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ORIGINAL ARTICLE

Exercise-induced oxidative stress: the effects of β-alanine supplementation in women

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Abstract The purpose of this study was to evaluate the effects of β-alanine supplementation on markers of oxidative stress. Twenty-four women (age: 21.7 ± 2.1 years; VO_2max: 2.6 ± 0.3 l min^{-1}) were randomly assigned, in a double-blind fashion, to a β-alanine (BA, 2 × 800 mg tablets, 3× daily; CarnoSyn®; $n = 13$) or placebo (PL, 2 × 800 mg maltodextrin tablets, 3× daily; $n = 11$) group. A graded oxygen consumption test (VO_2max) was performed to evaluate VO_2max, time to exhaustion, ventilatory threshold and establish peak velocity (PV). A 40-min treadmill run was used to induce oxidative stress. Total antioxidant capacity, superoxide dismutase, 8-isoprostane (8ISO) and reduced glutathione were measured. Heart rate and ratings of perceived exertion were recorded during the 40 min run. Separate three- [4 × 2 × 2; acute (base vs. IP vs. 2 vs. 4 h) × chronic (pre- vs. post-) × treatment (BA vs. PL)] and two- [2 × 2; time (pre-supplement vs. post-supplement) × treatment (BA vs. PL)] way ANOVAs were used for analyses. There was a significant increase in VO_2max ($p = 0.009$), independent of treatment, with no significant changes in TTE ($p = 0.074$) or VT ($p = 0.344$). Ratings of perceived exertion values were significantly improved from pre- to post-supplementation for the BA group only at 40 min ($p = 0.02$). The ANOVA model demonstrated no significant treatment effects on oxidative stress. The chronic effects of BA supplementation demonstrated little antioxidant potential, in women, and little influence on aerobic performance assessments.

Keywords Carnosine · Antioxidant · Sex · Running · Aerobic capacity · Supplement

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Introduction

As chemical species produced in all living cells, free radicals have the potential to be highly reactive and play a role in numerous 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 (Halliwell 1999). Due to production and accumulation of free radicals within the body, several antioxidant defenses have evolved. These defense systems utilize 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. In some circumstances, such as exercise, an overproduction of free radicals or a suppression of antioxidant defenses causes an imbalance, defined as oxidative stress (Packer 1997; Sen 1995). Various mechanisms support an exercise induced increase in free radicals, which, in turn confers a strong relationship between exercise and oxidative stress (Alessio 1993; Finaud et al. 2006). Among free radicals, reactive oxygen species (ROS) are derived from oxygen and metabolism, and previous research has suggested the mitochondria as

the major source of ROS (Di Meo and Venditti 2001; Nohl et al. 2003; Sjodin et al. 1990); Increases in central temperature, catecholamines and lactic acid all lead to an influx of ROS (Clarkson and Thompson 2000; Cooper et al. 2002). There is some evidence that ROS accumulation may have positive effects on the force of muscle contraction (Reid 2001a, b), however, ROS are potentially harmful provoking inflammation and altered cell function when surpassing antioxidant defenses.

The natural histidine-containing dipeptides, carnosine and anserine, are powerful antioxidants in vitro 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, to suppress peroxidation induced both enzymatically and non-enzymatically, and eliminating the products of free radical reactions (Boldyrev et al. 1997; Boldyrev and Severin 1990). However, in vivo antioxidant support of carnosine is scarce. A modest body of literature supports the role of carnosine, by way of β-alanine, as an agent to delay fatigue (Hill et al. 2007; Stout et al. 2006, 2007; Van Thienen et al. 2009; Zoeller et al. 2007). A number of explanations have been proposed for such an effect, such as H^+ buffering, an increase in the efficiency of the electromechanical coupling (Boldyrev et al. 1987), stimulation of ATPase activity of contractile proteins and activation of ATP producing enzymes (Boldyrev et al. 1992; Quinn et al. 1992) and may also be related to its proposed antioxidant capacity. In general, this compound has been reported to possess direct (carnosine) and indirect (β-alanine) 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 (Decker et al. 1992, 2001). Furthermore, carnosine has been shown to possess SOD activity and appears to regulate lipoxygenase activity (Decker et al. 1992; Boldyrev et al. 1999). While carnosine has demonstrated positive effects on oxidative stress biomarkers, all of the current studies have been conducted in vitro and in animal models. Previous human and animal studies suggest three possible mechanisms by which 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. Therefore, further research is warranted on the ability of carnosine to sequester free radicals in vivo and the potential performance effects of its constituent, β-alanine, yielding the purpose of this study: to evaluate the effects of 28 days of β-alanine supplementation on markers of oxidative stress. A secondary purpose was to investigate the effects of β-alanine supplementation on measures of aerobic performance, maximal oxygen consumption (VO_2max), time to exhaustion during VO_2max, and ventilatory threshold. Additionally, while this is the first study to evaluate the effects of β-alanine supplementation on in vivo antioxidant effects, this is also one of three studies to evaluate the properties of β-alanine in women.

Materials and methods

Subjects

Twenty-six women were recruited for this investigation; two participants dropped out due to the inability to complete the 40-min run and a musculoskeletal injury, respectively. Therefore, pre- and post-supplementation data resulted in 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. No participant reported current or ongoing musculoskeletal injury at the time of initiation. Participants were asked to refrain from caffeine, alcohol, analgesics (aspirin, acetaminophen, non-steroidal anti-inflammatories, and glucocorticoids) and cigarettes prior to testing weeks and specifically 48 h 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 subjects and all subjects completed a written informed consent form. Using the procedures described by Howell (2007) for estimating samples sizes for repeated measures designs, a minimum sample size of $n = 12$ was required for each

Table 1 Demographic characteristics

	Age (years)	Height (cm)	Weight (kg)	VO_2max (l min^{-1})
β-alanine ($n = 13$)	22.0 ± 2.5	165.0 ± 5.0	60.9 ± 6.3	2.6 ± 0.3
Placebo ($n = 11$)	21.4 ± 1.4	165.0 ± 6.5	62.9 ± 6.8	2.5 ± 0.2

Values reported as mean ± standard deviation (SD)

group to reach a statistical power (1-β) of 0.80 based on the findings of Derave et al. (2007), Stout et al. (2007), and Hill et al. (2007). A sample size of $n = 13$ per group was recruited to account for subject dropout.

