a velocity corresponding to 70%-75% of their previously determined PV for 40 minutes. This exercise protocol was chosen because it has been shown to induce oxidative stress without eliciting muscle damage [2, 12]. Heart rate and rating of perceived exertion (RPE) were monitored for intensity. If heart rate reached 'near-maximal' the velocity was lowered accordingly. Changes in velocity and total distance were recorded. Post-testing runs were completed at the same velocities as the pre-testing, with no differences over time (p=0.112-0.259) or between groups (0.563) in total distance run.

Blood Collection

Blood samples were collected prior to the exercise test, immediately following and 2h and 4h post-exercise by a person trained in phlebotomy. Plasma and whole blood

samples were obtained via vacutainers, gently inverted ten consecutive times and immediately centrifuged at 3000 rpm for 15 min before storing at -80˚C until completion of analyses.

Enzymatic Antioxidants

Total Antioxidant Capacity (TAC)

The antioxidant capacity (mM) was analyzed using a commercial colorimetric assay (Catalog # 709001, Cayman Chemical, Ann Arbor, MI) using the oxidation of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin in plasma. After a five-fold dilution of the sample, 10 µl of metamyoblobin, 150 µl of chromogen were added, followed by 40 µl of hydrogen peroxide to initiate the reaction. The plate was covered and incubated on an orbital plate shaker for five minutes and the absorbance was read at a wavelength of 405 nm using a Micro Plate Reader (Model #680, Bio Rad, Hercules, CA). Standard curves were generated for all measures using commercially developed standards with reported mean *r* values of 0.81 ± 0.14.

Superoxide Dismutase (SOD)

Using a commercial assay kit (Catalog # 706002,Cayman Chemical, Ann Arbor, MI) which utilizes a tetrazolium salt was used to detect superoxide radical generated by xanthine oxidase and hypoxanthine and measures three types of SOD (Cu/Zn, Mn, and FeSOD). According to the manufacturer's procedures, 200 µl of diluted radical detector and 10 µl of standard was added to designated wells. The diluted radical detector (200 µl) and 10 µl of sample (diluted five-fold) was added to the wells. 20 µl of diluted xanthine oxidase was added to all the wells to initiate the reactions and the plates were then shaken for mixing purposes and covered, on an oribital shaker for 20 minutes. The absorbance of the samples was measured using a micro plate reader (Model #680, Bio

Rad, Hercules, CA) at a wavelength of 450 nm. The precision of this assay previously resulted in an intra-assay coefficient of variation of 3.2% and the dynamic range of the kit is reported at 0.025-0.25 units/ml SOD. Standard curves were generated for all measures using commercially developed standards, reporting a mean r value of 0.85 ± 0.14. Intra-assay coefficients of variation for each assay were determined from each duplicate for all participants and resulted in a mean value of 7.07% ± 8.41%.

Lipid Peroxidation (*8-isoprostane*)

The analysis of lipid peroxidation by way of 8-isoprostane was conducted using a commercial EIA assay kit (Catalog # 516351.1, Cayman Chemical, Ann Arbor, MI) based on the competition between 9-isoprostane and an 8-isoprostane acetylcholinesterase (AChE) conjugate. The competitive binding yields an enzymatic reaction which can be measured spectrophotometrically. In short, 100 µl EIA buffer was added to non-specific binding (NSB) wells and 50 µl EIA buffer was added to Maximum Binding (B_0) wells. 50 µl of the 8-isoprostane EIA standard was added to the standard wells in order from lowest to highest using the same pipette tip. Subsequently, 50 µl of sample was added to each well, in duplicate. An additional 50 µl of 8-isoprostane AChE Tracer was added to all wells, excluding the Total Activity (TA) and Blank (Blk) wells. Also, 50 µl of 8-isoprostane EIA Antiserum was added in the same manner to all wells, except the TA, NSB and Blk wells. Each plate was then covered and incubated for 18 hours at 4˚C. Following incubation the wells were emptied and rinsed five times with Wash Buffer. 200 µl of Ellman's Reagent (Catalog # 400050) was added to each well and 5 µl of tracer was added to the TA wells. The plate was covered, secured on an orbital shaker in a dark room for 90-120 minutes. Absorbance of the plate was measured at a wavelength 405 nm (Micro Plate Reader Model #680, Bio Rad, Hercules, CA).

Intra-assay coefficient of variations were determined at multiple points on the standard curve yielding mean coefficients of 12.48% ± 12.3% and an average r value of 0.984 ± 0.01.

Glutathione (GSH)

GSH were analyzed according to procedures from a commercial assay kit (Catalog #703002, Cayman Chemical, Ann Arbor, MI). Fifty µL of standard was added to the designated wells, followed by an additional 50 µL of sample to each of the wells. 150 µL of a prepared Assay Cocktail consisting of MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml) and reconstituted DTNB (0.45 ml), was added to each of the wells. The plate was covered and placed on an orbital shaker, in the dark. The absorbance values were measured at 405-414 nm (Micro Plate Reader, Model #680; Bio Rad, Hercules, CA) at twenty five minutes (to estimate GSH in the sample. Performance characteristics of this assay have resulted in an inter-assay coefficient of variation of 3.6% (n=5) and 1.6% (n=84). Under the standardized conditions of the assay described for this assay, the dynamic range is 0-16 µM GSH and 0-8 µM GSSG. Standard curves were generated for all measures using commercially developed standards, reporting a mean r value of 0.994 ± 0.002. Intra-assay coefficients of variation for each assay were determined from each duplicate for all participants and resulted in a mean value of 1.6% ± 2.3%.

Statistical Analyses

Separate four-way mixed factorial ANOVAs [4 × 2 × 2 × 2; acute (base vs. IP vs. 2h vs. 4h) × chronic (pre- vs. post-) × treatment (placebo vs. beta-alanine) × sex (male vs. female)] were used to analyze oxidative stress markers. Two separate two-way mixed factorial ANOVAs [2 × 2; time (pre-supplement vs. post-supplement) × treatment

(placebo vs. beta-alanine)] were used to evaluate aerobic performance and time to exhaustion data (VO_2max, VO_2TTE, VT). When appropriate, post-hoc analyses for the ANOVA models were performed using lower-order ANOVAs and Bonferroni-corrected paired samples t-tests. An alpha level was set at $p \leq 0.05$, and all analyses were performed using PASW version 18.0 (SPSS, Inc., Chicago, IL).

In addition, percent change scores were calculated for each participant from pre- to post-supplementation. These percent change scores were average separately for the BA and PL groups and 95% confidence intervals were constructed around the mean percent change scores. When the 95% confidence interval included zero, the mean percent change score was not statistically different than zero. Additional independent samples t-tests were performed on the delta scores between groups. Results were interpreted using magnitude-based statistics, interpreted using Cohen's thresholds (<0.1, trivial; 0.1-0.3, small; 0.3-0.5, moderate; >0.5, large).[50] To make inferences on true effects of BA on performance and oxidative stress variables, the uncertainty in the effect was expressed as 90% confidence limits and the likelihood that the effect represents substantial change (harm/ergolytic or benefit/ergogenic). An effect was reported to be unclear if the confidence interval overlapped the thresholds for positive and negative substantiveness. These analyses were calculated using Microsoft Excel (Version 2007, Microsoft Corporation; The Microsoft Network, LLC, Richmond, VA)

CHAPTER IV

RESULTS

Compliance was reviewed from dosing journals and returned product bottles. All participants met the required supplement dosage (3.2 grams daily) and 8% of the participants demonstrated mild side effects of paresthesia. On average, the men dosed with 0.06 grams·kg·day^{-1} and the women consumed 0.08 grams·kg·day^{-1} of beta-alanine.

Aerobic Performance

Maximal Oxygen Consumption [*VO_2max ($l \cdot min^{-1}$)*]

There was no two-way interaction (time × treatment, p=0.728), and no main effect for treatment (p=0.681) but there was a main effect for time (p=0.020) for men (Figure 1A). The marginal means (collapsed across treatment) increased from pre- to post-testing (0.12 ± 0.04 l·min^{-1}; p=0.020). The mean percent change scores indicated that VO_2max increased following 28-days of beta-alanine supplementation (Figure 1C).

There was no two-way interaction (time × treatment, p=0.813), and no main effect for treatment (p=0.476) but there was a main effect for time (p=0.009) for women (Figure 1A). The marginal means (collapsed across treatment) increased from pre- to post-testing (0.80 ± 0.05 l·min^{-1}; p=0.009). The mean percent change scores indicated a significant increase in VO_2max for the BA group only (Figure 1D).

VO_2TTE (seconds)

There was no two-way interaction (time × treatment, p=0.515), and no main effect for treatment (p=0.617), but there was a main effect for time (p=0.018) for men (Figure 1B). The marginal means (collapsed across treatment) increased from pre- to post testing (19.42 ± 15.20 sec; p=0.018). The mean percent change scores yielded no significant

influence of either treatment on time to exhaustion (Figure 1C). Evaluation of magnitude inferences indicated a possible ergogenic effect of BA on VO_2TTE (Table 2).

There was no two-way interaction (time × treatment, p=0.074), and no main effects for time (p=0.962) or treatment (p=0.832) for women (Figure 1B). The marginal means (collapsed across treatment) indicated no significant change over time (-0.33 ± 1.92 sec; p=0.962). The mean percent change scores yielded no significant influence of either treatment on time to exhaustion (Figure 1D). Evaluation of magnitude inferences indicated a very likely ergogenic effect of BA on VO_2TTE (Table 2).

VT ($l \cdot min^{-1}$)

There was no two-way interaction (time × treatment, p=0.791), and no main effects for time (p=0.088) or treatment (p=0.713) for men (Figure 1A). There was no indication of change for VT in men for either group. The mean percent change scores indicated a significant increase for the BA group only (Figure 1C).

There was no two-way interaction (time × treatment, p=0.344), and no main effects for time (p=0.899) or treatment (p=0.119) for women (Figure 1A). There were no significant changes or differences related to the treatments. Mean percent change scores also demonstrated no change in VT following the treatment period (Figure 1D).

<u>40 min run</u>

Heart Rate (bpm)

There were no three-way (acute × chronic × treatment, p=0.419), two-way (acute × chronic, p=0.416), (acute × treatment, p=0.391), (chronic × treatment, p=0.189) interactions and no main effects for acute (p=0.642), chronic (p=0.189) or treatment (p=0.681) for men (Figure 2A). Marginal means collapsed across chronic and treatment

indicated HR values at 20 and 30 minutes were significantly lower than at 40 minutes (p=0.01). The mean percent change scores indicated that the PL group had a significant increase in heart rate at all four time points (10, 20, 30, and 40 min). Additionally, there was a significant decrease in heart rate at 40 min for the BA group (Figure 2E). The evaluation of magnitude inferences suggest a possible beneficial effect of BA on the HR response to a 40 min run (Table 3).

There was no three-way interaction (acute × chronic × treatment, p=0.330), no two-way interaction (acute × chronic, p=0.519), (acute × treatment, p=0.708) or (chronic × treatment, p=0.098), and no main effect for treatment (p=0.187), but there was a main effect for acute (p=0.001) for women (Figure 2B). The marginal means when collapsing across (chronic and treatment) demonstrated that heart rate values at 10 minutes were significantly lower than at 20 and 40 minutes (p<0.01). At 20 minutes, heart rate values were significantly greater than at 10 minutes and no different from any other time points. The mean percent change scores indicated that the PL group had a significant increase in heart rate at all four time points (10, 20, 30, and 40 min) (Figure 2F).

Rating of Perceived Exertion (RPE)

There were no three-way (acute × chronic × treatment, p=0.072), two-way (acute × chronic, p=0.416), (acute × treatment, p=0.652), or (chronic × treatment, p=0.122) interactions for men. Additionally, there were no main effects for chronic (p=0.694) or treatment (p=0.446) but there was a main effect for acute (p=0.01) (Figure 2C). Marginal means across the acute trial indicated significant lower RPE levels at 10 minutes than at 20, 30, and 40 minutes (p=0.01). RPE increased significantly across all time points (p=0.01). The mean percent change scores revealed a significant decrease in RPE at 40

min for the BA group (Figure 4E). Magnitude inferences suggest a likely ergogenic effect of BA on ratings of perceived exertion at 20-, 30- and 40 minutes of running (Table 3).

There was a three-way interaction (acute × chronic × treatment, p=0.036) for RPE for women. There was also a two-way interaction for acute × treatment (p=0.002) and a main effect for acute (p=0.001). The marginal means for acute (collapsed across chronic) indicated RPE for the BA group at 10 min was significantly less than 20-40 min and for the PL, each time point was significantly lower than the subsequent values. There was a significant difference at 40 minutes between the PL and BA groups (p=0.04). The mean percent change scores revealed a significant decrease in RPE at 30- and 40 minutes for the BA group only (Figure 2F). Magnitude inferences suggest a likely ergogenic effect of BA on ratings of perceived exertion at 30- and 40 minutes of running (Table 4).

Oxidative Stress Biomarkers

Total Antioxidant Capacity

There was no four-way interaction (acute × chronic × sex × treatment; p=0.955), no three-way interaction for acute × chronic × treatment (p=0.701), acute × chronic × sex (p=0.122), or acute × sex × treatment (p=0.056); however, there was a significant three-way interaction for chronic × sex × treatment (p=0.031). No two-way interaction resulted for chronic × sex (p=0.378), chronic × treatment (p=0.396), or sex × treatment (p=0.819), there was a significant main effect for sex (p=0.027) and a main effect for acute (p=0.001). There was no main effect for treatment (p=0.818). The marginal means for chronic (collapsed across sex and treatment) indicated a significant decrease in TAC from pre- to post-supplementing (p=0.015). The acute marginal means (collapsed across sex and treatment) indicated baseline values were significantly greater than IP (p=0.007), two

hour (p=0.041) and four hour values (p=0.001). There were no other significant acute values. The acute marginal means for sex (collapsed across chronic and treatment) indicated men had greater values than women (p=0.027). The mean percent change scores for men (Figure 3A) indicated a significant decrease in base and 4h TAC values for the BA group only. The mean percent change scores for women, revealed a significant decrease in TAC values for the placebo group only (Figure 3C). The evaluation of magnitude inferences suggested no effect of BA on Base and 4H post TAC values (Table 3).

Superoxide Dismutase

There was no four-way interaction (acute × chronic × sex × treatment; p=0.905), no three-way interaction for acute × chronic × sex (p=0.108), acute × chronic × treatment (p=0.683), acute × sex × treatment (p=0.488), chronic × sex × treatment (p=0.249), no two-way interactions for acute × chronic (p=0.374), acute × sex (p=0.263), acute × treatment (p=0.740), chronic × treatment (p=0.091), sex × treatment (p=0.466), but there was an interaction for chronic × sex (p=0.021). Additionally, there was a main effect for acute (p=0.031) and chronic (p=0.001), but no main effect for sex (p=0.862) or treatment (p=0.846). The marginal means for chronic SOD (collapsed across sex and treatment) demonstrated a significant decrease from pre- to post-supplement (p=0.001). The marginal means for acute, sex and treatment SOD values indicated no significant differences (p=0.195-1.0) (Figure 4A). The mean percent change scores for men (Figure 4B) indicated a significant decrease in SOD values for both PL and BA group at baseline and IP time points. The mean percent change scores for women revealed a significant decrease in SOD values for the BA group only at base, 2H post and 4H post (Figure 4C).

The evaluation of magnitude inferences suggested a likely harmful effect of BA on Base, IP and 2H post SOD values (Table 3).

8-Isoprostane

There was no four-way interaction (acute × chronic × sex × treatment; p=0.334), no three-way interaction for acute × chronic × treatment (p=0.142), acute × chronic × sex (p=0.743), chronic × sex × treatment (p=0.738), acute × sex × treatment (p=0.463), no two-way interactions for acute × sex (p=0.459), acute × treatment (p=0.239), chronic × sex (p=0.113), chronic × treatment (p=0.191), acute × chronic (p=0.151) or sex × treatment (p=0.386). There was a main effect for acute (p=0.001) and main effect for chronic (p=0.001), but no main effect for sex (p=0.058) or treatment (p=0.569). The marginal means for acute 8-isoprostane values indicated baseline values were significantly lower than IP (p=0.001) and greater than two hours post (p=0.034). Immediate post values were significantly greater than all time points (p=0.001-0.002). Values at two hours post were significantly lower than all time points (p=0.001-0.034). Values at four hours post were significantly lower than IP (p=0.002) and greater than two hours post (p=0.001). The marginal means for chronic 8-isoprostane levels indicated a significant decrease over time (p=0.001) (Figure 5A). The mean percent change scores for men demonstrated a significant decrease in 8-isoprostane at all time points for the BA group and at base, 2- and 4H time points for the PL group (Figure 5B). For women, the mean percent change scores demonstrated the same results (Figure 5C).

