Journal of Equine Veterinary Science 121 (2023) 104200

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Comparison of an Antioxidant Source and Antioxidant Plus BCAA on Athletic Performance and Post Exercise Recovery of Horses

Emily Kent[a], Stephen Coleman[a], Jason Bruemmer[b], Regan R. Casagrande[a], Christine Levihn[a], Grace Romo[a], Kevin Herkelman[c], Tanja Hess[a,*]

[a] Department of Animal Sciences, Colorado State University, Fort Collins CO
[b] USDA APHIS WS, National Wildlife Research Center, Fort Collins, CO
[c] Cooperative Research Farms, Richmond, VA

ARTICLE INFO

Article history:
Received 16 July 2022
Received in revised form 11 November 2022
Accepted 19 December 2022
Available online 25 December 2022

Keywords:
Antioxidants
BCAA
Horse
Muscle energy metabolism
Muscle hypertrophy

ABSTRACT

Antioxidant supplementation decreases postexercise oxidative stress but could also decrease muscle protein synthesis. This study compared the effects of three diets: low antioxidant (control, CON), high antioxidant (AO), and branched-chain amino acid high antioxidant (BCAO) supplementation on postexercise protein synthesis and oxidative stress. We hypothesized that supplementing antioxidants with branched-chain amino acids(BCAA) would reduce oxidative stress without hindering muscle protein synthesis. Eighteen mixed-breed polo horses (11 mares and 7 geldings, with age range between 5 and 18 years, were on CON diet for 30 days (from day -45 until day 0) and then were assigned to one of the treatments after the first lactate threshold test (day 0, LT). LT were also conducted on days 15 and 30 of supplemenation. Oxidative stress was assessed by measuring blood glutathione peroxidase, superoxide dismutase, and malondialdehyde concentrations before 2 and 4 hours after each LT. Muscle biopsies were taken before and 4 hours after each LT and analyzed for gene expression of protein synthesis by RTqPCR. Data were analyzed by ANOVA and compared by least-square means. A reduction in oxidative stress occurred over time ($P < .05$), from day 0 to day 30. An up-regulation in the abundance of muscle protein mRNA transcripts was found for CD36, CPT1, PDK4, MYF5, and MYOG ($P < .05$) after all lactate threshold tests, without a treatment effect. A treatment-by-exercise effect was observed for MYOD1 ($P = .0041$). Transcript abundance was upregulated in AO samples post exercise compared to other treatments. MYF6 exhibited a time-by-treatment effect ($P = .045$), where abundance increased more in AO samples from day 0 to day 15 and 30 compared to other treatments. Transcript abundance for metabolic and myogenic genes was upregulated in post exercise muscle samples with no advantage from supplementation of antioxidants with branched-chain amino acids compared to antioxidants alone.

1. Introduction

Exercise has been shown to cause oxidative stress, which induces cellular apoptosis in the body. An overabundance of reactive oxygen species (ROS) is categorized as oxidative stress reactions that cause damage to healthy cells. These reactions have been associated with permanent damage leading to issues such as aging and cancer in some patients [1]. In exercising horses, oxidative stress may cause muscle damage, increased lipid peroxidation levels [2] and is related to recurrent rhabdomyolysis indicated by increased plasma levels of creatine kinase (CK) and aspartate aminotransferase (AST) [3]. Previous studies demonstrated that oxidative stress can be moderated by antioxidant supplementation [4,5]. In a recent study in Thoroughbred horses [5], supplementation with N-Acetyl Cysteine and Coenzyme Q10 enhanced muscle glutathione concentrations and increased expression of proteins involved in the uptake of glutathione into mitochondria and the NAPDH-associated reduction of oxidized glutathione, showing that these AO supplements stimulated the ROS protective system. However, evidence suggests that antioxidant supplementation with vitamin E and lipoic acid may interfere with ROS mechanisms' beneficial effects, such as mitochondrial biogenesis [6]. In humans the supplementation of vitamin E and C hampered the cellular adaptation to endurance training, by showing no changes mitochondrial

Conflicts of interest: All authors declare that they have no conflicts of interest.
Animal care: The Institutional Animal care and use committee approved the current study (ID: 20-9958A).