Experimental design

A randomized, placebo controlled, mixed factorial design [acute (0 vs. IP vs. 2 vs. 4 h) × chronic (pre- vs. post-supplementation) × treatment (β-alanine vs. placebo)] was used to examine the effects of β-alanine loading on markers of oxidative stress. All other performance variables were assessed 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 peak velocity (PV). Within 2–3 days, participants returned to the lab for baseline blood draws followed by a non-damaging treadmill run (oxidative stress run) for 40 min at 70% PV (Nikolaidis et al. 2008). Additional blood samples were drawn immediately post (IP), 2 and 4 h following completion of the run. All samples were centrifuged and stored immediately. Hydration status was measured and controlled for using handheld refractometry (VEE GEE Refractometers, Model CLX-1) to determine specific gravity, before the oxidative stress run to decrease the risk of hydration and any potential impact on performance. Each participant was randomly assigned, by computer generated allocation, to either a placebo (PL: 800 mg/tablet of maltodextrin, 2 tablets 3 times daily) or β-alanine (BA: 800 mg/tablet (sustained release), 2 tablets 3 times daily; CarnoSyn®, Natural Alternatives Inc, San Marcos, CA) supplementing group. Participants were required to visit the lab after 2 weeks of supplementation to report product intake, any side effects and return completed dietary recalls. Following 28 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 completed a 3-day dietary recall prior to pre-testing and at the beginning of post-testing. At post-testing, participants were asked to consume a diet similar to pre-testing to control for macro and micronutrient intake before the oxidative stress testing.

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, USA) to determine VO_2max. Based on the protocol of Peake et al. (2004), the initial GXT velocity was set at 10 km h^{-1}at a 0% grade and increased 2 km h^{-1} every 2 min up to 16 km h^{-1}, followed by 1 km h^{-1} increments per minute up to 18 km 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 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 PV.

Exercise test (oxidative stress run)

During the experimental protocol, each subject ran on the treadmill at a velocity corresponding to 70–75% of their previously determined PV for 40 min. This exercise protocol was selected because it has been shown to induce oxidative stress without eliciting muscle damage (Alessio 1993; Bloomer et al. 2006b). 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 ($p = 0.563$) in total distance run.

Blood collection

Blood samples were collected prior to the exercise test, immediately following 2- and 4-h post-exercise. Plasma and whole blood samples were obtained via vacutainers, gently inverted ten consecutive times and immediately centrifuged at 3,000 rpm for 15 min before storing at −80°C until completion of analyses.

Antioxidant markers

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. The plate was covered and incubated on an orbital plate shaker for 5 min 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

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). 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. Absorbance of the plate was measured at a wavelength 405 nm (Micro Plate Reader Model #680, Bio Rad, Hercules, CA, USA). 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)

Reduced GSH was analyzed according to procedures from a commercial assay kit (Catalog #703002, Cayman Chemical, Ann Arbor, MI, USA). The absorbance values were measured at 405–414 nm (Micro Plate Reader, Model #680; Bio Rad, Hercules, CA) at 25 min 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 three-way mixed factorial ANOVAs [4 × 2 × 2; acute (base vs. IP vs. 2 vs. 4 h) × chronic (pre- vs. post-) × treatment (placebo vs. β-alanine)] 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. β-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, USA).

In addition, percent change scores were calculated for each participant from pre- to post-supplementation for the oxidative stress markers. These percent change scores were averaged 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. Recent statistical research has proposed the use of additional statistical tools to complement null hypothesis testing in an effort to reduce interpretation errors. To make inferences on true effects of β-alanine on performance and oxidative stress, a published spreadsheet using the unequal variances *t* statistic was used (Batterham and Hopkins 2006). The effect of BA was calculated as the change score by calculating the difference between the post- and pre-supplementation scores for the BA and PL groups. Each change score underwent a log-based transform and expressed as a percentage of baseline scores to reduce bias from nonuniformity error. The precision of the magnitude inference was set at 90% confidence limits using the *p* value corresponding to the *t*-statistic. The published spreadsheet calculated inferences whether the true population effect was substantially beneficial, harmful, or trivial based on the range of the confidence interval relative to the value for the smallest clinical worthwhile effect. An effect was reported to be unclear if the confidence interval overlapped the thresholds for positive and negative substantiveness (>5% chance that the value was both substantially positive and negative). Or, the chance that the value was positive or negative was evaluated by: <1%, almost certainly not; 1–5%, very unlikely; 5–25%, unlikely; 25–75%, possible; 75–95%, likely;

95–99% very likely; and >99% almost certain. These results were interpreted using magnitude-based statistics (Hopkins et al. 2009), using Cohen's thresholds (<0.1, trivial; 0.1–0.3, small; 0.3–0.5, moderate; >0.5 large) (Hopkins et al. 2009) and were used as supplementary analysis to the ANOVA.

A type I error rate that was less than or equal to 5% considered statistically significant for all analyses, except for the magnitude-based inferences, yielding a 10% error rate. ANOVA models and *t* tests were computed using SPSS (Version 18.0, PASW, Chicago, IL, USA). Additionally, all results were interpreted using the traditional null hypothesis testing; supplementary clinically based evaluations were provided according to inferential statistics to clarify the precision of estimation from the ANOVA model. Confidence limits were also used to evaluate whether each effect was true or un-true.