Glutathione (GSH)

There was no four-way interaction (acute × chronic × sex × treatment; p=0.338), no three-way interaction for acute × chronic × treatment (p=0.434), acute × chronic × sex

(p=0.923), acute × sex × treatment (p=0.774), chronic × sex × treatment (p=0.069), no two-way interaction for acute × sex (p=0.317), acute × treatment (p=0.798), chronic × sex (p=0.733), or chronic × treatment (p=0.219), sex × treatment (p=0.618), however, there was a significant two-way interaction for acute × chronic (p=0.003), and a main effect for acute (p=0.001) and chronic (p=0.001). There was no main effect for sex (p=0.245) or treatment (p=0.266). The acute marginal means (collapsed across sex and treatment) indicated baseline values were significantly greater than IP (p=0.001) and four hour values (p=0.005). IP values were significantly lower than all time points (p<0.001); values at two hours post were significantly greater than IP (p=0.001), and four-hour GSH values were significantly lower than baseline (p=0.005) but significantly greater than IP (p=0.001). The chronic marginal means indicated a significant increase from pre- to post-supplementing (p=0.001) (Figure 6A). The mean percent change scores, for the men, revealed a significant increase in GSH values for both groups, at all time points, except for IP values in the BA group, which indicated no change (Figure 6B). For women, the mean percent change values increased for all time points for the BA group, and increased for the PL group at base and 4H post (Figure 8C). The evaluation of magnitude inferences suggested a likely beneficial effect of BA on Base and 4H post GSH values (Table 3).

Nutrition & Exercise Status

Nutrition

There was no two-way interaction (time × treatment) for calories (p=0.469), carbohydrates (p=0.051), fat (p=0.314), protein (p=0.129), or protein quality (histidine;

p=0.130). Furthermore, there were no significant dietary differences between treatment groups before or after supplementing (p=0.155-0.752) for either men or women.

Exercise Status

There was a two-way interaction (time × treatment) for exercise duration (p=0.004) and a main effect for time (p=0.001) and treatment (p=0.003) for hours of exercise. Dependent samples t-tests indicated a significant decrease in time under exercise for both BA (-74.9%) and PL (-42.6%) groups (p=0.001) for men from hours of exercise pre-supplementation compared to an average of during-supplementing training hours.

There was no two-way interaction (time × treatment) for exercise duration (p=0.346) and no main effect for treatment (p=0.528), but there was a main effect for time (p=0.002) for women. The marginal means (collapsed across treatment) indicated a significant decrease in time spent exercising (p=0.001) from pre- to post-supplementing for women, with no difference between treatment groups.

CHAPTER V

DISCUSSION

The results of the present study suggest that twenty-eight days of beta-alanine supplementation has a slight, but significant ($p<0.05$), influence on aerobic performance while reducing perceived and actual rates of exertion during exercise, in both men and women (Figures 1-2) . Furthermore, the forty-minute protocol used in the present study was sufficient to induce oxidative stress. The markers of oxidative stress, 8-isoprostane and GSH, were also improved following beta-alanine supplementation, in both sexes (Figures 3-6). Specifically, lipid peroxidation marker, 8-isoprostane, was reduced for all time points, across sexes. Further, GSH levels were augmented for all time points for both men and women, excluding IP values in men.

Carnosine's role in exercise performance has been attributed to its physiological buffering abilities. As a cytoplasmic dipeptide characterized by its imidazole chemical group, it lends itself as an intracellular buffer, mirroring the intramuscular physiological pH. By virtue of a pKa of 6.83 and its high concentration in muscle, carnosine is more effective at sequestering protons, than either bicarbonate (pKa 6.37) or inorganic phosphate (pKa 7.2), the other two major physico-chemical buffers over the physiological pH range.[1, 8] However, as a result of the greater concentration of carnosine than either bicarbonate or inorganic phosphate in the initial stages of muscle contraction, its buffering contribution may be quantitatively more important. [89] In support, numerous studies have demonstrated the ergogenicity of beta-alanine supplementation in anaerobic [30, 48, 49, 57, 102, 106, 112] performance, with less support in aerobic activities.[30, 56, 98, 102, 112]

Aerobic Performance (VO_2max, TTE, VT)

Evaluation of aerobic performance, with beta-alanine supplementation alone, has consistently demonstrated no positive effects on VO_2peak measured during graded exercise tests.[100, 102, 112] However, this is the first study to evaluate the effects of supplementation alone, on a body-weight supported treadmill run. Maximal oxygen consumption increased over time for men and women in both beta-alanine (+2.64%) and placebo groups (+3.37%), with no significant interaction, supporting previous results and no influence on VO_2max. Evaluation of the confidence intervals demonstrated an increase in VO_2max for only the BA group, in both sexes (Figure 1C-1D). The increase in VO_2max may be attributed to the improvement in muscle buffering capacity. Even during relatively brief, high-intense exercise bouts, a considerable amount of proton and metabolite accumulation can occur. A greater muscle buffering capacity may yield improvements in aerobic capacity by lengthening the capacity of enzymatic reactions, such as, creatine kinase, hexokinase, lactate dehydrogenase and most influential, glyceraldehyde-3-phosphate-dehydrogenase.[52, 107]

Time to exhaustion is a common measure of aerobic capacity that has been evaluated during a GXT. Stout et al. [102] reported a slight, but significant 2.5% increase in TTE during a GXT on a cycle ergometer. The authors attributed these improvements to anaerobiosis and greater reliance on intracellular buffering observed toward the end of a GXT. In contrast, Zoeller et al.[112] showed no positive influence of BA on TTE. The present study is in line with Stout et al, demonstrating a non-significant 3.11% increase for the BA supplementing women compared to a -3.4% decrease in the placebo group. Both male groups demonstrated a significant increase in TTE, at 4.61% (BA) and 2.8% (PL). Although not significant, the magnitude of the increases give

further support for the possible beneficial effects of BA supplementation on TTE, for both men and women.

Increasing skeletal muscle carnosine concentration with BA supplementation may improve the ability to stabilize intramuscular pH during intense exercise by buffering accumulating H^+. Offsetting the indirect effect of proton accumulation on contractile function with the use of BA, has been shown to be effective in delaying neuromuscular fatigue, improving ventilatory threshold (VT) and time to exhaustion in both trained and untrained individuals.[47, 60, 101, 111] Delaying CO_2 by-production, due to a reduced reliance on extracellular buffering can stimulate an improvement in VT and delay fatigue.[39] Ventilatory threshold represents the non-linear point at which ventilation begins to increase disproportionately with VO_2 during a graded exercise test [77] and also corresponds to an increase in anaerobic metabolism.[71] Previous improvements in VT, following 28-days of beta-alanine supplementation, have been reported.[100, 102, 112] Following a similar supplementation protocol as the current study, Stout et al.[102] demonstrated a 13.9% increase in VT in untrained women, while Zoeller et al.[112] reported a 7% increase in untrained men. The current study also demonstrated a significant 4.8% increase for the male BA group, and a non-significant 3.0% increase for the female BA group compared to a -2.2% decrease in VT for the PL group.

40 minute Run

To date, the influence of augmented muscle carnosine levels on longer duration exercise has yet to be evaluated. Beta-alanine has been shown to be ergogenic for short, high-intensity work bouts,[4, 29, 90] while single longer duration events have been suggested to reflect no improvements, due to a negligible reliance on muscle buffer

capacity. However, there is evidence demonstrating a similar association between lactate and H^+ accumulation during graded exercise test.[99] Soller et al. [99] reported a significant increase in $[H^+]$ at 50% VO_2max in untrained subjects (VO_2max: 2.39 ±1.04 $l \cdot min^{-1}$). Therefore, the moderate intensity (70%) of the current exercise protocol would be sufficient to initiate a lactate and H^+ response. Although TTE was pre-determined, HR and RPE were recorded to establish exertion. For men in the current study, the PL supplementing group demonstrated a significant increase in HR for all time points (Figure 2A), while the BA group showed a significant decrease at 40 min post-supplementing (Figure 2E). Similarly, the women in the PL group showed a significant increase in HR across all time points (Figure 2F). In addition, RPE followed a similar pattern, with only the BA group showing a decreased level of exertion at 40 min and 30 and 40 min for the men and women, respectively (Figure 2). In the current study, VT increased for both men and women suggesting an enhanced buffering capacity. Increasing VT may be associated with improved submaximal performance. Indeed, the subjects in the current study demonstrated a significant decrease in level of exertion.

Exercise-Induced Oxidative Stress

Moderate and exhaustive exercise stimulates the increase of ROS production, exceeding the capacity of antioxidant defenses.[2, 55, 66] With an exercised-induced depletion of ATP, catabolism of high ADP intracellular levels trigger the conversion of xanthine dehydrogenase to xanthine oxidase causes an increase in free radicals. [74] ROS concentration may be further elevated due to an increased respiration rate and concomitant increased flow of electrons in the electron transport chain.[69] In addition,

ROS formation has been shown to be dependent on pH, while lactate has also been shown to scavenge the $HO^{\cdot}$ radical and O_2^{-}.[93]

Severin was the first to demonstrate an increase in muscle working capacity when carnosine was added to an *in vitro* exhausted muscle.[96] Additional experimental *in vitro* evidence provided verification of the detrimental effects of free radicals on altered contractile function, decreased maximal force and augmented fatigue rates in animal skeletal muscle. Boldyrev and colleagues [20] have suggested that the antioxidant protective effects of carnosine can relieve some of the fatigue induced effects due to its binding affinity for reactive species, such as hydroxyl radicals and ROS, therefore preventing compounded damage. Ergov et al. [35] provided further evidence for an antioxidant effect of carnosine attributing its ability to quench singlet oxygen molecules to its functional imidazole structure. Kohen et al. suggest that a drop in pH hinders the scavenging abilities of histidine-related compounds,[63] however, the high-buffering capacity of carnosine allows for a unique antioxidant ability by prolonging physiological pH values.[79] More so, human skeletal muscle holds some of the highest levels of carnosine, as a potential physiological adaptation to cope with oxidative stress. The hydrophilic structure of carnosine plays a large role in its protective characteristic within the cytosol. High muscle carnosine levels have been found in other tissues, including nerve, eye and brain tissue. With this knowledge, the suggested primary role of carnosine as a pH buffer, may be inconsistent with other potential functions in subsidiary tissues, such as an antioxidant.

Total Antioxidant Capacity

Multiple studies have reported a significant elevation in TAC following an acute bout of exercise.[12, 37, 58, 72] The resulting increase in TAC following exercise suggests an increase in antioxidant defenses, and typically reflects mobilization of tissue antioxidant stores into plasma.[37] Antioxidant capacity, an overall sum of all antioxidants, may be difficult to interpret because it can increase as a result of numerous internal antioxidants, as well as to adaptations in nutrition and/or the adaptablity to a state of oxidative stress.[62]

The current study yielded conflicting results with previous studies, presenting a significant reduction in TAC levels, from baseline, remaining suppressed for up to 4H. This trend was the same for all groups and genders. While most studies demonstrate an increase in TAC following exercise, the present results could potentially be explained from a decreased synthesis of hepatic urate. Uric acid is a specific tissue antioxidant product that assists with the mobilization of tissue antioxidants into the plasma, synthesis of urate is blunted during periods of acidosis.[25] This could potentially reduce the muscle-plasma antioxidant flux, and if there is a greater suppression of intramuscular oxidative markers, there would likely be a reduction in plasma TAC levels. Chronic values demonstrated a slightly greater influx in TAC levels from pre- to post-supplementation. Although non-significant, the women revealed a greater increase in TAC IP exercise than the PL group, prospectively attributed to a greater muscle buffering capacity and simultaneous urate-TAC plasma efflux.

Superoxide Dismutase

A single bout of treadmill running and other acute exercise has been shown to increase SOD as a result of increased O_2^-,[83] potentially offering greater antioxidant

protection.[55] It was hypothesized that SOD values would increase following exercise and return to baseline within the 4H recovery time. However, while acute values increased IP running, SOD levels did not return to normal during the recovery time, and the PL group demonstrated a -5% decrease from baseline. Pepe et al. reported a similar non-significant decrease in SOD following various running bouts (800m-3,000m), with similar responses for men and women.[80]

Carnosine has been shown to possess superoxide dismutase activity, *in vitro*.[43] It is important to identify that although plasma markers are a reliable indication of oxidative stress, less stable molecules may be completely oxidized within other tissues, such as skeletal muscle and other organs and tissues.[81] It has been shown that many reactive species are membrane permeable and have a longer half-life, in particular the superoxide anion (O_2^-) rapidly dismutates to H_2O_2 with a longer half life within the blood and plasma.[75] Consequently, a large decrease in SOD has been attributed to the presence of H_2O_2, which inhibits SOD activity.[14] That being said, the significant decrease in chronic values for both groups, with the BA group showing a greater blunted SOD response (Figure 6), may be attributed to the reduced stability and permeability of SOD transmission from skeletal muscle to plasma. Additionally, exercise severely increases free radicals, specifically H_2O_2, production in vascular tissues, that have a longer half-life and more permeable to macromolecules within the blood.[75] This attribute may have caused a greater decrease in SOD values over time. More so, it has been suggested that following acute exercise, increased production of ROS could potentially denature the enzyme.[105]

Isoprostanes

Isoprostanes materialize from the oxidation of arachadonic acid, and their appearance from strenuous cycling and running is well documented.[36, 108] Although the duration and intensity of previous running protocols differ from the current study, all studies demonstrated a significant increase (22-80%) in lipid peroxidation, in the form of isoprostanes.[108] The effects of exercise on blood lipid peroxidation may be heavily reliant upon oxygen partial pressure, and therefore increased under more intense hypoxic activities.[75] The present study demonstrated an average 15% increase in IP 8-isoprostane levels following 40 minutes of running, validating the exercise protocol in the current protocol, initiated some degree of oxidative stress. Results from the present study indicate that lipid peroxidation, as measured by 8-isoprostane, was elevated immediately returning to near baseline values at 2H post. This increase likely represents an increased oxidation of unsaturated fatty acid, which is supported following aerobic exercise.[108, 110]

In vitro, Boldyrev gave substantial support for the role of carnosine in lipid peroxidation inhibition, protecting biological membranes.[17] Furthermore, Kohen et al. [63] previously demonstrated a decrease in the oxidation of linoleic acid from carnosine, and Salim-Hanna supported this by revealing 53% protection against lipid oxidation at a concentration of 10 mM carnosine. [91] The change in 8-isoprostane levels from pre- to post-supplementation suggest a possible decrease in resting lipid peroxidation, as well as minimizing oxidative damage IP strenuous exercise. However, additional consideration must be given to the decrease in 8-isoprostane levels in the PL group (Figure 7), although the response was not as great as the BA group. This marker has been shown to be fairly stable, under freezing conditions and all samples were stored under the same conditions.

Furthermore, the prevention of unsaturated fatty acids, within the mitochondria, may protect membrane permeability and ion pumps.[82] Also, maintaining calcium-ATPase within the sarcoplasmic reticulum may maintain the integrity of the excitation-contraction coupling process in vivo,[17, 34, 95] creating a possible link between carnosine's ability to reduce lipid oxidation and potential improvements in performance.

Glutathione

The most frequently used antioxidant marker of exercise-induced oxidative stress is the glutathione redox status, which demonstrates a decreased ratio following exercise.[38, 55, 108] In the current study, due to the purification of the samples and the assay technique, only reduced glutathione (GSH) was measured. The exercise protocol in the current study initiated a decrease in GSH values, which is consistent with other exercise protocols. In fact, Gopal et al. [42] instituted a duplicate protocol to the current study, running for 30 minutes at 75%-80% VO_2max, reporting a significant decrease in GSH. Glutathione levels IP exercise significantly decreased ($p<0.05$) and approached near baseline levels at 4H, however, remained statistically different ($p=0.01$). This study provides more data on the timing of GSH analysis, showing that GSH levels remain depressed at 4H and 5H, [72] but return to baseline at 6H.[13] The decrease in GSH following intense exercise reflects an increased consumption by muscle as well as a lag in hepatic GSH supply pulling thiols from the blood supply.[72]

High levels of carnosine have been found in the lens of the human eye, in addition to high glutathione levels. These elevated optic levels have been attributed to protective characteristics with anti-inflammatory, wound healing, and cataract diminishing effects.[17] The differences between optic and skeletal muscle physiology are evident,

although the relationship between carnosine's antioxidant effects in both are uncertain. Carnosine has been shown to reduce peroxidation processes and GSH within the lens, decreasing cataracts.[19, 61] The only *in vitro* data with carnosine is related to cataract processes. The present *in vivo* results demonstrate a significant elevation in chronic GSH values ($p<0.05$). Although there were no significant gender effects, the women BA group had a more prominent response, significantly increasing GSH values at all time points, post-supplementation (Figure 6A). This increase in GSH following supplementation may signify free radical scavenging abilities within the muscle.

Limitations

Plasma levels of antioxidant markers and enzymes, which was the site of markers in the current study, may represent a redistribution between tissue and plasma.[55] While the majority of literature interprets blood and muscle antioxidant markers in tandem, and it is reasonable to assume there is bidirectional movement across membranes, the half-life and permeability of specific enzymes and markers may need further interpretation. All previous antioxidant research with carnosine has been done *in vitro* or *in vivo* mouse models, lacking carnosinase. Therefore a strict interpretation of available literature must be viewed with caution. However, markers with greater stability and trans-tissue permeability may provide insight. Stable lipid oxidative markers, and possibly GSH may provide the most valuable perspective of the antioxidant capabilities of carnosine in humans. Future research should re-evaluate these markers in a more controlled population. The alterations in time under training could have potentially influenced some of the more sensitive markers (SOD, TAC), due to the various reported effects of training status on oxidative stress.[2, 11] A further limitation is a result of the subject sample.