* Corresponding author at: Tanja Hess, Department of Animal Sciences, Colorado State University, 735 S Overland Trl, Fort Collins CO 80523-1679.
E-mail address: Tanja.hess@colostate.edu (T. Hess).

https://doi.org/10.1016/j.jevs.2022.104200
0737-0806/

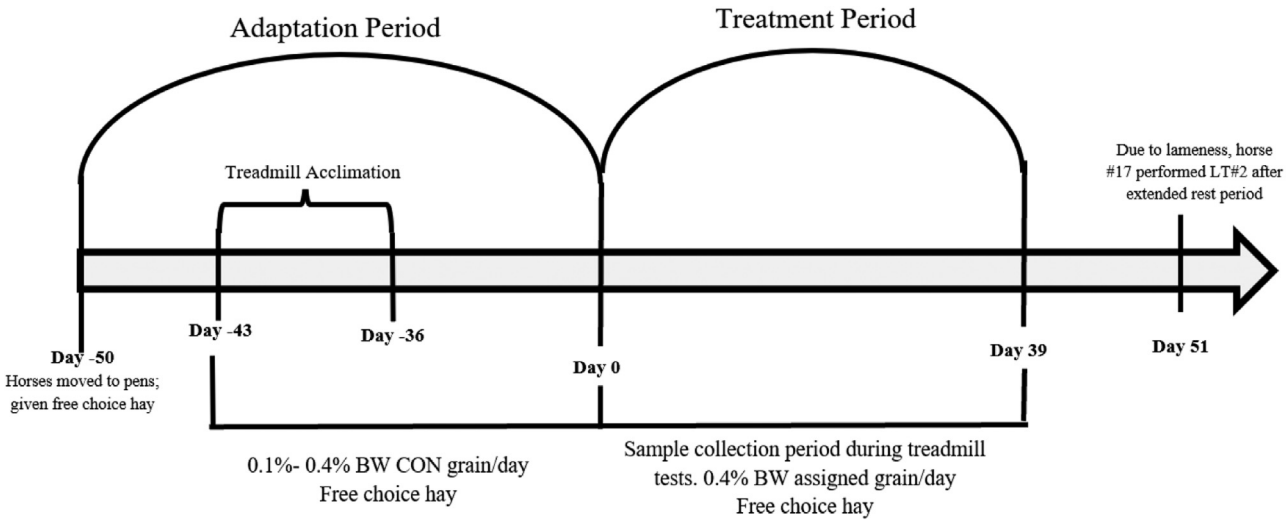

Fig. 1. Timeline of Study beginning July 27, 2020.

markers of cytochrome oxidase, and mRNA levels of a mitogen derived protein kinase compared to placebos [7].

The supplementation of branched-chain amino acids (BCAA) has helped to reduce muscle soreness and fatigue during intense exercise in humans [8]. The supplementation with BCAA to Standardbred in training did not affect metabolites such as lactate and glucose, however it increased α-ketoisocaproic acid, a metabolite of alanine, one of the BCAA supplemented to these horses [9,10]. In recent studies, [10,11] lower leucine and valine levels and higher tryptophan has been found after a show jumping competition, indicating that supplementation would be beneficial to exercising horses. Several findings suggest a direct correlation between BCAA supplementation and an upregulation of the mammalian target of rapamycin (mTOR) pathway responsible for muscle protein synthesis in humans, cell cultures, and horses respectively [12–15]. A recent study showed that mTOR pathways in resting horses are stimulated by increasing levels of crude protein supplementation levels, and that the mTOR pathway responds in a dose-dependent manner up to a plateau, when the enzymatic pathway is saturated [16]. The protein type can also influence in post prandial mTOR activation [17] .The mTOR pathway is responsible for muscle protein synthesis and hypertrophy, and can be stimulated by exercise, presence of cellular glucose and amino acids, and activation of the neuronal nitric oxide synthase (nNOS) enzyme. This enzyme produces free radical nitric oxide, which is upregulated during exercise. Several stepwise reactions identified in mice have shown a direct relationship between nitric oxide production and muscle hypertrophy [18].

The hypothesis for the current study was that horses supplemented with AO and BCAA would inhibit excessive ROS production while stimulating muscle protein synthesis postexercise, thus preventing the possible reduction in muscle growth associated with AO only supplementation. Our objective involved supplementation with AO and BCAA to improve muscle protein synthesis while keeping oxidative stress levels at a minimum (BCOA). We compared this treatment to low antioxidant supplementation (CON) and high antioxidant supplementation alone (AO).