Results

Compliance was reviewed from dosing journals and returned product bottles. All participants met the required supplement dosage (3.2 g daily) and 2 participants (8%) in the BA supplementation group reported mild side effects of paresthesia documented in dosing journals and during dosing compliance checks mid-way through and during post-testing of the study. Symptoms were reported as a slight prickling on the back of the hands and face, with only mild sensations. All subjects, despite symptoms, were maintained in the analyses cohort.

Exercise performance

Maximal oxygen consumption [VO_2max (l min^{-1})]

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 ($p = 0.009$) (Fig. 1a). The marginal means (collapsed across treatment) increased from pre- to post-testing (0.80 ± 0.05 l min^{-1}; $p = 0.009$).

Ventilatory threshold [VT (l min^{-1})]

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 (Fig. 1a). There were no significant changes or differences related to the treatments.

VO_2-time-to-exhaustion [VO_2TTE (s)]

There was no two-way interaction (time × treatment, $p = 0.074$), and no main effects for time ($p = 0.962$) or

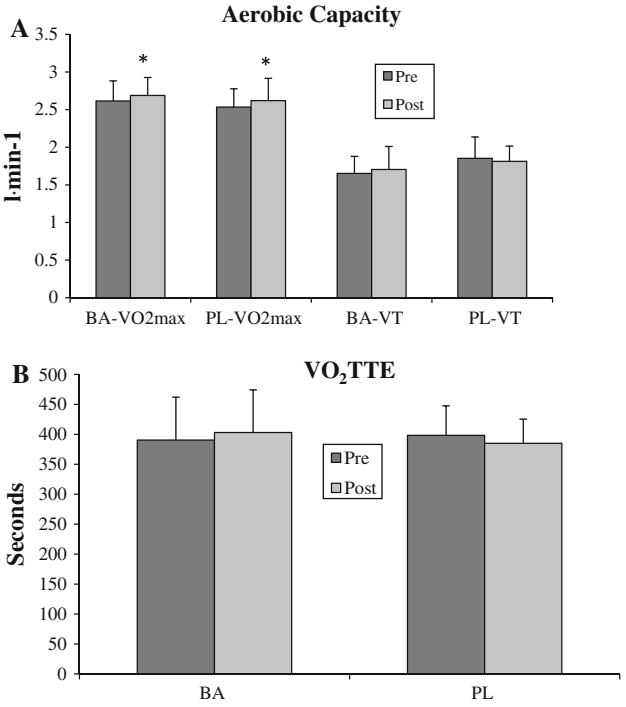

Fig. 1 Marginal means for aerobic capacity for **a** maximal oxygen consumption (VO_2max) and ventilatory threshold (VT) for pre- to post-supplementation, **b** time to exhaustion during the GXT (VO_2TTE). Mean percent change scores from pre- to post-supplementation with 95% confidence intervals for VO_2max, VO_2TTE, VT for for the β-alanine (BA: *black*) and placebo (PL: *shaded*) groups. **a** *Asterisks* indicates a significant increase from pre- to post-supplementation ($p < 0.05$). Values are marginal means ± SEM. **b** *Asterisks* indicates a significant increase from pre- to post-supplementation ($p < 0.05$). Values are marginal means ± SEM

treatment ($p = 0.832$) (Fig. 1b). The marginal means (collapsed across treatment) indicated no significant change over time (−0.33 ± 1.92 s; $p = 0.962$). Evaluation of magnitude inferences indicated a very likely ergogenic effect of BA on VO_2TTE (Table 2).

40-min run

Heart rate [beats per minute (bpm)]

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 (Fig. 2a). The marginal means when collapsing across (chronic and treatment) demonstrated that heart rate values at 10 min were significantly lower than at 20 and 40 min ($p < 0.01$). At 20 min, heart rate values were significantly greater than at 10 min and no different from any other time points.

Table 2 Effect of β-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 (%)
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

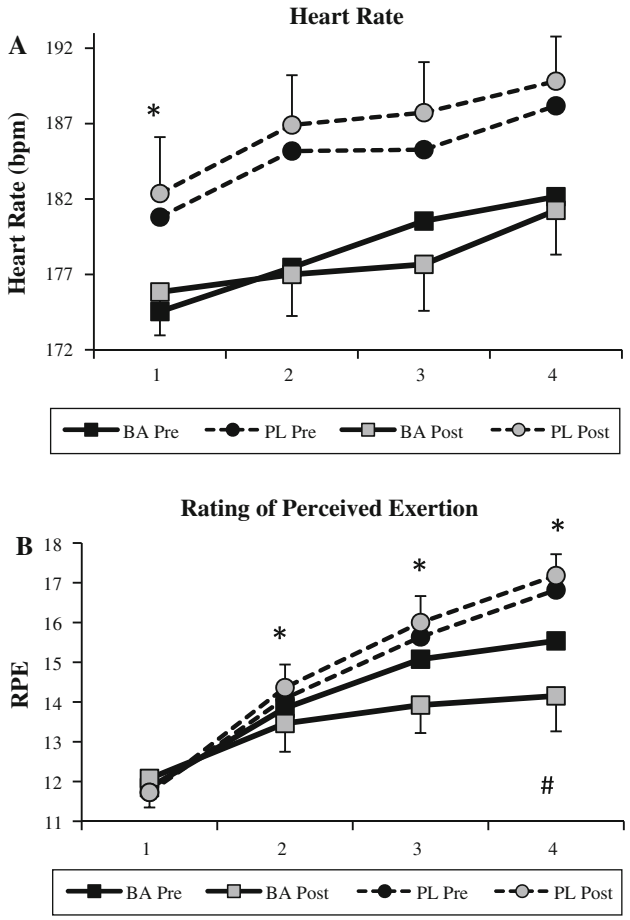

Fig. 2 Marginal means for heart rate (**a**) and ratings of perceived exertion (**b**) taken at time intervals of 10 min (*1*), 20 min (*2*), 30 min (*3*) and 40 min (*4*) during the oxidative stress run. **a** *Asterisks* indicates values at 10 min were significantly lower than HR values at 20 and 40 min ($p < 0.01$). Main effect for time. **b** *Asterisks* indicates RPE values at 20, 30, and 40 min were significantly different from each other, with each time point significantly lower than the subsequent value ($p < 0.05$). *Hash* indicates a significant difference between groups at 40 min ($p < 0.05$). Values are marginal means