Several studies have used a similar cohort of moderately trained men and women; however, the PL group in the current study resulted in some similar, if not better, adaptations than the BA group. The presence of this potential confounder is beyond the control of the investigators. It is important to discuss the change in exercise status from the beginning to the end of the supplementation period. Both men and women demonstrated a reduction in time under training from pre- to post-supplementation. Specifically, there was a significant decrease for men (-75%, -43%) and women (-70%, -57%) for the BA and PL groups, respectively. Despite the reduction in training time, there could be a potential influence of BA supplementation to help maintain aerobic capacity under periods of reduced training, demonstrated by an increase in VO_2max in the current study. Maximum oxygen uptake and TTE during a GXT has not been shown to reflect a detraining effect in several studies,[26, 51, 68] which is supported by the current study. However, HR may be a better indication of detraining.[26] While both BA groups yielded greater reductions in pre-post training durations [-70%-75% (BA) vs. -43%-57% (PL)], only the PL groups revealed a significant increase in HR. This may demonstrate a slight influence of BA supplementation minimizing the effects of detraining.

An additional limitation was the lack of direct carnosine measurements. Although muscle carnosine concentration was not measured directly, several recent studies have shown significantly elevated carnosine levels (+60%) after 28 days of BA supplementation.[46, 47] Furthermore, the dosing strategy was similar to those used in the studies of Harris et al.[46] and Hill et al.[47] at 3 g-6 g per day (126 g given the first 21 days and 63 g given over the second set of 21 days), suggesting that muscle carnosine

levels were increased. Dosing recommendations for beta-alanine are somewhat broad and generalized, recommended in absolute amounts from 6 grams per day, in divided doses for a loading phase, to 3 grams per day for maintenance, for all individuals, independent of age, height, weight and sex. The present study utilized the time release formula at 4.8 grams daily in divided doses (CarnoSyn®, Natural Alternatives Inc, San Marcos, CA) which has been demonstrated to significantly augment muscle carnosine levels by 27-39% in fast- and slow-twitch muscle fibers, respectively [6] under a similar dosing scenario. When evaluating dosage on a per kg basis, men consumed an average intake of 0.06 grams·kg·day^{-1}, while women consumed an average of 0.08 grams·kg·day^{-1}. Additionally, there were no significant correlations between body weight dosing and all variables (p=0.179-0.954). While the current study and previous reviews support the ergogenicity of a generalized supplementation regime,[4, 10, 29, 46, 90] future recommendations should evaluate the physiological effects of body weight specific consumption of beta-alanine on both carnosine augmentation, performance and oxidative stress.

Conclusions/Practical Applications

In a large number of studies, antioxidant supplementation has not been shown to be directly ergogenic on performance variables.[23, 64] Beta-alanine on the other hand has substantial support for its role in anaerobic performance, training volume, and body composition. The current study adds to the ergogenic effect, demonstrating increases in VT, as well as a potential increase in VO_2max. This is the first study to report quantitative evidence for a feeling of reduced effort during exercise, giving a foundation for use in both aerobic and anaerobic activities (Figure 2). Future research should further

evaluate feelings of reduced efforts during various exercise environments, as well as the influence of BA on maintaining training adaptations during a period of detraining.

A biological antioxidant is characterized by its abilities to prevent oxidative damage to lipids, proteins, DNA and other macromolecules.[62] There is some specificity, in that most antioxidants provide only one type of protection. The present results are consistent with the physiological role for carnosine, demonstrated *in vitro*.[82] Our data suggest that carnosine, *in vivo*, may have an influence on attenuating lipid peroxidation and thus protecting biological membranes. Although there is still much to be elucidated with the chronic attenuation of lipid peroxidation, this study gives insight into potential clinical and therapeutic uses. Our data, along with previous human and animal studies, suggest three possible mechanisms that carnosine may attenuate exercise induced oxidative stress: 1) regulating the accumulation of H^+, thereby controlling pH (indirectly reducing oxidative stress); 2) serving as a scavenger of free radicals, and indirectly maintaining stability of the cell membrane and enzyme integrity; and 3) a potential influence on augmenting calcium kinetics by maintaining contraction-induced muscular fatigue, although this has not yet been evaluated *in vivo*.

Additionally, as a secondary variable the question of gender based oxidative responses to exercise are not completely evident. As hypothesized, there were no significant gender differences for SOD, isoprostanes or GSH. In contrast, there was a significant difference between men and women for TAC values. While this study is original in its approach to evaluate the antioxidant effects of BA supplementation in men and women, the results provide a foundation for future research examining other markers

of oxidative stress localized within the skeletal muscle, as well as chronic effects on performance and recovery.

REFERENCES

1. Abe H. Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. Biochemistry 2000; 65:757-765

2. Alessio HM. Exercise-induced oxidative stress. Medicine and science in sports and exercise 1993; 25:218-224

3. Alessio HM, Hagerman AE, Fulkerson BK, Ambrose J, Rice RE, Wiley RL. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. Medicine and science in sports and exercise 2000; 32:1576-1581

4. Artioli GG, Gualano B, Smith A, Stout J, Lancha AH, Jr. Role of beta-alanine supplementation on muscle carnosine and exercise performance. Medicine and science in sports and exercise 2010; 42:1162-1173

5. Babizhayev MA, Seguin MC, Gueyne J, Evstigneeva RP, Ageyeva EA, Zheltukhina GA. L-carnosine (beta-alanyl-L-histidine) and carcinine (beta-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. The Biochemical journal 1994; 304 (Pt 2):509-516

6. Baguet A, Reyngoudt H, Pottier A, Everaert I, Callens S, Achten E, Derave W. Carnosine loading and washout in human skeletal muscles. J Appl Physiol 2009; 106:837-842

7. Bakardjiev A, Bauer K. Transport of beta-alanine and biosynthesis of carnosine by skeletal muscle cells in primary culture. European journal of biochemistry / FEBS 1994; 225:617-623

8. Bate-Smith EC. The buffering of muscle in rigor: Protein, phosphate and carnosine. The Journal of physiology 1938; 92:336-343

9. Bauer K, Schulz M. Biosynthesis of carnosine and related peptides by skeletal muscle cells in primary culture. European journal of biochemistry / FEBS 1994; 219:43-47

10. Begum G, Cunliffe A, Leveritt M. Physiological role of carnosine in contracting muscle. International journal of sport nutrition and exercise metabolism 2005; 15:493-514

11. Bloomer RJ, Falvo MJ, Fry AC, Schilling BK, Smith WA, Moore CA. Oxidative stress response in trained men following repeated squats or sprints. Medicine and science in sports and exercise 2006; 38:1436-1442

12. Bloomer RJ, Goldfarb AH, McKenzie MJ. Oxidative stress response to aerobic exercise: comparison of antioxidant supplements. Medicine and science in sports and exercise 2006; 38:1098-1105

13. Bloomer RJ, Goldfarb AH, Wideman L, McKenzie MJ, Consitt LA. Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. J Strength Cond Res 2005; 19:276-285

14. Blum J, Fridovich I. Inactivation of glutathione peroxidase by superoxide radical. Arch Biochem Biophys 1985; 240:500-508

15. Boldyrev A, Leotsakos A, Quinn P. The effect of membrane stabilizing agents on Ca-pump of the sarcoplasmic reticulum. Ukrainskii biokhimicheskii zhurnal 1992; 64:54-58

16. Boldyrev A, Song R, Lawrence D, Carpenter DO. Carnosine protects against excitotoxic cell death independently of effects on reactive oxygen species. Neuroscience 1999; 94:571-577

17. Boldyrev AA, Dupin AM, Bunin A, Babizhaev MA, Severin SE. The antioxidative properties of carnosine, a natural histidine containing dipeptide. Biochemistry international 1987; 15:1105-1113

18. Boldyrev AA, Dupin AM, Pindel EV, Severin SE. Antioxidative properties of histidine-containing dipeptides from skeletal muscles of vertebrates. Comp Biochem Physiol B 1988; 89:245-250

19. Boldyrev AA, Severin SE. The histidine-containing dipeptides, carnosine and anserine: distribution, properties and biological significance. Advances in enzyme regulation 1990; 30:175-194

20. Boldyrev AA, Stvolinsky SL, Tyulina OV, Koshelev VB, Hori N, Carpenter DO. Biochemical and physiological evidence that carnosine is an endogenous neuroprotector against free radicals. Cellular and molecular neurobiology 1997; 17:259-271

21. Brutto M, Nosek T. Hydrogen peroxide disrupts calcium release from the sarcoplasmic reticulum of rat skeletal muscle fibers. J Appl Physiol 1996; 81:731-737

22. Chan WK, Decker EA, Chow CK, Boissonneault GA. Effect of dietary carnosine on plasma and tissue antioxidant concentrations and on lipid oxidation in rat skeletal muscle. Lipids 1994; 29:461-466

23. Clarkson PM, Thompson HS. Antioxidants: what role do they play in physical activity and health? The American journal of clinical nutrition 2000; 72:637S-646S

24. Cooper CE, Vollaard NB, Choueiri T, Wilson MT. Exercise, free radicals and oxidative stress. Biochemical Society transactions 2002; 30:280-285

25. Craan AG, Lemieux G, Vinay P, Gougoux A. The kidney of chicken adapts to chronic metabolic acidosis: in vivo and in vitro studies. Kidney Int 1982; 22:103-111

26. Cullinane EM, Sady SP, Vadeboncoeur L, Burke M, Thompson PD. Cardiac size and VO2max do not decrease after short-term exercise cessation. Medicine and science in sports and exercise 1986; 18:420-424

27. Decker EA, Crum AD, Calvert JT. Differences in the antioxidant mechanism of carnosine in the presence of copper and iron. Journal of agricultural and food chemistry 1992; 40:756-759

28. Decker EA, Ivanov V, Zhu BZ, Frei B. Inhibition of low-density lipoprotein oxidation by carnosine histidine. Journal of agricultural and food chemistry 2001; 49:511-516

29. Derave W, Everaert I, Beeckman S, Baguet A. Muscle carnosine metabolism and beta-alanine supplementation in relation to exercise and training. Sports Med 2010; 40:247-263

30. Derave W, Ozdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, Wise JA, Achten E. beta-Alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. J Appl Physiol 2007; 103:1736-1743

31. Di Meo S, Venditti P. Mitochondria in exercise-induced oxidative stress. Biological signals and receptors 2001; 10:125-140

32. Dunnett M, Harris RC. Influence of oral beta-alanine and L-histidine supplementation on the carnosine content of the gluteus medius. Equine veterinary journal 1999; 30:499-504

33. Dunnett M, Harris RC, Dunnett CE, Harris PA. Plasma carnosine concentration: diurnal variation and effects of age, exercise and muscle damage. Equine veterinary journal 2002:283-287

34. Dupin AM, Stvolinskii SL. [Changes in carnosine levels in muscles working in different regimens of stimulation]. Biokhimiia (Moscow, Russia) 1986; 51:160-164

35. Egorov S, Kurella EG, Boldyrev AA, Krasnovsky AA, Jr. Quenching of singlet molecular oxygen by carnosine and related antioxidants. Monitoring 1270-nm phosphorescence in aqueous media. Biochemistry and molecular biology international 1997; 41:687-694

36. Fam SS, Morrow JD. The isoprostanes: unique products of arachidonic acid oxidation-a review. Curr Med Chem 2003; 10:1723-1740

37. Fatouros IG, Chatzinikolaou A, Douroudos, II, Nikolaidis MG, Kyparos A, Margonis K, Michailidis Y, Vantarakis A, Taxildaris K, Katrabasas I, Mandalidis D, Kouretas D, Jamurtas AZ. Time-course of changes in oxidative stress and antioxidant status responses following a soccer game. J Strength Cond Res 2010; 24:3278-3286

38. Finaud J, Lac G, Filaire E. Oxidative stress : relationship with exercise and training. Sports medicine (Auckland, NZ 2006; 36:327-358

39. Fitts RH, Holloszy JO. Lactate and contractile force in frog muscle during development of fatigue and recovery. The American journal of physiology 1976; 231:430-433

40. Gariballa SE, Sinclair AJ. Carnosine: physiological properties and therapeutic potential. Age and ageing 2000; 29:207-210

41. Goldfarb AH, McKenzie MJ, Bloomer RJ. Gender comparisons of exercise-induced oxidative stress: influence of antioxidant supplementation. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 2007; 32:1124-1131

42. Gopal I. The effect of gender on exercise-induced oxidative stress [disseration]. In. Greensboro, NC: University of North Carolina; 1997

43. Gulyaeva NV, Obidin AB, Marinov BS. Modulation of superoxide dismutase by electron donors and acceptors. FEBS letters 1987; 211:211-214

44. Halliwell B. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free radical research 1999; 31:261-272

45. Harris RC, Jones G, Hill CH, Kendrick IP, Boobis L, Kim CK, Kim HJ, Dang VH, Edge J, Wise JA. The carnosine content of vastus lateralis in vegetarians and omnivores. FASEB J 2007; 21:76.20

46. Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill CA, Sale C, Wise JA. The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis. Amino acids 2006; 30:279-289

47. Hill CA, Harris RC, Kim HJ, Harris BD, Sale C, Boobis LH, Kim CK, Wise JA. Influence of beta-alanine supplementation on skeletal muscle carnosine concentrations and high intensity cycling capacity. Amino acids 2007; 32:225-233

48. Hoffman J, Ratamess N, Faigenbaum A, Ross R, Kang J, Stout J, Wise JA. Short-duration beta-alanine supplementation increases training volume and reduces subjective feelings of fatigue in college football players. Nutrition Research 2007; 28:31-35

49. Hoffman J, Ratamess NA, Ross R, Kang J, Magrelli J, Neese K, Faigenbaum AD, Wise JA. beta-Alanine and the Hormonal Response to Exercise. International journal of sports medicine 2008;

50. Hopkins WG, Marshall SW, Batterham AM, Hanin J. Progressive statistics for studies in sports medicine and exercise science. Medicine and science in sports and exercise 2009; 41:3-13

51. Houmard JA, Hortobagyi T, Johns RA, Bruno NJ, Nute CC, Shinebarger MH, Welborn JW. Effect of short-term training cessation on performance measures in distance runners. International journal of sports medicine 1992; 13:572-576

52. Houston ME. Biochemistry primer for exercise science. 3rd ed. Champaign, IL: Human Kinetics; 2006:xi, 267 p.

53. Howell D. Statistical Methods for Psychology. Belmont, CA: Wadsworth; 2007

54. Jenkins RR. Free radical chemistry. Relationship to exercise. Sports medicine (Auckland, NZ 1988; 5:156-170

55. Ji LL. Antioxidants and oxidative stress in exercise. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY 1999; 222:283-292

56. Kendrick IP, Harris RC, Kim HJ, Kim CK, Dang VH, Lam TQ, Bui TT, Smith M, Wise JA. The effects of 10 weeks of resistance training combined with beta-alanine supplementation on whole body strength, force production, muscular endurance and body composition. Amino acids 2008; 34:547-554

57. Kendrick IP, Kim HJ, Harris RC, Kim CK, Dang VH, Lam TQ, Bui TT, Wise JA. The effect of 4 weeks beta-alanine supplementation and isokinetic training on carnosine concentrations in type I and II human skeletal muscle fibres. European journal of applied physiology 2009;

58. Kerksick C, Taylor Lt, Harvey A, Willoughby D. Gender-related differences in muscle injury, oxidative stress, and apoptosis. Medicine and science in sports and exercise 2008; 40:1772-1780

59. Khassaf M, Child RB, McArdle A, Brodie DA, Esanu C, Jackson MJ. Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. J Appl Physiol 2001; 90:1031-1035

60. Kim HJ, Kim CK, Lee YW, Harris RC, Sale C, Harris BD, Wise JA. The effect of a supplement containing B-alanine on muscle carnosine synthesis and exercise capacity, during 12 week combined endurance and weight training. J Int Soc Sports Nutr 2006; 3:S9

61. Klebanov GI, Sorokovoi VI, Vladimirov YA. Investigation of the conformation of biological membranes by the protein luminescence method. Mol Biol 1972; 6:143-148

62. Kohen R, Vellaichamy E, Hrbac J, Gati I, Tirosh O. Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. Free Radic Biol Med 2000; 28:871-879

63. Kohen R, Yamamoto Y, Cundy KC, Ames BN. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proceedings of the

National Academy of Sciences of the United States of America 1988; 85:3175-3179

64. Laursen JB, Boesgaard S, Trautner S, Rubin I, Poulsen HE, Aldershvile J. Endothelium-dependent vasorelaxation in inhibited by in vivo depletion of vascular thiol levels: role of endothelial nitric oxide synthase. Free radical research 2001; 35:387-394

65. Leeuwenburgh C, Hansen P, Shaish A, Holloszy JO, Heinecke JW. Markers of protein oxidation by hydroxyl radical and reactive nitrogen species in tissues of aging rats. The American journal of physiology 1998; 274:R453-461

66. Lovlin R, Cottle W, Pyke I, Kavanagh M, Belcastro AN. Are indices of free radical damage related to exercise intensity. European journal of applied physiology and occupational physiology 1987; 56:313-316