Rating of perceived exertion (RPE)

There was a three-way interaction (acute × chronic × treatment, $p = 0.036$) for RPE. 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 min between the PL and BA groups ($p = 0.04$). Magnitude inferences suggest a likely ergogenic effect of BA on ratings of perceived exertion at 30 and 40 min of running (Table 3).

Oxidative stress biomarkers

Total antioxidant capacity

There was no three-way interaction for acute × chronic × treatment ($p = 0.656$) and no two-way interaction resulted for acute × treatment ($p = 0.234$). There was a significant two-way interaction for chronic × treatment ($p = 0.050$), as well as a main effect for acute ($p = 0.001$). There was no main effect for treatment ($p = 0.616$). The marginal means for chronic (collapsed across acute) indicated a significant decrease in TAC from pre- to post-supplementing ($p = 0.031$). The acute marginal means (collapsed across treatment) indicated baseline values were significantly greater than 4-h post-values ($p = 0.036$) (Fig. 3a). There were no other significant acute values. The mean percent change scores, revealed a significant decrease in TAC values for the placebo group only (Fig. 3b). The evaluation of magnitude inferences suggested negligible effects of BA on all TAC values (Table 4).

Superoxide dismutase

There was no three-way interaction for acute × chronic × treatment ($p = 0.907$) and no two-way interactions for acute × chronic ($p = 0.242$), or acute × treatment ($p = 0.513$), but there was an interaction for chronic × treatment ($p = 0.050$). Additionally, there was no main effect for acute ($p = 0.475$), chronic ($p = 0.135$), or treatment ($p = 0.757$). The marginal means for chronic SOD (collapsed across acute and treatment) demonstrated no change from pre- to post-supplement ($p = 0.135$). The

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

BA vs. PL	Mean improvement (%) and 90% CI	Clinical inference	Beneficial/ ergogenic (%)	Negligible/ trivial (%)	Harmful/ ergolytic (%)
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 beneficial	99.0	0.4	0.6

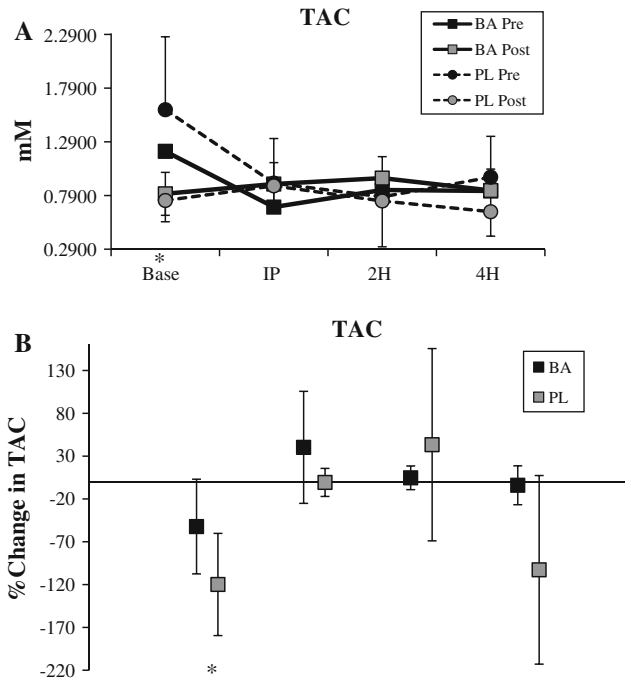

Fig. 3 Marginal means for **a** total antioxidant capacity (*TAC*) from pre- to post-supplementation for β-alanine (*black*) and placebo (*shaded*). Values are marginal means. Mean percent change scores from pre- to post-supplementation with 95% confidence intervals (**b**). **a** *Asterisks* indicates baseline values (collapsed across treatment) were significantly greater than 4 h ($p = 0.036$). Values are marginal means. **b** *Asterisks* indicates the percent change for baseline TAC values were significantly less than zero ($p < 0.05$). Values are means ± 95% confidence intervals

marginal means for acute and treatment SOD values indicated no significant differences ($p = 0.363$–1.0) (Fig. 4a). The mean percent change scores revealed a significant decrease in SOD values for the BA group only at base, 2- and 4-h post (Fig. 4b). The evaluation of magnitude inferences suggested a likely negligible effect of BA on all post SOD values (Table 4).

8-Isoprostane

There was no three-way interaction for acute × chronic × treatment ($p = 0.111$), no two-way interactions for acute × chronic ($p = 0.403$), acute × treatment ($p = 0.429$), or chronic × treatment ($p = 0.309$). There was a main effect for acute ($p = 0.001$) and main effect for chronic ($p = 0.001$), but no main effect for treatment ($p = 0.305$). The marginal means for acute 8-isoprostane values indicated baseline values were significantly lower than IP ($p = 0.001$). Immediate post values were significantly greater than all time points ($p = 0.001$–0.033). Values at 2-h post were significantly lower than IP ($p = 0.001$) and 4-h points ($p = 0.005$). Values at 4-h post were significantly lower than IP ($p = 0.033$) and greater than 2-h post ($p = 0.005$). The marginal means for chronic 8-isoprostane levels indicated a significant decrease over time ($p = 0.001$) (Fig. 5a). The mean percent change scores demonstrated a significant decrease in 8-isoprostane at all time points for the BA group, and at base, 2- and 4-h time points for the PL group (Fig. 5b).

Glutathione

There was no three-way interaction for acute × chronic × treatment ($p = 0.182$) and no two-way interactions for acute × chronic ($p = 0.138$), acute × treatment ($p = 0.534$), or chronic × treatment ($p = 0.657$). There was, however, a main effect for acute ($p = 0.001$) and chronic ($p = 0.001$), but no main effect for treatment ($p = 0.634$). The acute marginal means (collapsed across treatment) indicated baseline values were significantly greater than IP ($p = 0.001$); IP values were significantly lower than all time points ($p < 0.001$); values at 2-h post were significantly greater than IP ($p = 0.001$). The chronic marginal means indicated a significant increase from pre- to post-supplementing ($p = 0.001$) (Fig. 6a). The mean percent change values increased for all time points for the BA

Table 4 Effect of β-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 women

Women BA vs. PL	Mean improvement (%) and 90% CI	Clinical inference	Beneficial/ ergogenic (%)	Negligible/ trivial (%)	Harmful/ ergolytic (%)
TAC					
Base	0.39 ± 7.7	Negligible	37.0	62.4	0.6
IP	0.22 ± 0.29	Negligible	5.3	94.7	0.0
2 h	16 ± 7.7	Negligible	1.7	98.3	0.0
4 h	0.39 ± 0.29	Negligible	26.9	73.1	0.0
SOD					
Base	0.15 ± 7.7	Negligible	0.0	99.1	0.8
IP	0.17 ± 0.3	Negligible	0.0	96.4	3.6
2 h	0.27 ± 7.7	Negligible	0.0	93.4	6.6
4 h	0.17 ± 0.28	Negligible	0.0	97.2	2.8
8-ISO					
Base	0.58 ± 7.7	Possibly beneficial	51.3	15.6	33.1
IP	11 ± 30	Likely beneficial	83.6	6.3	10.1
2 h	3.0 ± 7.7	Likely beneficial	86.9	7.1	6.0
4 h	4.4 ± 4.1	Likely beneficial	94.1	3.2	2.7
GSH					
Base	0.22 ± 7.7	Unclear	49.4	2.3	48.4
IP	52 ± 80	Likely beneficial	86.2	0.4	13.3
2 h	51 ± 7.7	Likely beneficial	87.8	0.4	11.8
4 h	−52.0 ± 82	Likely harmful	14.0	0.4	85.5

group, and increased for the PL group at base and 4-h post (Fig. 6b). The evaluation of magnitude inferences suggested a likely beneficial effect of BA on IP and 2-h values, and a likely harmful effect on 4-h values (Table 4).

Nutrition and exercise status

Nutrition

There was no two-way interaction (time × treatment) for calories ($p = 0.099$), carbohydrates ($p = 0.173$), fat ($p = 0.093$), protein ($p = 0.096$), or protein quality (histidine; $p = 0.097$). Furthermore, there were no significant dietary differences between treatment groups before or after supplementing ($p = 0.136$–0.965).

Exercise status

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 ($p = 0.002$). The marginal means (collapsed across treatment) indicated a significant decrease in time spent exercising ($p = 0.001$) from pre- to post-supplementing (BA: −69%; PL: −56%), with no difference between treatment groups.

Discussion

The results of the present study suggest 28 days of β-alanine supplementation has a slight, non-significant ($p > 0.05$), influence on aerobic performance (TTE, VT), while reducing perceived rates of exertion during a 40-min treadmill run. The oxidative stress markers demonstrated little change following 28 days of β-alanine supplementation. A collective evaluation of the ANOVA and confidence interval statistics, suggest a slight reduction in 8ISO levels, for all time points following β-alanine supplementation.

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 (Abe 2000; Bate-Smith 1938). 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 (Robergs et al. 2004). In support, numerous

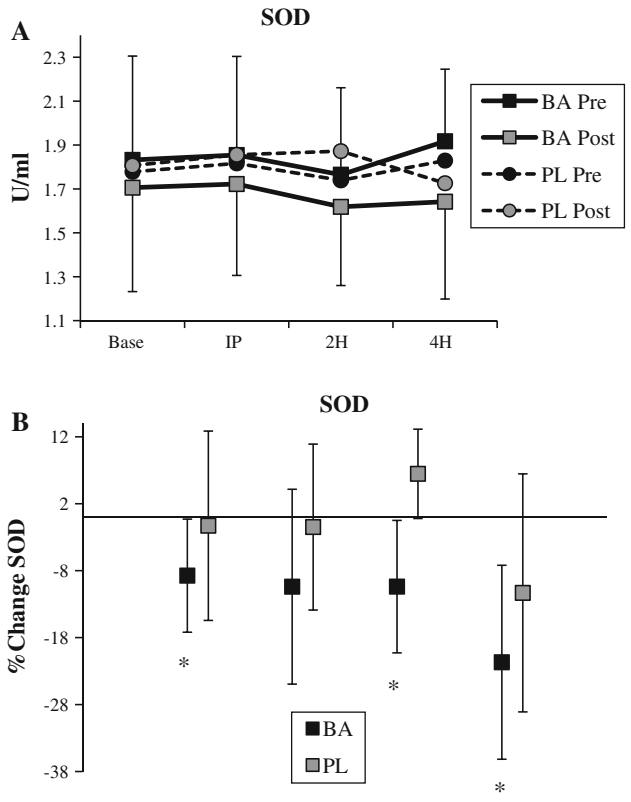

Fig. 4 Marginal means for **a** superoxide dismutase (SOD) from pre- to post-supplementation for β-alanine (*black*) and placebo (*shaded*) groups. Values are marginal means. Mean percent change scores from pre- to post-supplementation with 95% confidence intervals (**b**). **a** There were no significant differences ($p > 0.05$) Values are marginal means. **b** *Asterisks* indicates the percent change for baseline, 2- and 4-h SOD values were significantly less than zero ($p < 0.05$). Values are means ±95% confidence intervals