67. MacIntyre DL, Reid WD, McKenzie DC. Delayed muscle soreness. The inflammatory response to muscle injury and its clinical implications. Sports medicine (Auckland, NZ 1995; 20:24-40

68. Madsen K, Pedersen PK, Djurhuus MS, Klitgaard NA. Effects of detraining on endurance capacity and metabolic changes during prolonged exhaustive exercise. Journal of applied physiology 1993; 75:1444-1451

69. Mastaloudis A, Leonard SW, Traber MG. Oxidative stress in athletes during extreme endurance exercise. Free radical biology & medicine 2001; 31:911-922

70. Matthews MM, Traut TW. Regulation of N-carbamoyl-beta-alanine amidohydrolase, the terminal enzyme in pyrimidine catabolism, by ligand-induced change in polymerization. The Journal of biological chemistry 1987; 262:7232-7237

71. McArdle WD, Katch FI, Katch VL. Exercise physiology: energy, nutrition, and human performance. . 6th Ed ed. Baltimore: Lippincott, Williams & Wilkins; 2006

72. Michailidis Y, Jamurtas AZ, Nikolaidis MG, Fatouros IG, Koutedakis Y, Papassotiriou I, Kouretas D. Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. Medicine and science in sports and exercise 2007; 39:1107-1113

73. Misra HP, Fridovich I. The generation of superoxide radical during the autoxidation of hemoglobin. The Journal of biological chemistry 1972; 247:6960-6962

74. Moller P, Wallin H, Knudsen LE. Oxidative stress associated with exercise, psychological stress and life-style factors. Chem Biol Interact 1996; 102:17-36

75. Nikolaidis MG, Jamurtas AZ, Paschalis V, Fatouros IG, Koutedakis Y, Kouretas D. The effect of muscle-damaging exercise on blood and skeletal muscle

oxidative stress: magnitude and time-course considerations. Sports medicine (Auckland, NZ 2008; 38:579-606

76. Nohl H, Kozlov AV, Gille L, Staniek K. Cell respiration and formation of reactive oxygen species: facts and artefacts. Biochemical Society transactions 2003; 31:1308-1311

77. Orr GW, Green HJ, Hughson RL, Bennett GW. A computer linear regression model to determine ventilatory anaerobic threshold. J Appl Physiol 1982; 52:1349-1352

78. Packer L. Oxidants, antioxidant nutrients and the athlete. Journal of sports sciences 1997; 15:353-363

79. Parkhouse WS, McKenzie DC, Hochachka PW, Ovalle WK. Buffering capacity of deproteinized human vastus lateralis muscle. J Appl Physiol 1985; 58:14-17

80. Pepe H, Balci SS, Revan S, Akalin PP, Kurtoglu F. Comparison of oxidative stress and antioxidant capacity before and after running exercises in both sexes. Gend Med 2009; 6:587-595

81. Powers SK, Lennon SL. Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. The Proceedings of the Nutrition Society 1999; 58:1025-1033

82. Quinn PJ, Boldyrev AA, Formazuyk VE. Carnosine: its properties, functions and potential therapeutic applications. Molecular aspects of medicine 1992; 13:379-444

83. Radak Z, Asano K, Inoue M, Kizaki T, Oh-Ishi S, Suzuki K, Taniguchi N, Ohno H. Superoxide dismutase derivative reduces oxidative damage in skeletal muscle of rats during exhaustive exercise. J Appl Physiol 1995; 79:129-135

84. Radak Z, Kaneko T, Tahara S, Nakamoto H, Ohno H, Sasvari M, Nyakas C, Goto S. The effect of exercise training on oxidative damage of lipids, proteins, and DNA in rat skeletal muscle: evidence for beneficial outcomes. Free radical biology & medicine 1999; 27:69-74

85. Reid MB. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. J Appl Physiol 2001; 90:724-731

86. Reid MB. Nitric oxide, reactive oxygen species, and skeletal muscle contraction. Medicine and science in sports and exercise 2001; 33:371-376

87. Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, West MS. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. J Appl Physiol 1992; 73:1797-1804

88. Renke J, Popadiuk S, Korzon M, Bugajczyk B, Wozniak M. Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in

plasma of children with juvenile chronic arthritis. Free radical biology & medicine 2000; 29:101-104

89. Robergs RA, Ghiasvand F, Parker D. Biochemistry of exercise-induced metabolic acidosis. American journal of physiology 2004; 287:R502-516

90. Sale C, Saunders B, Harris RC. Effect of beta-alanine supplementation on muscle carnosine concentrations and exercise performance. Amino acids 2010; 39:321-333

91. Salim-Hanna M, Lissi E, Videla LA. Free radical scavenging activity of carnosine. Free radical research communications 1991; 14:263-270

92. Schneider CD, Barp J, Ribeiro JL, Bello-Klein A, Oliveira AR. Oxidative stress after three different intensities of running. Can J Appl Physiol 2005; 30:723-734

93. Selivanov VA, Zeak JA, Roca J, Cascante M, Trucco M, Votyakova TV. The role of external and matrix pH in mitochondrial reactive oxygen species generation. The Journal of biological chemistry 2008; 283:29292-29300

94. Sen CK. Oxidants and antioxidants in exercise. J Appl Physiol 1995; 79:675-686

95. Severin SE, Boldyrev AA. Effects of carnosine, a specific component of striated muscle, on muscle and other tissues. Biomedical science 1991; 2:91-94

96. Severin SE, Glemzha AA. [Effect of Imidazole Derivatives on Pyruvate Dehydrogenase in Muscle Tissue.]. Biokhimiia (Moscow, Russia) 1964; 29:1170-1176

97. Sjodin B, Hellsten Westing Y, Apple FS. Biochemical mechanisms for oxygen free radical formation during exercise. Sports medicine (Auckland, NZ 1990; 10:236-254

98. Smith AE, Walter AA, Graef JL, Kendall KL, Moon JR, Lockwood CM, Fukuda DH, Beck TW, Cramer JT, Stout JR. Effects of beta-alanine supplementation and high-intensity interval training on endurance performance and body composition in men; a double-blind trial. J Int Soc Sports Nutr 2009; 6:5

99. Soller BR, Yang Y, Lee SM, Wilson C, Hagan RD. Noninvasive determination of exercise-induced hydrodgen ion threshold through direct optical measurement. J Appl Physiol 2008; 104:837-844

100. Stout JR, Cramer JT, Mielke M, O'Kroy J, Torok DJ, Zoeller RF. Effects of twenty-eight days of beta-alanine and creatine monohydrate supplementation on the physical working capacity at neuromuscular fatigue threshold. Journal of strength and conditioning research / National Strength & Conditioning Association 2006; 20:928-931

101. Stout JR, Cramer JT, Zoeller RF, Torok D, Costa P, Hoffman JR, Harris RC. Effects of beta-alanine supplementation on the onset of neuromuscular fatigue and ventilatory threshold in women. Amino acids 2006;

102. Stout JR, Cramer JT, Zoeller RF, Torok D, Costa P, Hoffman JR, Harris RC, O'Kroy J. Effects of beta-alanine supplementation on the onset of neuromuscular fatigue and ventilatory threshold in women. Amino acids 2007; 32:381-386

103. Suzuki Y, Ito O, Mukai N, Takahashi H, Takamatsu K. High level of skeletal muscle carnosine contributes to the latter half of exercise performance during 30-s maximal cycle ergometer sprinting. The Japanese journal of physiology 2002; 52:199-205

104. Suzuki Y, Nakao T, Maemura H, Sato M, Kamahara K, Morimatsu F, Takamatsu K. Carnosine and anserine ingestion enhances contribution of nonbicarbonate buffering. Medicine and science in sports and exercise 2006; 38:334-338

105. Tauler P, Aguilo A, Gimeno I, Fuentespina E, Tur JA, Pons A. Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. Eur J Nutr 2006; 45:187-195

106. Van Thienen R, Van Proeyen K, Vanden Eynde B, Puype J, Lefere T, Hespel P. Beta-alanine improves sprint performance in endurance cycling. Medicine and science in sports and exercise 2009; 41:898-903

107. Vinnakota KC, Kushmerick MJ, Robergs RA. Point: Counterpoint "Muscle lactate and H+ production do / do not have a 1:1 association in skeletal muscle". J Appl Physiol 2011;

108. Vollaard NB, Shearman JP, Cooper CE. Exercise-induced oxidative stress:myths, realities and physiological relevance. Sports medicine (Auckland, NZ 2005; 35:1045-1062

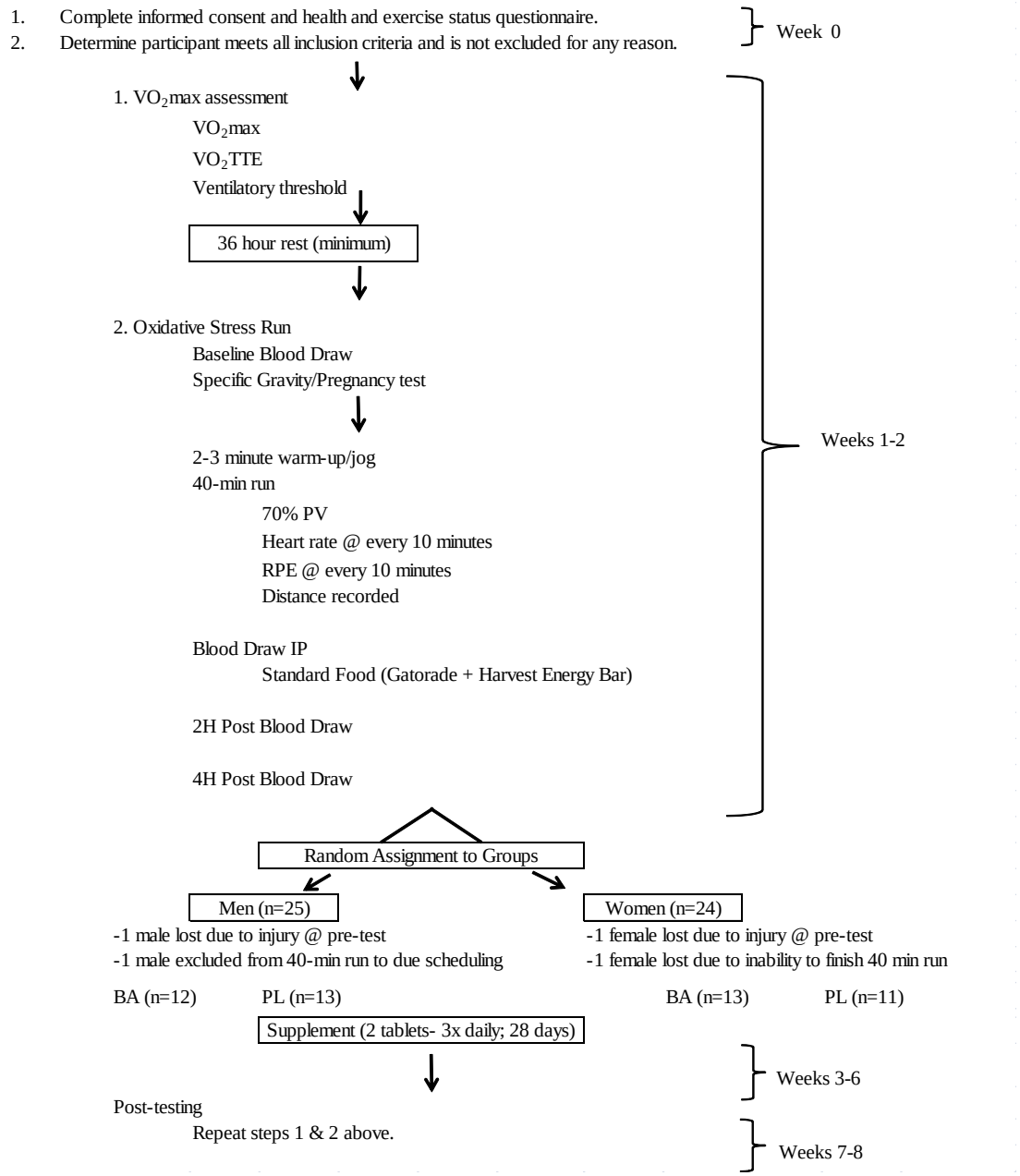
109. Wallace SS. Biological consequences of free radical-damaged DNA bases. Free radical biology & medicine 2002; 33:1-14

110. Watson TA, Callister R, Taylor RD, Sibbritt DW, MacDonald-Wicks LK, Garg ML. Antioxidant restriction and oxidative stress in short-duration exhaustive exercise. Medicine and science in sports and exercise 2005; 37:63-71

111. Zoeller RF, Stout JR, O'Kroy J A, Torok DJ, Mielke M. Effects of 28 days of beta-alanine and creatine monohydrate supplementation on aerobic power, ventilatory and lactate thresholds, and time to exhaustion. Amino acids 2006;

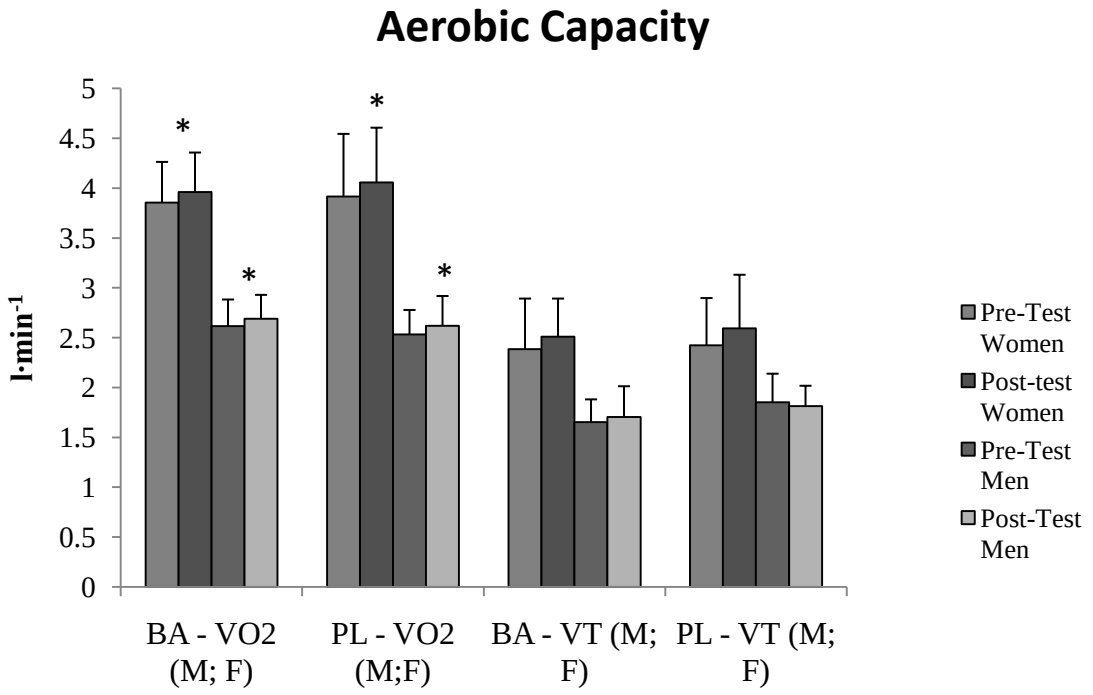
112. Zoeller RF, Stout JR, O'Kroy J A, Torok DJ, Mielke M. Effects of 28 days of beta-alanine and creatine monohydrate supplementation on aerobic power, ventilatory and lactate thresholds, and time to exhaustion. Amino acids 2007; 33:505-510

Appendix A.

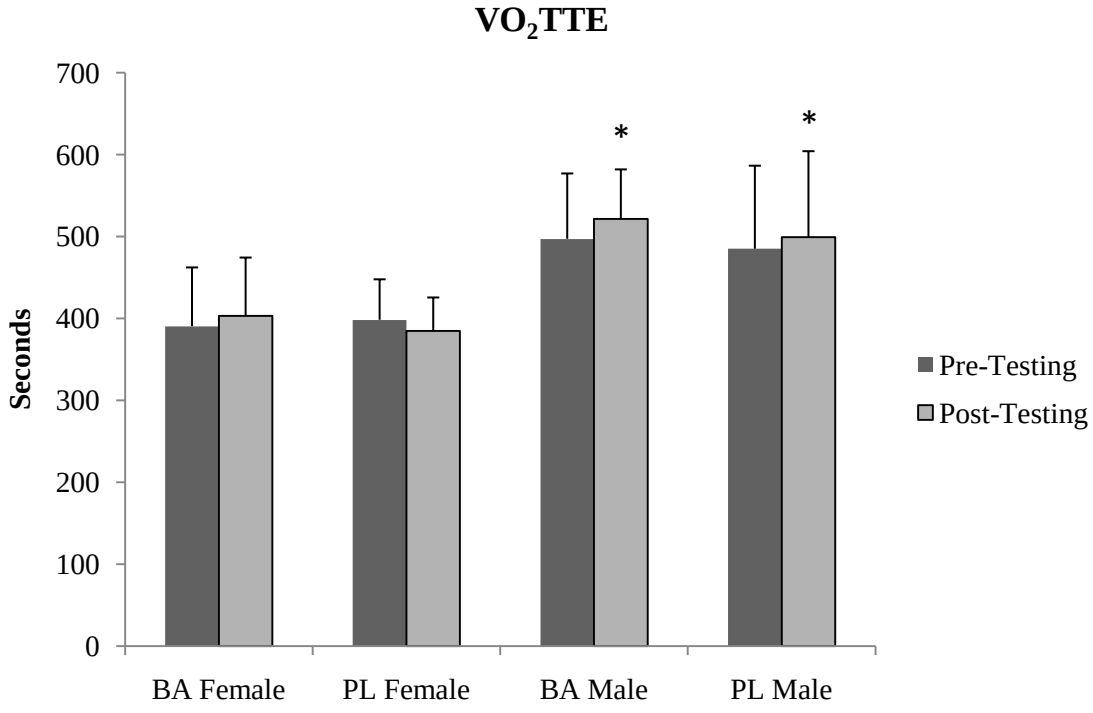

1. Complete informed consent and health and exercise status questionnaire.
2. Determine participant meets all inclusion criteria and is not excluded for any reason.
Week 0
1. VO2max assessment
VO2max
VO2TTE
Ventilatory threshold
36 hour rest (minimum)
2. Oxidative Stress Run
Baseline Blood Draw
Specific Gravity/Pregnancy test
2-3 minute warm-up/jog
40-min run
70% PV
Heart rate @ every 10 minutes
RPE @ every 10 minutes
Distance recorded
Blood Draw IP
Standard Food (Gatorade + Harvest Energy Bar)
2H Post Blood Draw
4H Post Blood Draw
Weeks 1-2
Random Assignment to Groups
Men (n=25)
Women (n=24)
-1 male lost due to injury @ pre-test
-1 male excluded from 40-min run to due scheduling
-1 female lost due to injury @ pre-test
-1 female lost due to inability to finish 40 min run
BA (n=12)
PL (n=13)
BA (n=13)
PL (n=11)
Supplement (2 tablets- 3x daily; 28 days)
Weeks 3-6
Post-testing
Repeat steps 1 & 2 above.
Weeks 7-8

Appendix B.

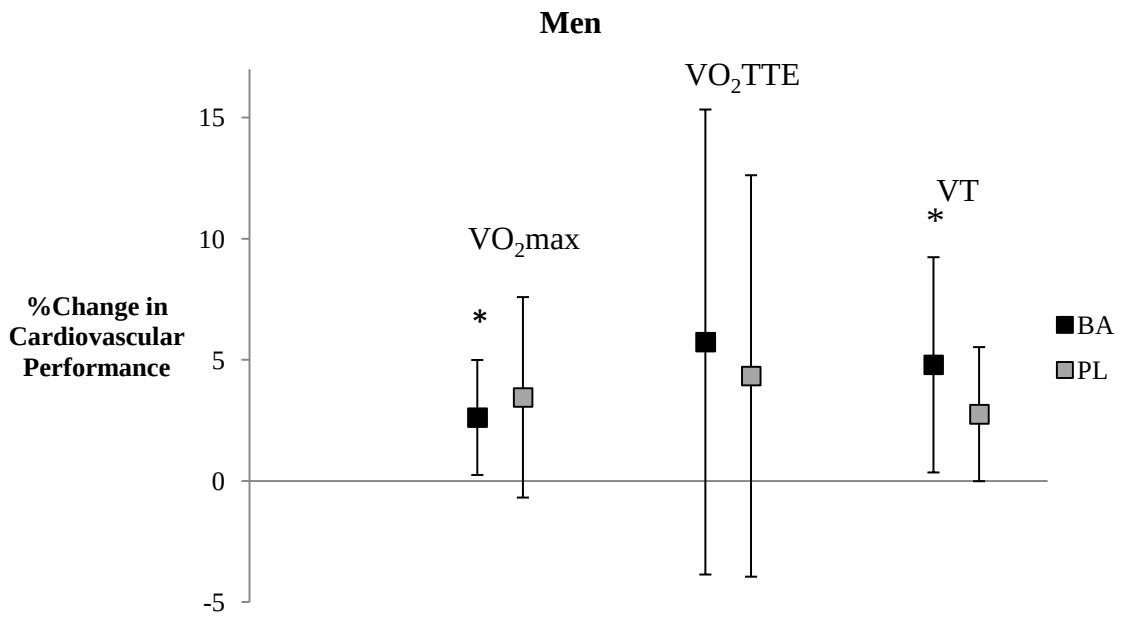
Figure 1.
A)



B)



C)


Men
VO2TTE
VT
VO2max
*
*
15
10
5
0
-5
%Change in Cardiovascular Performance
BA
PL

D)

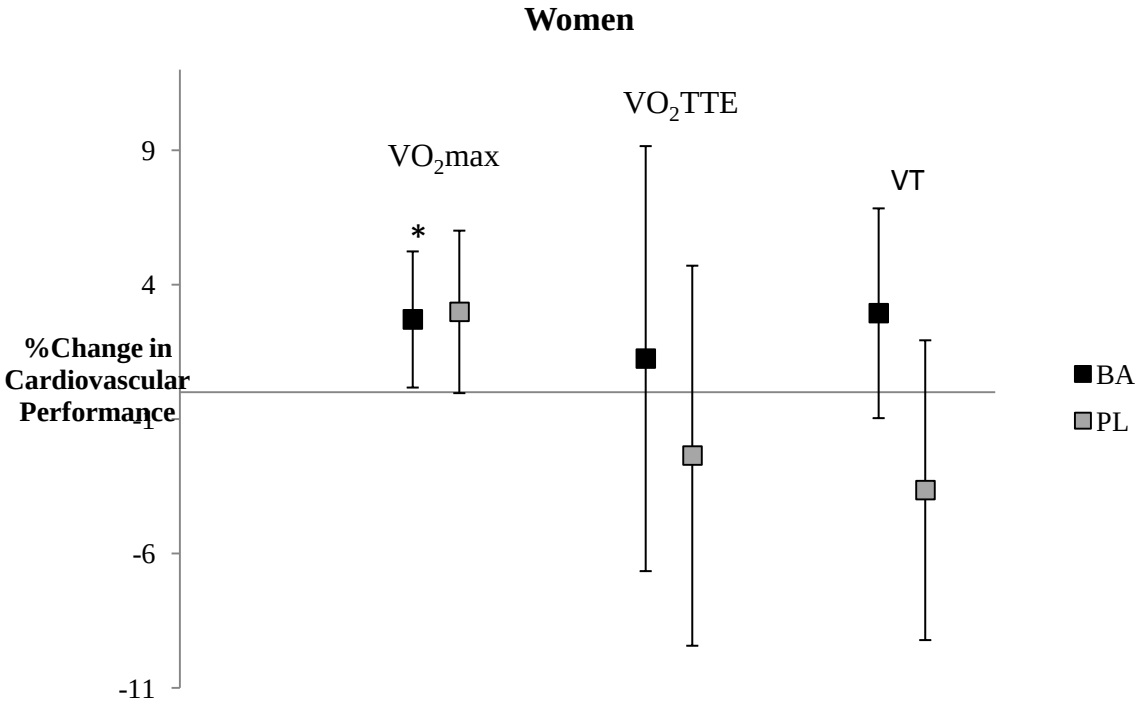
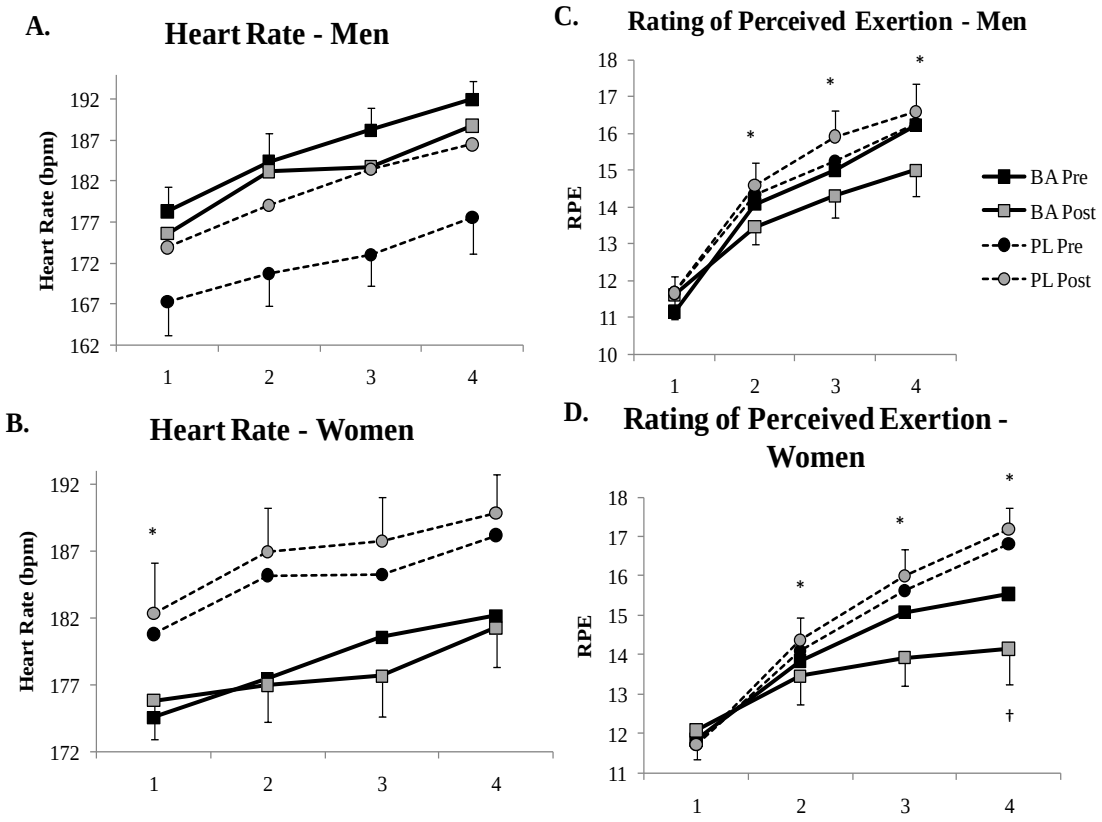
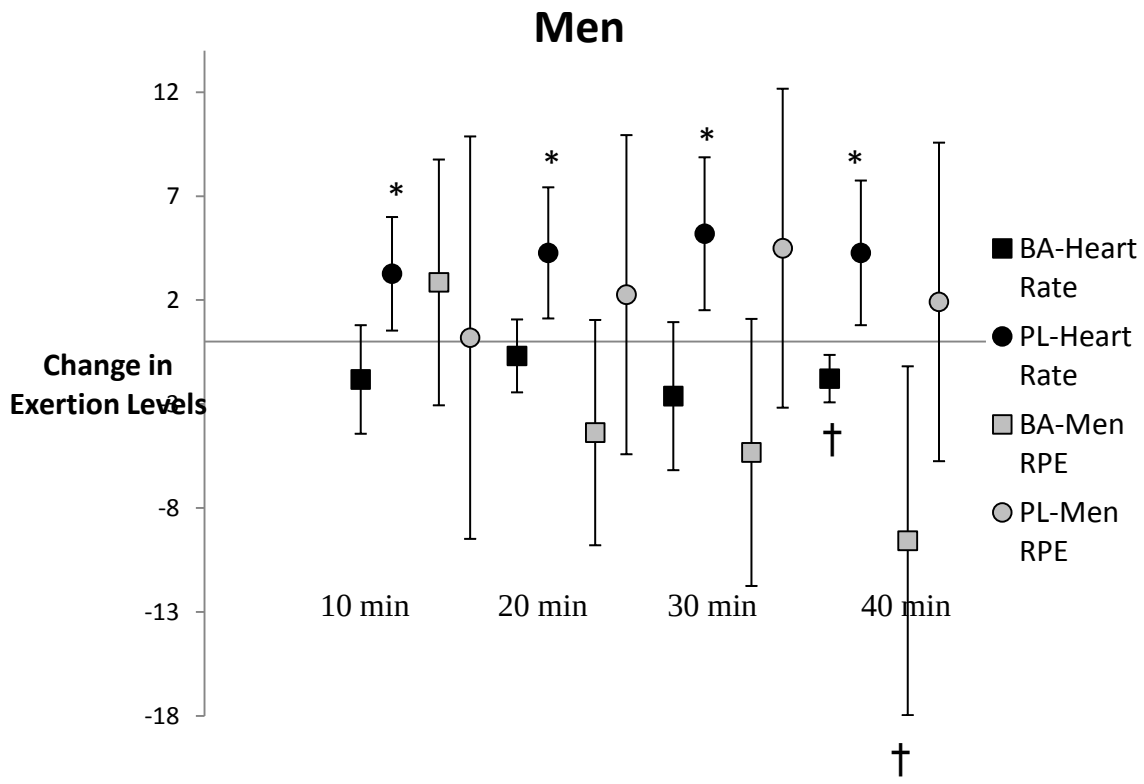

Women
VO2TTE
VO2max
VT
*
9
4
-1
-6
-11
%Change in Cardiovascular Performance
BA
PL

Figure 2.


A.
Heart Rate - Men
Heart Rate (bpm)
192
187
182
177
172
167
162
1
2
3
4
C.
Rating of Perceived Exertion - Men
RPE
18
17
16
15
14
13
12
11
10
*
BA Pre
BA Post
PL Pre
PL Post
B.
Heart Rate - Women
Heart Rate (bpm)
192
187
182
177
172
D.
Rating of Perceived Exertion - Women
RPE
†

E)


Men
12
7
2
-3
-8
-13
-18
Change in Exertion Levels
10 min
20 min
30 min
40 min
*
†
BA-Heart Rate
PL-Heart Rate
BA-Men RPE
PL-Men RPE

F)