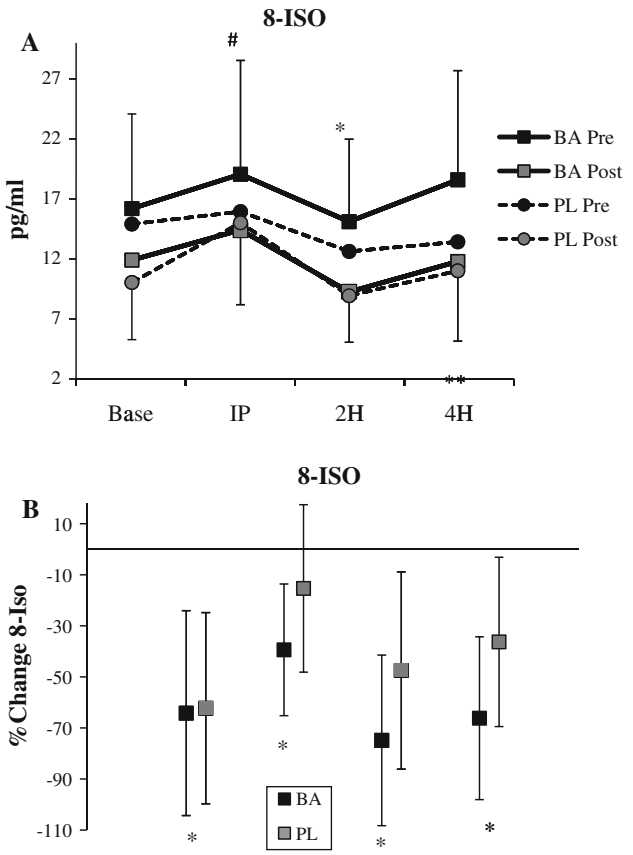

Fig. 5 Marginal means for **a** 8-isoprostanes (8ISO) from pre-to post-supplementation for β-alanine (*black*) and placebo (*shaded*) groups. Values are marginal means. Mean percent change scores from pre-to post-supplementation with 95% confidence intervals (**b**). **a** *Asterisks* indicates a significant lower value for 2 h vs. IP and 4 h ($p < 0.01$). *Hash* indicates a significant greater value than all time points ($p < 0.01$). *Double asterisks* indicates a significant decrease from pre to post for all values ($p = 0.001$). Values are marginal means. **b** *Asterisks* 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

studies have demonstrated the ergogenicity of β-alanine supplementation in anaerobic performance (Derave et al. 2007; Hoffman et al. 2007, 2008; Kendrick et al. 2009; Stout et al. 2007; Van Thienen et al. 2009; Zoeller et al. 2007), with less support in aerobic activities (Derave et al. 2007; Kendrick et al. 2008; Smith et al. 2009; Stout et al. 2007; Zoeller et al. 2007) and only two previous studies evaluating the effects in women (Stout et al. 2007; Walter et al. 2010). The present results suggest a slight antioxidant potential, with a reduction in lipid peroxidation, which falls in line with the functional role related to the imidazole structure and further supports the role as a pH buffer. With a possible direct effect on oxidative stress by the scavenging of free radicals, an indirect effect on cell membrane stabilization may add to the benefits of β-alanine supplementation. While this may not have a large role in performance, there could be benefits relative to recovery and enhanced training volume.

Exercise capacity (VO_2max, TTE, VT, 40-min run)

Evaluation of aerobic performance, with β-alanine supplementation alone, has consistently demonstrated no positive effects on VO_2peak measured during GXTs (Stout et al. 2006, 2007; Zoeller et al. 2007). However, this is the first study to evaluate the effects of supplementation on aerobic treadmill running. Maximal oxygen consumption increased from pre- to post-testing in both β-alanine (+2.67%) and placebo groups (+3.55%), with no significant interaction, supporting previous results, yielding no effects on VO_2max. Time to exhaustion is another common measure that has been evaluated during a GXT, with β-alanine supplementation. Stout et al. (2007) 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

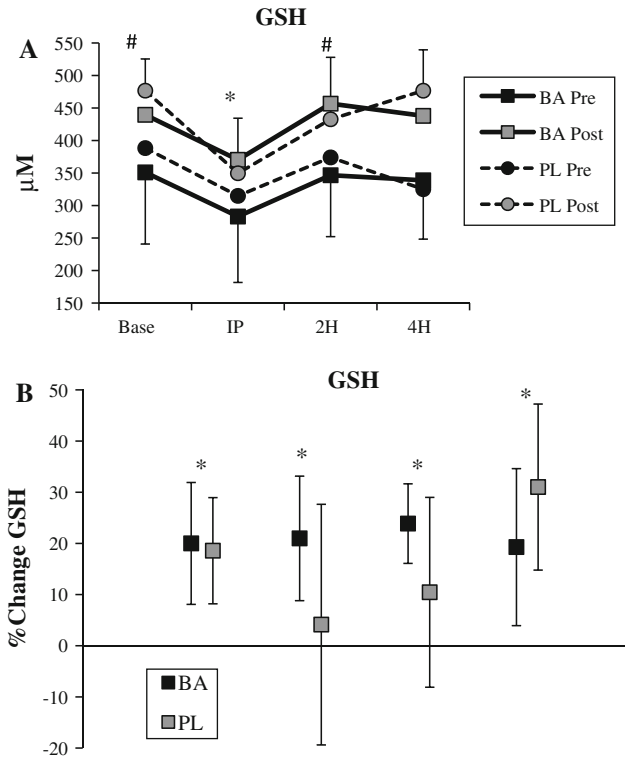

Fig. 6 Marginal means for **a** glutathione (GSH) from pre-to post-supplementation for β-alanine (*black*) and placebo (*shaded*) groups. Values are marginal means. Mean percent change scores from pre-to post-supplementation with 95% confidence intervals (**b**). **a** *Asterisks* indicates a significant lower value between all time points ($p < 0.01$). *Hash* indicates a significant greater value than IP and 4 h values ($p < 0.01$). Values are marginal means. **b** *Asterisks* 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