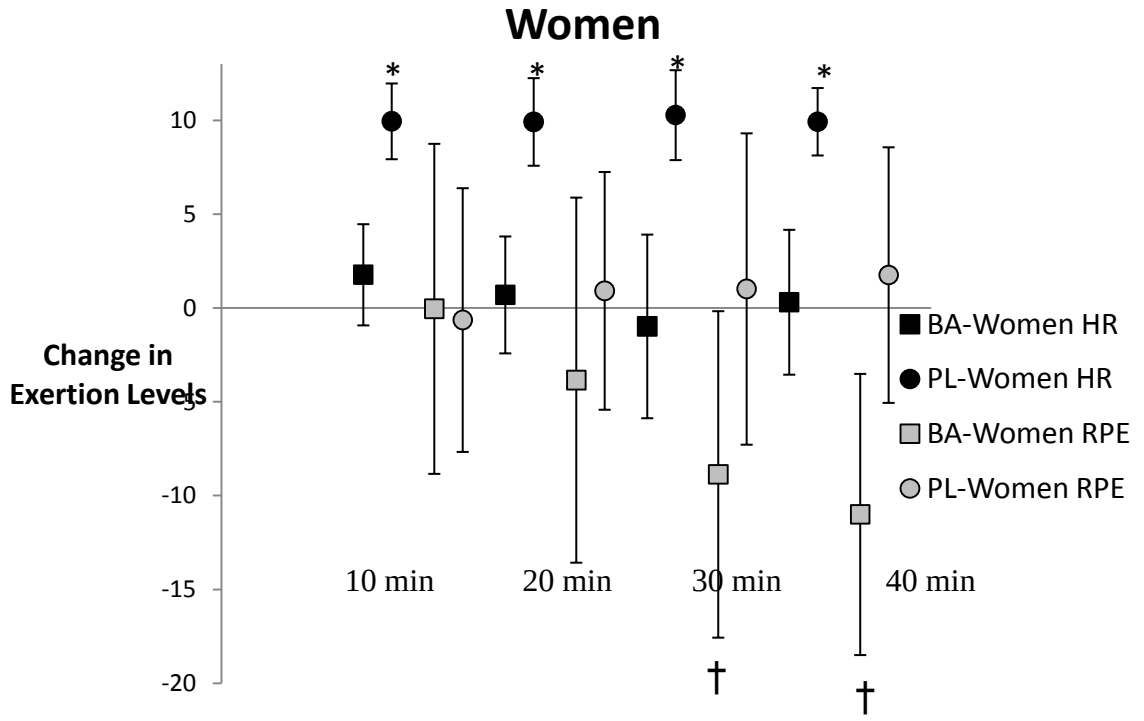
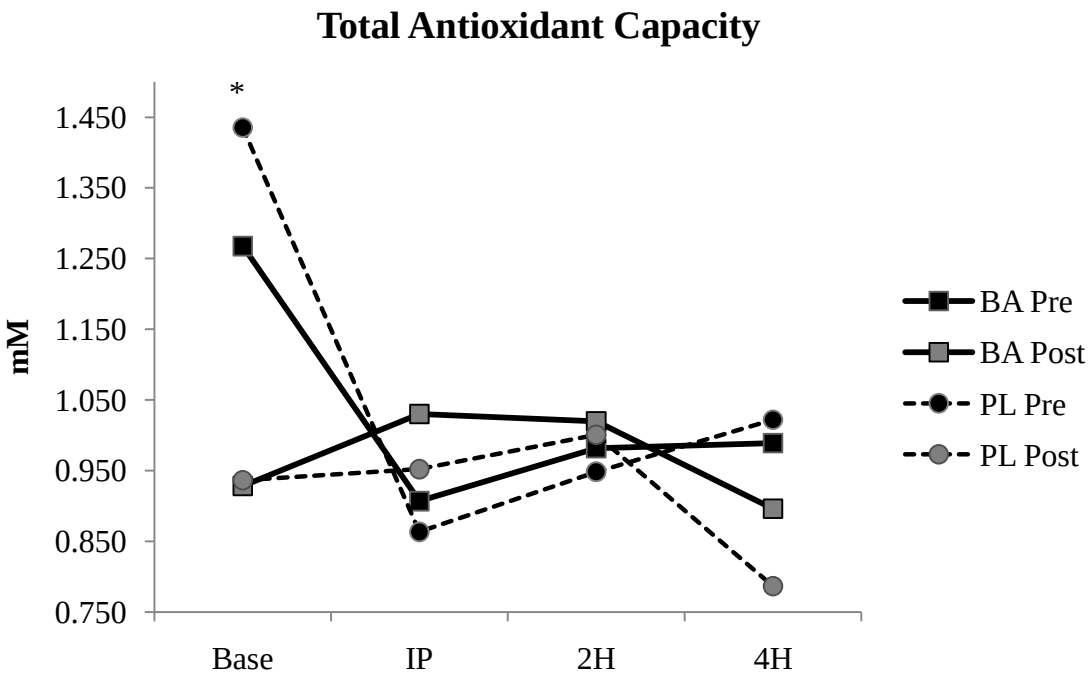
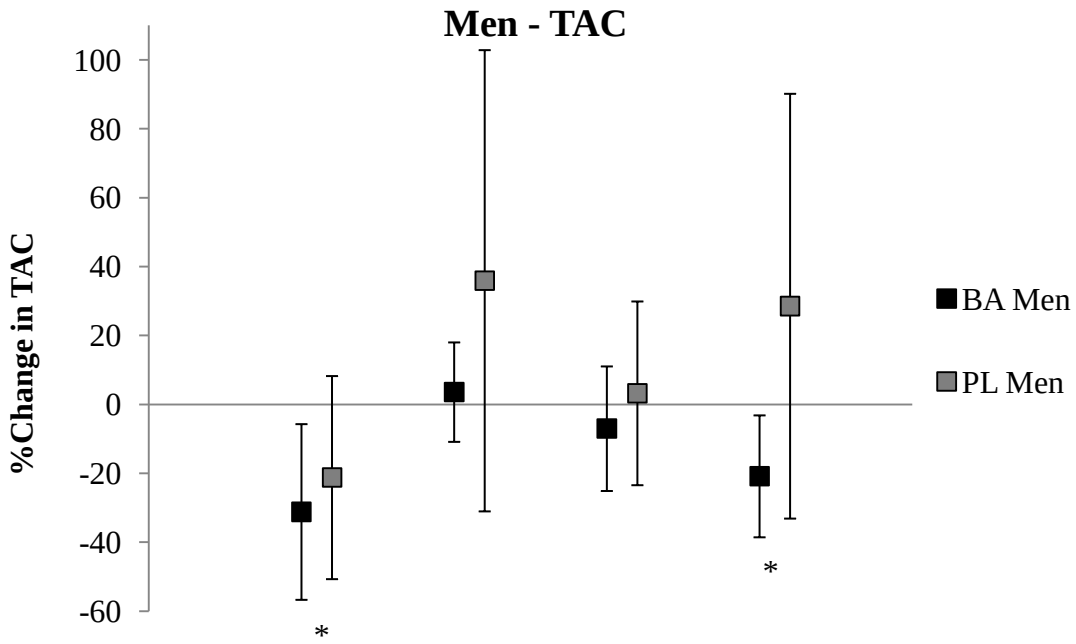

Women
10
5
0
-5
-10
-15
-20
Change in Exertion Levels
10 min
20 min
30 min
40 min
*
†
BA-Women HR
PL-Women HR
BA-Women RPE
PL-Women RPE

Figure 3.

A)


Total Antioxidant Capacity
mM
1.450
1.350
1.250
1.150
1.050
0.950
0.850
0.750
*
Base
IP
2H
4H
BA Pre
BA Post
PL Pre
PL Post

B)


Men - TAC
%Change in TAC
100
80
60
40
20
0
-20
-40
-60
*
*
BA Men
PL Men

C)

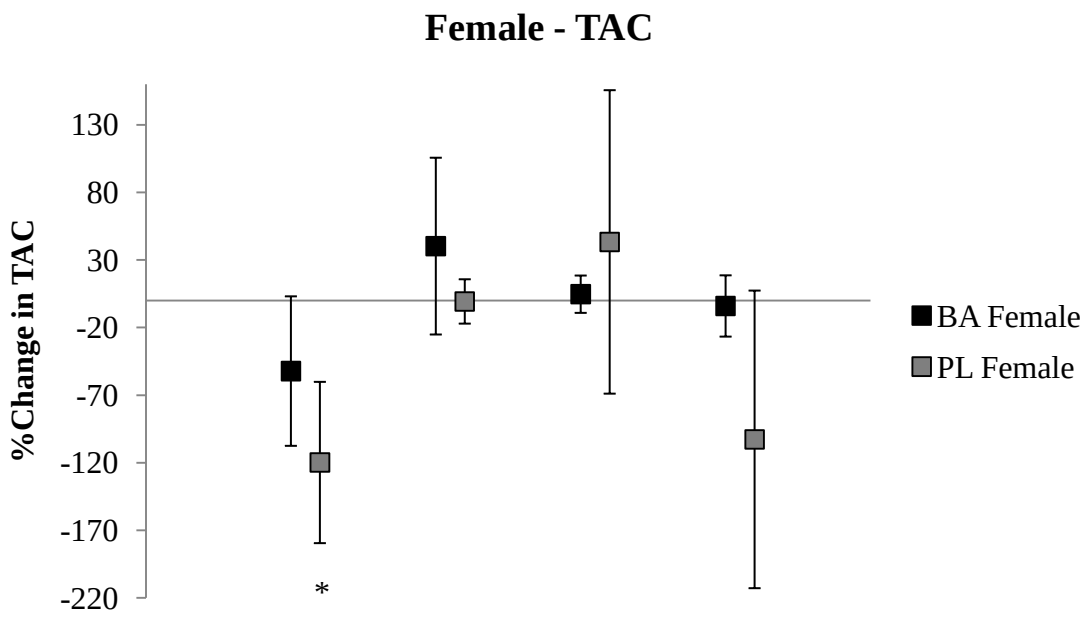
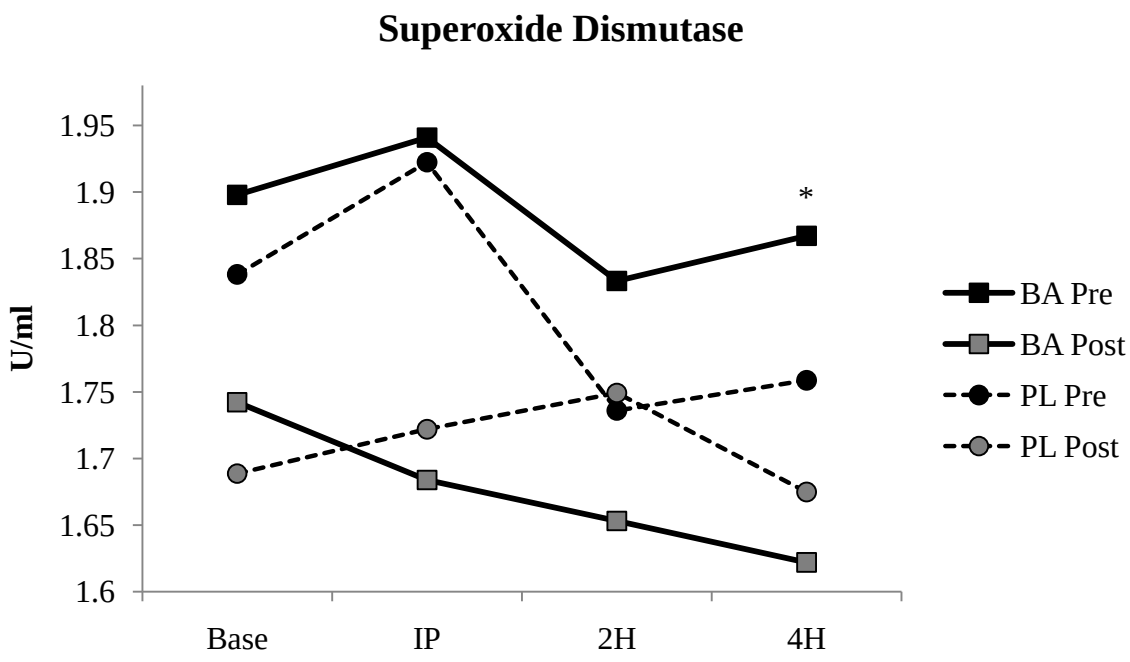
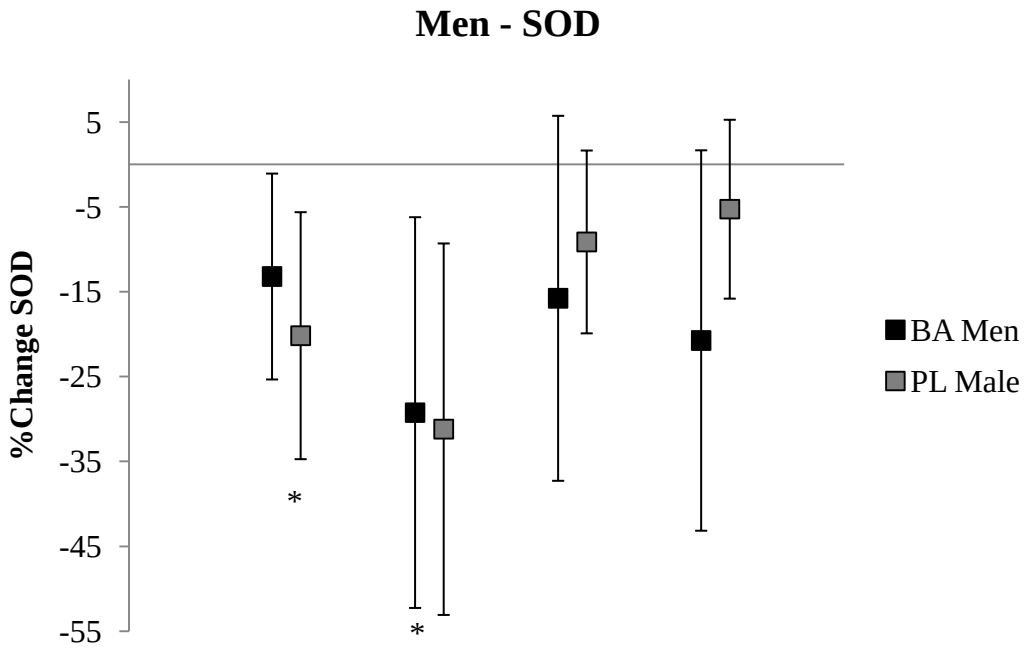

Female - TAC
%Change in TAC
130
80
30
-20
-70
-120
-170
-220
*
BA Female
PL Female

Figure 4.

A)


Superoxide Dismutase
U/ml
1.95
1.9
1.85
1.8
1.75
1.7
1.65
1.6
Base
IP
2H
4H
*
BA Pre
BA Post
PL Pre
PL Post

B)


Men - SOD
%Change SOD
5
-5
-15
-25
-35
-45
-55
*
*
BA Men
PL Male

C)

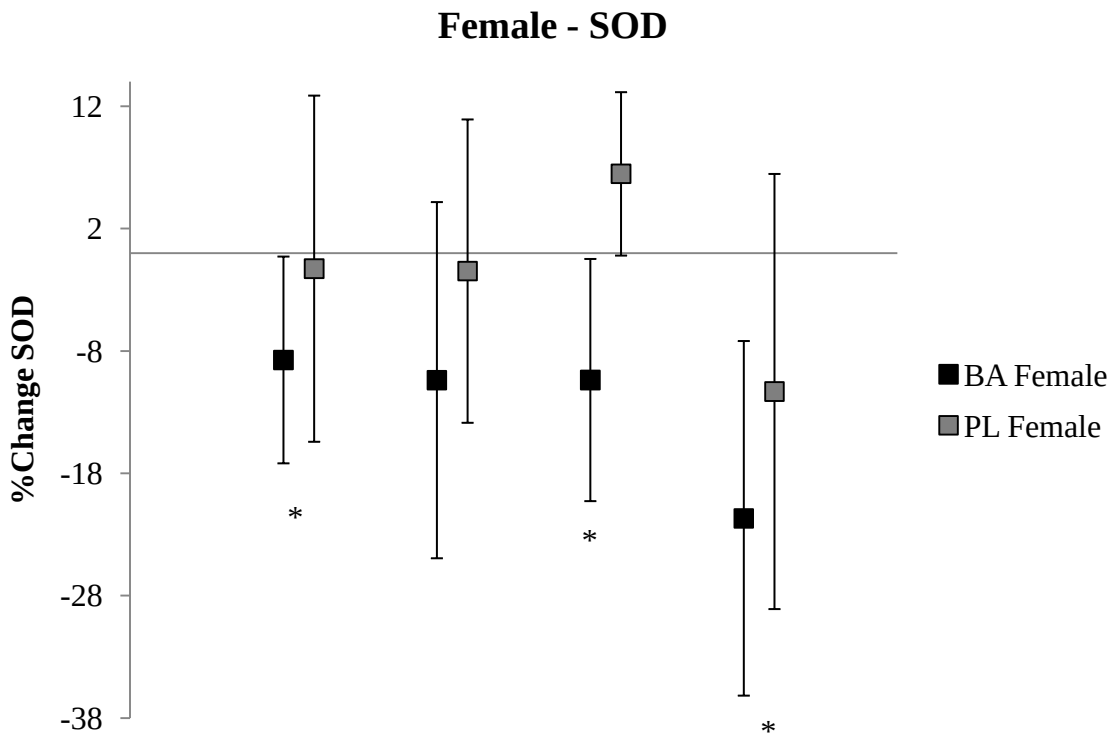
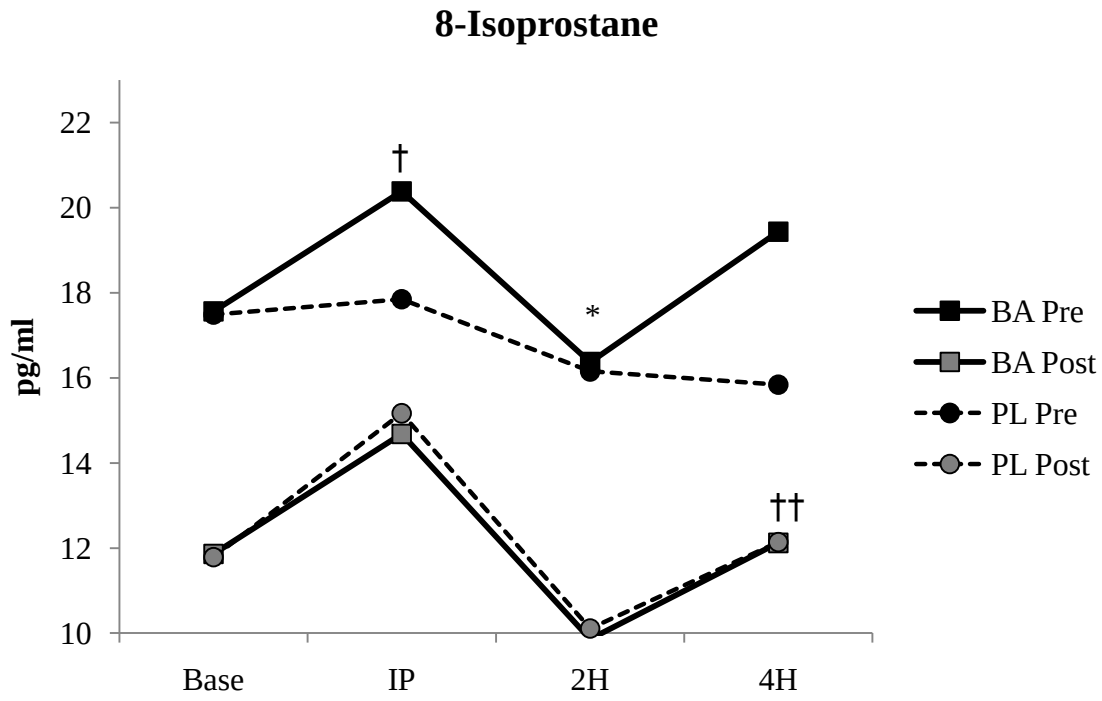
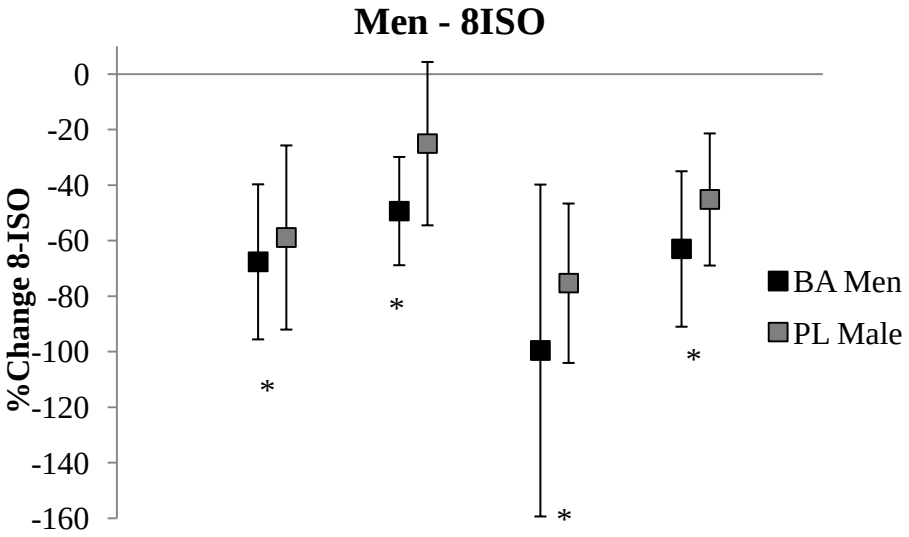


Figure 5.

A)


8-Isoprostane
pg/ml
22
20
18
16
14
12
10
Base
IP
2H
4H
†
*
††
BA Pre
BA Post
PL Pre
PL Post

B)


Men - 8ISO
%Change 8-ISO
0
-20
-40
-60
-80
-100
-120
-140
-160
*
*
*
*
BA Men
PL Male

C)

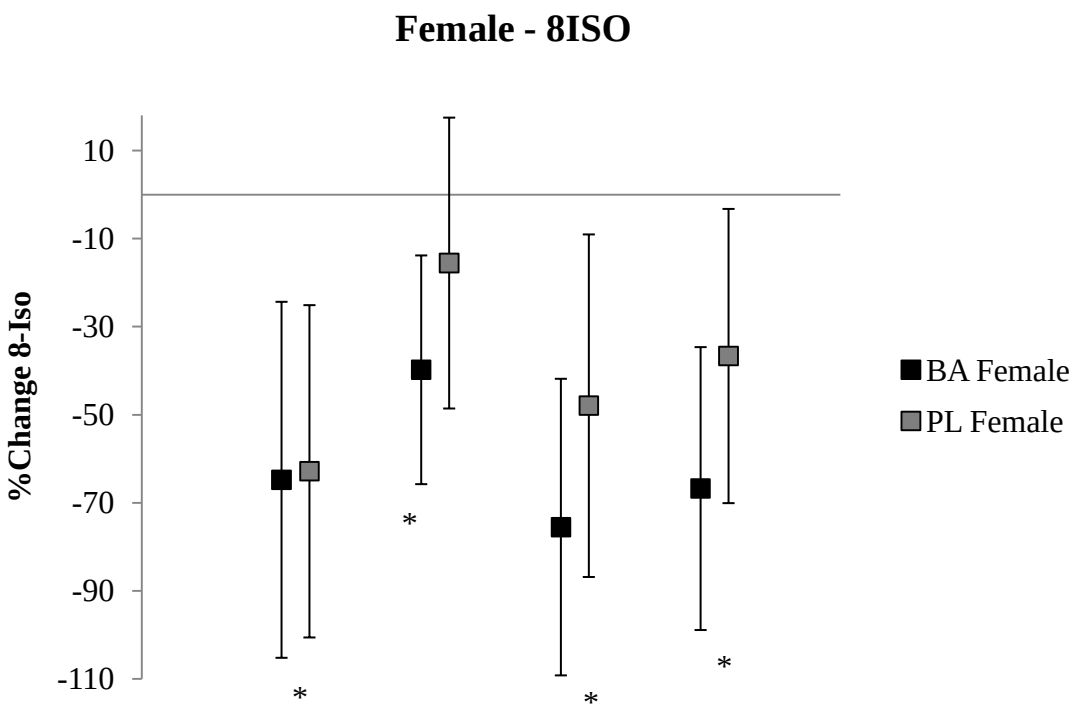
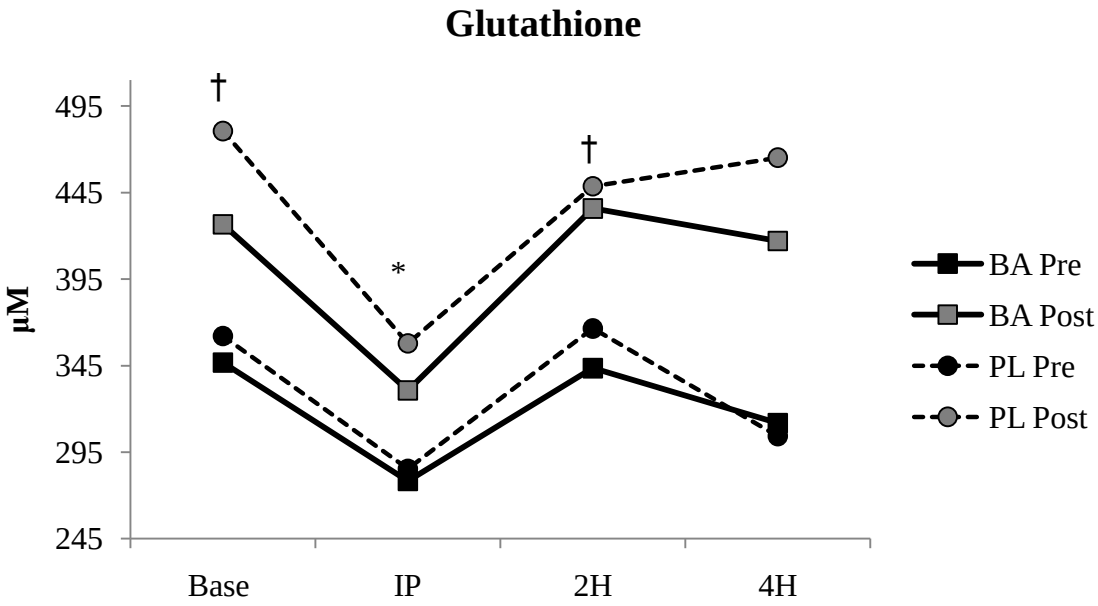
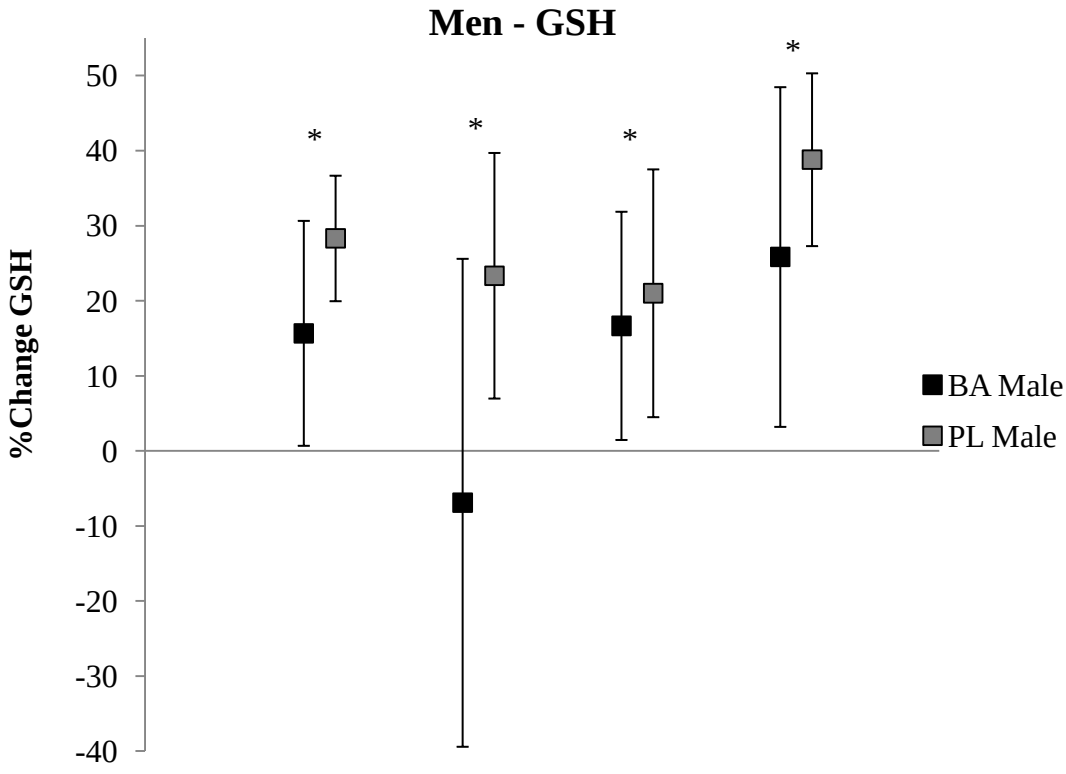

Female - 8ISO
%Change 8-Iso
10
-10
-30
-50
-70
-90
-110
*
*
*
BA Female
PL Female

Figure 6.

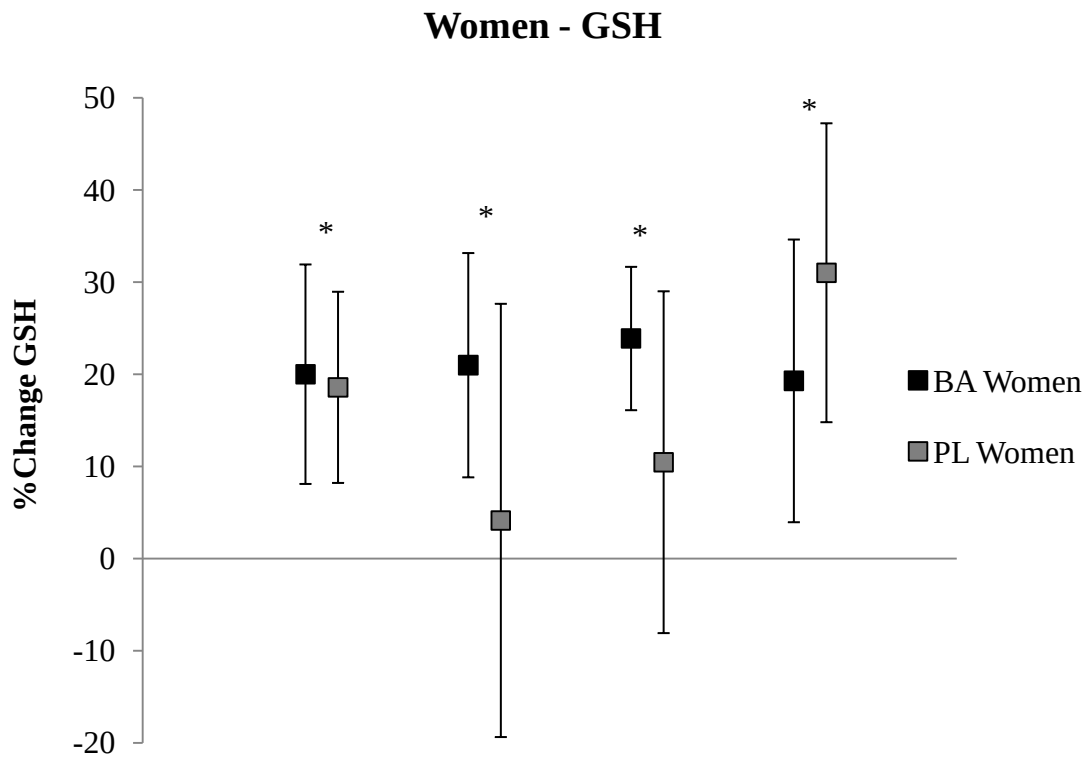
A)


Glutathione
495
445
395
345
295
245
µM
Base
IP
2H
4H
†
*
†
BA Pre
BA Post
PL Pre
PL Post

B)


Men - GSH
50
40
30
20
10
0
-10
-20
-30
-40
%Change GSH
*
*
*
*
BA Male
PL Male

C)



Appendix C.

Table 1. Demographic characteristics for men and women.

	Age (yrs)	Height (cm)	Weight (kg)	VO_2max ($l \cdot min^{-1}$)
Men (n=26)	22.0 ± 3.3	177.8 ± 5.5	79.0 ± 9.7	3.9 ± 0.5
Women (n=24)	21.7 ± 2.1	165.0 ± 5.4	61.9 ± 6.4	2.6 ± 0.3

Table 2. Effect of beta-alanine supplementation on maximal oxygen consumption (VO_2max), time to exhaustion during a graded exercise test (VO_2TTE) and ventilatory threshold (VT) and qualitative practical significance.

BA vs. PL	Mean improvement (%) and 90%CI	Clinical Inference	Beneficial/ Ergogenic	Negligible/ Trivial	Harmful/ Ergolytic
Men					
VO_2max	0.83, ±4.2	Unclear	55.3 %	15.0 %	29.8 %
VO_2TTE	2, ±4.5	Possibly	71.6 %	10.9 %	17.4 %
VT	-0.36, ±12	Unclear	49.2 %	5.5 %	45.2 %
Women					
VO_2max	0.28, ±7.7	Unclear	45.7 %	19.3 %	35.0 %
VO_2TTE	6.6, ±7.7	Very Likely	96.0 %	1.8 %	2.2 %
VT	3.7, ±7.7	Possibly	72.5 %	5.8 %	21.7 %

Table 3. Effect of beta-alanine supplementation on heart rate (HR) and ratings of perceived exertion (RPE) at 10, 20, 30 and 40 minutes and qualitative practical significance.

BA vs. PL	Mean improvement (%) and 90%CI	Clinical Inference	Beneficial/ Ergogenic	Negligible/ Trivial	Harmful/ Ergolytic
Men					
HR10	-5.7, ±7.7	Very Likely	99.4 %	0.5 %	0.2 %
HR20	-5.3, ±3	Very Likely	99.4 %	0.4 %	0.2 %
HR30	-8.4, ±7.7	Most Likely	99.8 %	0.1 %	0.1 %
HR40	-6.8, ±3.4	Most Likely	99.8 %	0.1 %	0.1 %
RPE10	2.7, ±7.7	Unclear	29.2 %	6.2 %	64.6 %
RPE20	-6.6, ±8.1	Likely	89.6 %	3.1 %	7.2 %
RPE30	-9.8, ±7.7	Very Likely	96.1 %	1.3 %	2.7 %
RPE40	-11, ±10	Very Likely	96.4 %	1.0 %	2.6 %
Women					
HR10	0.63, ±7.7	Unclear	52.7 %	20.4 %	26.9 %
HR20	0.06, ±3.6	Unclear	41.6 %	19.0 %	39.4 %
HR30	-2.8, ±7.7	Unclear	77.5 %	8.5 %	14.0 %
HR40	0.48, ±3.9	Unclear	33.6 %	16.8 %	49.7 %
RPE10	0.6, ±7.7	Unclear	42.1 %	7.2 %	50.7 %
RPE20	-4.8, ±9.8	Unclear	76.7 %	4.8 %	18.4 %
RPE30	-9.9, ±7.7	Likely Beneficial	94.0 %	1.6 %	4.3 %
RPE40	-13, ±8.4	Very Likely Bene	99.0 %	0.4 %	0.6 %

Table 4. Effect of beta-alanine supplementation on oxidative stress markers measured as total antioxidant capacity (TAC), superoxide dismutase (SOD), 8-isoprostanes (8-ISO), glutathione (GSH) and the qualitative practical significance for the entire group (A), men (B) and women (C).

A.

BA vs. PL		Mean improvement (%) and 90%CI	Clinical Inference	Beneficial/	Negligible/	Harmful/
TAC	Base	-54, ±40	Possibly Ergolytic	1.3 %	0.1 %	98.6 %
	IP	2, ±44	Unclear	52.2 %	1.5 %	46.2 %
	2H	23, ±45	Unclear	79.7 %	1.0 %	19.2 %
	4H	9.9, ±14	Unclear	87.2 %	2.3 %	10.5 %
SOD	Base:	13 ±25	Likely Ergogenic	80.6 %	1.8 %	17.6 %
	IP	27, ±15	Likely Ergogenic	99.7 %	0.1 %	0.3 %
	2H	13, ±11	Likely Ergogenic	95.9 %	1.1 %	3.0 %
	4H	-8.6, ±14	Likely Ergolytic	14.4 %	2.8 %	82.9 %
8-ISO	Base	-5.3, ±30	Unclear	60.7 %	2.1 %	37.1 %
	IP	8, ±24	Unclear	27.5 %	2.4 %	70.1 %
	2H	25, ±50	Unclear	12.2 %	0.7 %	87.2 %
	4H	5.2, ±25	Unclear	35.4 %	2.5 %	62.2 %
GSH	Base	-3.2 ±9.9	Unclear	26.8 %	5.8 %	67.4 %
	IP	4.1 ±19	Unclear	62.6 %	3.3 %	34.1 %
	2H	-0.98, ±12	Unclear	42.0 %	5.4 %	52.6 %
	4H	-8.5, ±14	Possibly Ergolytic	14.4 %	2.8 %	82.8 %

B.