greater reliance on intracellular buffering observed toward the end of a GXT. In contrast, Zoeller et al. (2007) 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. Although not significant, the magnitude of the increase gives further support for the possible beneficial effects of BA supplementation on TTE (Table 2).

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 VT and TTE in both trained and untrained individuals (Hill et al. 2007; Stout et al. 2007; Zoeller et al. 2007). Delaying CO_2 by-production, due to a reduced reliance on extracellular buffering may increase VT and delay fatigue (Fitts and Holloszy 1976). Ventilatory threshold represents the non-linear point at which ventilation begins to increase disproportionately with VO_2 during a GXT (Orr et al. 1982) and also corresponds to an increase in anaerobic metabolism (McArdle et al. 2006). Previous improvements in VT, following 28 days of β-alanine supplementation, have been reported (Stout et al. 2006, 2007; Zoeller et al. 2007). Following a similar supplementation protocol as the current study, Stout et al. (2007) demonstrated a 13.9% increase in VT in untrained women, while Zoeller et al. (2007) reported a 7% increase in untrained men. The current study also demonstrated a non-significant 3.0% increase for the BA group compared to a −2.2% decrease in VT for the PL group. Differences between the current study and previous studies examining VT may be attributed to the modes of testing, treadmill versus cycling.

To date, the influence of augmented muscle carnosine levels, by way of β-alanine supplementation, on longer duration exercise has yet to be evaluated. β-alanine has been shown to be ergogenic for short, high-intensity work bouts limited by acidosis (Artioli et al. 2010; Derave et al. 2010; Sale et al. 2010), 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 testing (Soller et al. 2008). Soller et al. (2008) reported a significant increase in $[H^+]$ at 50% VO_2max in untrained subjects (VO_2max: 2.39 ± 1.04 l min^{-1}). Therefore, the moderate intensity (70%) of the current exercise protocol would be sufficient to initiate a lactate and H^+ response. Although the duration of the run was pre-determined, HR and RPE were recorded to establish exertion. While not significantly different between groups, HR stratifications can be observed (Fig. 2a). In addition, RPE followed a similar pattern, with only the BA group showing a decreased level of exertion at 30- and 40-min time points. In the current study, VT increased non-significantly for the BA group, while decreasing for the PL group, possibly suggesting an enhanced buffering capacity following supplementation, and also indicative of an improvement in submaximal performance. Furthermore, the significant decrease in perceived exertion in the current study would coincide with similar adaptations and perhaps be indicative of the contrasting VT changes (+ for BA and − for PL).

Exercise-induced oxidative stress

Moderate and exhaustive exercise has previously demonstrated an increased production of ROS, exceeding the

capacity of antioxidant defenses (Lovlin et al. 1987; Alessio 1993; Ji 1999). With an exercise-induced depletion of ATP, high ADP intracellular levels trigger the conversion of xanthine dehydrogenase to xanthine oxidase causing an increase in free radicals (Moller et al. 1996). ROS formation has been shown to be dependent on pH, while lactate has also been shown to scavenge the HO· radical and O_2^- (Selivanov et al. 2008). A single bout of treadmill running and other acute exercise has been shown to increase TAC (Bloomer et al. 2006b; Fatouros et al. 2010; Kerksick et al. 2008; Michailidis et al. 2007) and SOD (Radak et al. 1995), as a result of increased O_2^-, potentially offering greater antioxidant protection (Ji 1999). In contrast to previous findings, the current study resulted in a significant reduction in TAC levels, from baseline, remaining suppressed for up to 4 h. This trend was the same for both treatment groups. The reduction in TAC values in the present study may have been due to 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, and synthesis of urate is blunted during periods of acidosis (Craan et al. 1982) More so, it is suggested that urate be a marker measured in future evaluations of in vivo antioxidant properties of carnosine. Antioxidant capacity (TAC), 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 adaptability to a state of oxidative stress (Kohen et al. 2000). Similar to TAC, the other antioxidant enzyme measured in this study, SOD, yielded no significant changes from pre- to post-supplementation or between groups. Confidence interval values demonstrate an effect of exercise and no influence of supplementation (Fig. 4).

Other antioxidant byproducts have been blunted with higher antioxidant responses including isoprostanes and the glutathione redox status, the most frequently used antioxidant marker of exercise-induced oxidative stress. Isoprostanes materialize from the oxidation of arachadonic acid, and their appearance from strenuous cycling and running is well documented (Fam and Morrow 2003; Vollaard et al. 2005). The present study demonstrated an average 24% increase in IP 8-isoprostane levels following 40 min of running, validating the current protocol resulted in oxidative stress. In addition, chronic values demonstrated a significant increase from pre- to post-supplementation for both groups ($p = 0.001$; Fig. 5). Evaluation of the confidence intervals, combined with the magnitude inferences, seems as if BA supplementation may be somewhat beneficial on reducing lipid peroxidation (Table 4; Fig. 5). Additionally, the exercise protocol in the current study initiated a decrease in GSH values, which is consistent with other exercise protocols (Gopal 1997). The decrease in GSH following intense exercise likely reflects an increased consumption by muscle as well as a lag in hepatic GSH supply pulling thiols from the blood supply (Michailidis et al. 2007). The present in vivo results, interpreted from the ANOVA model demonstrate a significant elevation in chronic GSH values irregardless of treatment ($p = 0.001$; Fig. 6). Although there were no significant treatment effects, the inferential statistics and confidence intervals suggest the BA group had a more prominent response, significantly increasing GSH values at all time points, post-supplementation (Fig. 6). Of note, the PL group also demonstrated significantly elevated base and 4 h values. Due to the changes in both groups, caution of these results is warranted, likely demonstrating a negligible effect on GSH.