MEN BA vs. PL		Mean improvement (%) and 90%CI	Clinical Inference	Beneficial/ Ergogenic	Negligible/ Trivial	Harmful/ Ergolytic
TAC	Base	-89, ±54	Possibly Ergolytic	0.5 %	0.0 %	99.5 %
	IP	-34, ±62	Possibly Ergolytic	17.8 %	0.7 %	81.6 %
	2H	36, ±90	Unclear	75.1 %	0.6 %	24.4 %
	4H	-6.5, ±20	Unclear	27.6 %	2.9 %	69.5 %
SOD	Base:	20 ±17	Likely Ergogenic	96.8 %	0.6 %	2.6 %
	IP	31, ±22	Likely Ergogenic	98.6 %	0.2 %	1.1 %
	2H	18, ±12	Likely Ergogenic	99.1 %	0.3 %	0.7 %
	4H	-11, ±18	Likely Ergolytic	15.2 %	2.2 %	82.6 %
8-ISO	Base	-5.3, ±7.7	Unclear	60.7 %	2.1 %	37.1 %
	IP	8, ±24	Unclear	70.1 %	2.4 %	27.5 %
	2H	25, ±7.7	Unclear	87.0 %	0.9 %	12.0 %
	4H	5.2, ±25	Unclear	62.2 %	2.5 %	35.4 %
GSH	Base	-9.7 ±12	Likely Ergolytic	7.2 %	2.1 %	90.8 %
	IP	-19 ±25	Unclear	9.1 %	1.1 %	89.8 %
	2H	-11, ±22	Unclear	19.6 %	2.2 %	78.2 %
	4H	-8.6, ±43	Unclear	35.8 %	1.5 %	62.7 %

C.

WOMEN BA vs. PL		Mean improvement (%) and 90%CI	Clinical Inference	Beneficial/ Ergogenic	Negligible/ Trivial	Harmful/ Ergolytic
TAC	Base	-19, ±57	Unclear	28.2 %	1.0 %	70.8 %
	IP	39, ±64	Unclear	84.6 %	0.6 %	14.8 %
	2H	12, ±21	Unclear	83.0 %	1.9 %	15.1 %
	4H	25, ±19	Likely Ergogenic	98.0 %	0.3 %	1.7 %
SOD	Base:	6.8, ±13	Unclear	79.6 %	3.5 %	17.0 %
	IP	23, ±24	Likely Ergogenic	94.0 %	0.7 %	5.3 %
	2H	9.5, ±20	Unclear	77.6 %	2.4 %	20.0 %
	4H	-5.2, ±23	Unclear	33.6 %	2.7 %	63.7 %
8-ISO	Base	-1.8, ±46	Unclear	51.9 %	1.5 %	46.6 %
	IP	11, ±30	Unclear	25.9 %	1.8 %	72.3 %
	2H	28, ±61	Unclear	21.9 %	0.8 %	77.2 %
	4H	2.1, ±38	Unclear	52.8 %	1.8 %	45.5 %
GSH	Base	4.3 ±17	Unclear	65.2 %	3.7 %	31.2 %
	IP	28, ±28	Likely Ergogenic	94.8 %	0.6 %	4.6 %
	2H	7.2, ±14	Unclear	79.1 %	3.2 %	17.7 %
	4H	-6.6, ±23	Unclear	30.3 %	2.6 %	67.1 %

Appendix D.

Figure Legends

Figure 1. Marginal means for aerobic capacity for (A) maximal oxygen consumption (VO_2max) and ventilatory threshold (VT) for pre- to post-supplementation for men and women, (B) time to exhaustion during the GXT (VO_2TTE). Mean percent change scores from pre- to post-supplementation with 95% confidence intervals for VO_2max, VO_2TTE, VT for men (C) and women (D) for the beta-alanine [BA; black] and placebo [PL; shaded] groups.

(A) * indicates a significant increase from pre- to post-supplementation ($p<0.05$). Values are marginal means ± SEM.

(B) * indicates a significant increase from pre- to post-supplementation ($p<0.05$). Values are marginal means ± SEM.

(C) * indicates the percent change for VO_2max and VT was significantly greater than zero ($p<0.05$). Values are means ± 95% confidence intervals.

(D) * indicates the percent change for VO_2max was significantly greater than zero ($p<0.05$). Values are means ± 95% confidence intervals.

Figure 2. Marginal means for heart rate for men (A) and women (B) and marginal means for ratings of perceived exertion for men (C) and women (D) taken at time intervals of 10 minutes (1), 20 minutes (2), 30 minutes (3) and 40 minutes (4) during the oxidative stress run. Mean percent change scores from pre- to post-supplementation with 95% confidence intervals for HR (Black) and RPE (Shaded) for men (E) and women (F) for the beta-alanine (BA; square) and placebo (PL; circle) groups.

(A) * indicates a significant difference between values ($p<0.05$). Values are marginal means.

(B) * indicates a significant difference between values ($p<0.05$). Values are marginal means.

(C) * indicates a significant difference between values ($p<0.05$). Values are marginal means.

(D) * indicates a significant difference between values ($p<0.05$). † indicates a significant difference between groups ($p<0.05$). Values are marginal means.

(E) * indicates the percent change for placebo HR was significantly greater than zero at all time points ($p<0.05$). † indicates the percent change for HR and RPE at 40 minutes was significantly less than zero ($p<0.05$).Values are means ± 95% confidence intervals.

(F) * indicates the percent change for placebo HR was significantly greater than zero at all time points ($p<0.05$). † indicates the percent change for RPE at 30 and 40 minutes was significantly less than zero ($p<0.05$).Values are means ± 95% confidence intervals.

Figure 3. Marginal means for (A) total antioxidant capacity (TAC) from pre- to post-supplementation for beta-alanine (black) and placebo (shaded). Values are marginal means. Mean percent change scores from pre- to post-supplementation with 95% confidence intervals for men (B) and women (C).

(A) * indicates a significant difference between values ($p<0.05$). Values are marginal means.

(B) * indicates the percent change for baseline and 4H TAC values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

(C) * indicates the percent change for baseline TAC values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

Figure 4. Marginal means for (A) superoxide dismutase (SOD) from pre-to post-supplementation for beta-alanine (black) and placebo (shaded) groups. Values are marginal means. Mean percent change scores from pre-to post-supplementation with 95% confidence intervals for men (B) and women (C).

(A) * indicates a significant difference between values ($p<0.05$). Values are marginal means.

(B) * indicates the percent change for baseline and IP SOD values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

(C) * indicates the percent change for baseline, 2H and 4H SOD values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

Figure 5. Marginal means for (A) 8-isoprostanes (8ISO) from pre-to post-supplementation for beta-alanine (black) and placebo (shaded) groups. Values are marginal means. Mean percent change scores from pre-to post-supplementation with 95% confidence intervals for men (B) and women (C).

(A) * indicates a significant lower value between all time points ($p<0.01$). † indicates a significant greater value than all time points ($p<0.01$). †† indicates a significant decrease over time ($p<0.01$). Values are marginal means.

(B) * indicates the percent change for all time points for 8ISO values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

(C) * indicates the percent change for all time points for 8ISO values were significantly less than zero ($p<0.05$). Values are means ± 95% confidence intervals.

Figure 6. Marginal means for (A) glutathione (GSH) from pre-to post-supplementation for beta-alanine (black) and placebo (shaded) groups. Values are marginal means. Mean percent change scores from pre-to post-supplementation with 95% confidence intervals for men (B) and women (C).

(A) * indicates a significant lower value between all time points ($p<0.01$). † indicates a significant greater value than IP and 4H values ($p<0.01$). Values are marginal means.

(B) * indicates the percent change for all time points for GSH values were significantly greater than zero ($p<0.05$). Values are means ± 95% confidence intervals.

(C) * indicates the percent change for all time points for GSH values were significantly greater than zero ($p<0.05$). Values are means ± 95% confidence intervals.

Appendix E.

Informed Consent

CONSENT FORM

Version 3, 7/6/10 ***IRB No: 15244***

Consent Form
University of Oklahoma Health Sciences Center (OUHSC)
University of Oklahoma, Norman Campus

The antioxidant effects of a non-essential amino acid, beta-alanine, on oxidative stress in men and women

Dr. Jeffrey R. Stout

This is a research study. Research studies involve only individuals who choose to participate. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to take part in this trial/study because **you are a healthy, moderately active man or woman between the ages of 18-35.**

Why Is This Study Being Done?
The purpose of this study is to **evaluate the effect of beta-alanine, compared to a placebo (an inactive substance) on the acute effects of exercise on the body.**

What is the Status of the Drugs (Devices or Procedures) involved in this study?
Beta-Alanine is a naturally occurring amino acid produced in the liver and in muscle tissue as a component of carnosine. It is found in common meats such as turkey and chicken. The total amount that you might consume in this study is comparable to 150 grams of chicken breast (i.e. about 4 chicken breasts). The placebo tablets are made up of maltodextrin, a sweet starch used as a common additive, containing fewer calories than sugar. Total daily consumption would be ~4-8 grams totaling 24 calories per day. The supplement in this study has not been evaluated or approved by the US Food and Drug Administration. Beta-Alanine is characterized by the food and Drug Administration as Generally Regarded as Safe (GRAS). The use of this supplement is considered investigational and not intended to diagnose, treat, cure, or prevent any disease.

How Many People Will Take Part In The Study?
About ***72*** people will take part in this study, all at this location.

What Is Involved In The Study?

If you agree to be in this study, you will be asked to do the following:

1. Fill out an ENROLLMENT FORM and a PRE-EXERCISE TESTING HEALTH and EXERCISE STATUS QUESTIONNAIRE, which may determine your ability to participate in this study.
2. Complete the entire study, including the pre-testing (Week 1- Visits 1, 2, and 3), the supplementing period at home (Weeks 2-5) and post-testing (Week 6; Visits 4, 5, and 6).
3. Abstain from caffeine, supplements, multi-vitamin and anti-inflammatories during the two weeks of testing and maintain your current exercise routine through the duration of the study.
4. During Visits 1 (Week 1) and 4 (Week 6) you will be tested for cardiorespiratory fitness (VO_2max). You will be required to run on a treadmill with a mouthpiece attached to your head and wear a heart rate monitor, for 7-15 minutes. Difficulty level and speed of running will increase every 2 minutes. You will be required to run until you can no longer comfortably keep up with the treadmill.
5. Within 24-48 hours of your VO_2max test, during Visits 2 (Week 1) and 5 (Week 6) you will be asked to perform a critical velocity test by completing three runs to exhaustion on a treadmill. The intensities of the three runs will be based on you initial fitness level at 110% and 90% and 100% of your final velocity achieved during the VO_2max test. Each run will last between 1 and 15 minutes, with at least 15 minutes of recovery between each of the runs. Your finger will also be pricked before and after each run to measure lactate levels. This prick is small and does not require a band-aid.
6. During Visits 3 (Week 1) and 6 (Week 6) occurring 24-48 hours after the previous visit, you will also be asked to perform a submaximal run on a treadmill for 40-45 minutes at 70-75% of your final velocity achieved during the VO_2max test. Your hydration levels will be assessed prior to your run with a urine sample, in order to prevent dehydration. You will also have your blood drawn before, immediately after, 2 hours after and 4 hours after your run.
7. During Week 1 you will be asked to write down what you eat for three total days. You will be asked to eat a similar diet during Week 6 in order to keep your vitamin and mineral intake similar between weeks.
8. Following Visit 3 you will be randomly assigned to a supplement group with either beta-alanine (2 capsules of 800 mg Beta-Alanine, 3 times daily) or placebo (2 capsules of 800 mg Maltodextrin (a common starch), 3 times daily). This assignment will be random and neither you nor the investigator will know which supplement you are assigned to. You will consume the product three times a day for 28 days orally. You will also be asked to record your consumption on a provided supplement log.
9. Post-testing (Visits 4, 5, and 6) will occur following consumption of all of your study product.

For Randomized Trials:

You will be randomized to receive either study supplement *(a non-essential amino acid, Beta-Alanine)* or placebo (inactive substance, which will look like the study drug). Randomization means that you are put in a group by chance. If you have an **equal; 50/50** chance use the following comparison, like the flip of a coin. A computer program at the study sponsor will make this random assignment. Neither you nor the investigator will choose which group you will be in. Also, neither you nor the investigator will know which group you have been assigned to.

How Long Will I Be In The Study?
We think that you will be in the study for a total of **six weeks with a total of 6 lab testing visits**. There will be a set of three pre-testing days, followed by 28 days (about four weeks) of consuming your assigned supplement, and three post-testing days after consumption of all of your assigned supplement. You will also be required to visit the lab one time per week during the supplementation period to submit your supplement log (for a total of 4 times).
There may be anticipated circumstances under which your participation may be terminated by the investigator without regard to your consent. The investigators may terminate your participation if you fail to follow the pre-testing instructions (i.e. consume caffeine and anti-inflammatories prior to testing) and if you fail to consume your assigned supplement.

What Are The Risks of The Study?
There is minimal risk from consumption of beta-alanine, a flushing/tingling of the skin may occur. This flushing/tingling has not been reported with the time-release form of beta-alanine that is given in this study. Beta-alanine can cause a flushing/tingling sensation that may be uncomfortable but usually lasts less than 60 minutes.

There is a possible risk of muscle strain or injury during graded exercise test (GXT) protocol and high-speed runs on the treadmill. During these tests you will be asked to give maximal effort which may lead to some discomfort. To reduce the chance of these risks, preliminary screenings (Health History Questionnaire) are evaluated. Physical risks will be minimized by having each testing session conducted by qualified investigators and all speeds will be established based on your initial fitness level. All testing procedures will be done in a controlled manner. All additional research staff members directly involved with testing of the subjects are familiar with the American College of Sports Medicine standards and protocols for exercise testing and emergency management. Rise in heart rate and blood pressure associated with exercise may also occur. You may also experience minor pain, bruising, and arm soreness from having your blood drawn. To minimize your risks, you blood will be drawn by an individual trained in phlebotomy.

If you are a female, you must <u>not be</u> and should <u>not become</u> pregnant nor breast-feed an infant while on this study. Taking the study drug(s), undergoing a particular procedure or treatment involved in this study while pregnant or breastfeeding may involve risks to an embryo, fetus or infant, including birth defects which are currently unforeseeable. In order to reduce your risk of pregnancy, you or your partner should use one or more of the acceptable methods of birth control <u>listed below,</u> regularly and consistently while you are in this study.
Acceptable methods of birth control (continuing throughout the study and for one month after the study) include:

- An approved oral contraceptive (birth control pill)
- Intra-uterine device (IUD)

- Hormone implants (Norplant)
- Contraceptive injection (Depo-Provera)
- Barrier methods (diaphragm with spermicidal gel or condoms with contraceptive foam)
- Transdermal contraceptives (birth control patch)
- Vaginal contraception ring (birth control ring)
- Sterilization (tubal ligation, hysterectomy or vasectomy)

If you become pregnant or suspect that you are pregnant you should immediately inform the study personnel. If you become pregnant or suspect that you are pregnant while in this study, a pregnancy test will need to be done. If pregnancy is confirmed, you will need to withdraw from the study. Payment for all aspects of obstetrical, child, or related care will be your responsibility.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.

Are There Benefits to Taking Part in The Study?
If you agree to take part in this study, there is no direct medical/physical benefit to you. We hope that the information learned from this study will benefit other patients and athletes in the future.
The possible benefits of taking part in the study are the same as receiving Beta-Alanine without being in the study.

What About Confidentiality?
Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the US Food & Drug Administration, the OU Health and Exercise Science Metabolic and Body Composition Laboratory and the OUHSC Institutional Review Board.
What Are the Costs?
There is no cost to you for participating in this study.

Will I Be Paid For Participating in This Study?
You will be reimbursed for your time and participation in this study in the amount of $40.00. Upon full-completion of the study, you will receive a check in the amount of $40.00. If you are not able to complete the entire study, the amount will be pro-rated. Completion of the first or second visit during pre-testing will warrant $5.00 per completed visit. If the third visit of the pre-testing sessions is completed, you will

receive $10.00, for a total of $20.00 for completing all of pre-testing. Post-testing sessions will be pro-rated in a similar manner. Completion of visits four and five will warrant $5.00 per completed visit, and $10.00 for the final ($6^{th}$ visit).

What if I am Injured or Become Ill While Participating in this Study?
In the case of injury or illness resulting from this study, emergency medical treatment is available. However, you or your insurance company will be responsible for the costs of this treatment. No funds have been set aside by The University of Oklahoma to compensate you in the event of injury.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. You may discontinue your participation at any time without penalty or loss of benefits, to which you are otherwise entitled. Whether or not you participate in this study will have no affect whatsoever on your academic status in any of the investigators classes. You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher and your regular doctor first.

We will provide you with any significant new findings developed during the course of the research that may affect your health, welfare or willingness to continue your participation in this study.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.

Whom Do I Call If I have Questions or Problems?
If you have questions, concerns, or complaints about the study or have a research-related injury, contact Jeffrey R. Stout, PhD at **405-325-9023 or** Abbie Smith at **515-681-5842.**

If you cannot reach the Investigator or wish to speak to someone other than the investigator, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

For questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

Signature:

By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

_____ _____ _____

PARTICIPANT SIGNATURE (age ≥18) Printed Name Date

(*Or Legally Authorized Representative)*

_____ _____ _____

SIGNATURE OF PERSON Printed Name Date

OBTAINING CONSENT