Boldyrev et al. (1997) 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. Egorov et al. (1997) 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. (1988) suggests that a drop in pH hinders the scavenging abilities of histidine-related compounds, however, the high-buffering capacity of carnosine allows for a unique antioxidant ability by prolonging physiological pH values (Parkhouse et al. 1985). The hydrophilic structure of carnosine plays a large role in its protective nature within the cytosol. The present study shows some potential of β-alanine, by way of carnosine, to act as antioxidant, most clearly related to a reduction in lipid peroxidation. More in vivo data is needed to support this finding. In vitro, Boldyrev (1987) gave substantial support for the role of carnosine in lipid peroxidation inhibition, protecting biological membranes. Furthermore, Kohen et al. (1988) previously demonstrated a decrease in the oxidation of linoleic acid from carnosine, and Salim-Hanna supported this by reporting a 53% increase in protection against lipid oxidation at a concentration of 10 mM carnosine (Salim-Hanna et al. 1991). The change in 8-isoprostane levels from pre- to post-supplementation suggests a possible decrease in resting lipid peroxidation, as well as minimizing oxidative damage IP strenuous exercise. A reduction in lipid peroxidation has also been linked to a maintenance in the integrity of the excitation–contraction coupling process in vivo (Boldyrev et al. 1987; Severin and Boldyrev 1991; Dupin and Stvolinskii 1986), creating a possible link between carnosine's ability to reduce lipid oxidation and potential improvements in performance, in addition to its muscle buffering capabilities.

Limitations

The majority of literature interprets blood and muscle antioxidant markers in tandem, and it is reasonable to assume there is bidirectional movement across membranes. However, 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 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 reduction 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 (Alessio 1993; Bloomer et al. 2006a). A further limitation is a result of the subject sample. Several studies have used a similar cohort of moderately trained men, with very little research on women; more so, 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, but should be further evaluated. It is important to discuss the change in exercise status from the beginning to the end of the supplementation period. Both groups demonstrated a reduction in time under training from pre- to post-supplementation. Specifically, there was a significant decrease for the BA (−70%) and PL (−57%) groups. 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 a maintenance in VO_2max across the 28-day supplementation period. Maximum oxygen uptake and TTE during a GXT has not been shown to reflect a detraining effect in several studies (Cullinane et al. 1986; Houmard et al. 1992; Madsen et al. 1993) which is supported by the current study. However, HR may be a better indication of detraining (Cullinane et al. 1986). While both BA and PL groups yielded reductions in pre–post training durations only the PL groups revealed a significant increase in HR.

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 (Harris et al. 2006, 2007). Furthermore, the dosing strategy was similar to those used in the studies of Harris et al. (2006, 2007) at 3–6 g per day suggesting that muscle carnosine levels were increased. The present study utilized the time release formula at 4.8 g daily in divided doses (CarnoSyn®, Natural Alternatives Inc, San Marcos, CA, USA) which has been demonstrated to significantly augment muscle carnosine levels by 27–39% in fast- and slow-twitch muscle fibers, respectively (Baguet et al. 2009) under a similar dosing scenario. Although there are limited evaluations of the influence of BA in women, all studies directly measuring muscle carnosine levels have shown an increase in 100% of the subjects. To date, there are no studies strictly evaluating women, but the aging muscle, both fiber types, have demonstrated an increase with supplementation, and therefore yields a strong assumption that the female muscle will also respond.

Summary

In a large number of studies, antioxidant supplementation has not been shown to be directly ergogenic on performance variables. β-alanine on the other hand has substantial support for its role in anaerobic performance, training volume, and body composition. This is the first study to report quantitative evidence for a feeling of reduced effort during exercise, suggesting a potential for use in both aerobic and anaerobic activities (Fig. 2). Future research should further evaluate self-reported perception of reduced efforts during various exercise environments, as well as the influence of BA on maintaining training adaptations during a period of detraining.

The present results demonstrate that carnosine, in vivo, yields minimal effects on antioxidant markers. There was a slight attenuation of lipid peroxidation and thus possible protection of biological membranes. However, there is still much to be elucidated with the chronic attenuation of lipid peroxidation and the role in exercise induced oxidative stress. While it is undisputable that carnosine acts as an effective physiological H^+ buffer, there is much in vitro data suggesting other various roles. This current study is original in its approach to evaluate the antioxidant effects of BA supplementation in women and it is the first in vivo study to evaluate one of these claimed roles. The results suggest a slight antioxidant potential, with a minor reduction in lipid peroxidation. However, this does not oppose the role as a buffer. Instead, it falls in line with the functional role related to the imidazole structure and further supports the role as a pH buffer, indirectly reducing oxidative stress by scavenging free radicals and therefore maintaining stability of the cell membrane. While this may not have a large role in performance, there could be benefits relative to recovery and training volume. More so, these results provide a foundation for future research examining other markers of oxidative stress localized

within skeletal muscle, as well as the chronic effects on exercises limited by acidosis and recovery.

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Conflict of interest The authors declare no conflict of interest.

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