2. Materials and Methods

2.1. Subjects

IACUC approved all procedures used in this study (ID: 20-9958A). Eighteen mixed thoroughbred mares (11) and geldings (7) with an age range of 5–18 years, from the CSU intercollegiate polo team were used for this study. CSU polo horses are all donated retired polo horses with some being used in other equestrian disciplines as well. In a normal year, the intercollegiate polo season lasts 9 months with horses conditioned at varying intensity anywhere between 2 and 5x per week with a polo match once a month. Due to COVID-19, conditions were not normal and the horses were maintained on pasture with minimal conditioning for 3 months prior to the study. The horses were transferred to group pens in July. Horses were fed to meet the requirement for light working horses [19] and adapted to the control research diet by supplementation with grain in feed bags at 0.1% body weight per day for forty three days preceeding the LT (diet composition is described below). This allotment was slowly increased over 2 weeks to 0.4% BW in grain/day, 5 days before the first lactate threshold (LT) test. Horses were conditioned at low intensity for 2–3 weeks to regain their aerobic fitness level following time on pasture before interval training, sprint training and treadmill training for the study as described below.

2.2. Conditioning Program

Horses were conditioned with low-intensity exercise and interval training that continued through the adaptation and treatment periods (Fig. 1). Sessions lasted 30–45 minutes. All horses were ridden by the same four experienced riders, which exercised twice a week at low intensity and once a week at high intensity. The low-intensity exercise involved walk and trot intervals (walk both directions for 7 minutes, trot both directions for 10 minutes, walk both directions). The interval training exercise involved a 7 minutes warmup at a walk and trot, followed by interval training consisting of 3 repetitions of a 3–5 minutes gallop at heart rates between 165 and 190 beats per minute (bpm) alternating with a 3–4 minutes recovery period, where the heart rate returned to 120 bpm, followed by a 10 minute cool down at the walk at the end. Horses were fitted with *Polar (*Polar Electro, NY) heart rate monitors to ensure the appropriate intensity was reached and maintained during the interval training sessions [20]. The principal investigator made sure that all training was done similarly for all horses, being one of the riders. Starting in mid-August, the study horses participated in polo practices with experienced riders twice per week, working on drills and practicing field movements in addition to their weekly low-intensity conditioning. The polo practices involved straight-line sprinting exercises that substituted the

Table 1
Treatment groups based on Age, BCS, and BW.

Treatments			
Item	CON	AO	BCAO
Age, yr	14.6 ± 1.1	13 ± 1.1	12.2 ± 1.1
BCS, 1 to 9	5.94 ± 0.23	5.93 ± 0.23	5.85 ± 0.23
BW, kg	544.9 ± 17.3	525.4 ± 17.3	521 ± 17.3

Table 2
Daily Average Intake of Nutrients from research grain per horse.

Nutrients	CON	AO	BCAO
DE**, Mcal	25.2	24.5	24.3
Crude Fat, kg	0.15	0.15	0.15
Crude Fiber, kg	0.38	0.38	0.38
ADF, kg	0.36	0.36	0.36
NDF, kg	0.70	0.70	0.70
Vit E, IU	840	1580	1580
CP, kg	0.23	0.23	0.23
Amino Acids[b]			
Lys, g	17.1	17.1	17.1
Met, g	5.7	5.7	5.7
Met + Cys, g	9.5	9.5	9.5
Thr, %	11.4	11.4	11.4
Leu g/day*	–	–	16
Ile g/day*	–	–	8
Val g/day*	–	–	10

interval training day. Horses did 4–5 sprints of 1 minute each intercalated with walking.

Between 43 and 36 days before the treatments began, horses were acclimatized to treadmill exercise (EquiGym, KY, USA). When horses were able to gallop at two increasing steps/speeds on the treadmill, they were deemed ready to perform the LT tests and assigned to AO and BCAA supplementation treatment groups (described below) blocked by age, body condition score (BCS) [21], and body weight (BW). Body weight was assessed by weighing horses on an electronic scale (True-test Inc., Mineral Wells,TX) (Table 1). Initial and biweekly assessment of BCS was made by three trained and unbiased independent scorers. BCS was based on a scale of 1–9 [21].

2.3. Feeding Protocol

During the adaptation period (Fig. 1), horses were fed a daily average 10.6 kg of hay (DM) in a feed bunk with enough space for each horse, supplemented with the control treatment diet (trt = CON) two times daily in individual feed bags. The CON treatment was based on a commercial feed (Tribute, Equine Nutrition) containing a small amount of vitamin E. The experimental treatments were both based on the control diet with the addition of AO in the form of vitamin E (dl-tocopherol) (trt = AO), or AO plus BCAA (trt = BCAO), pelleted at the manufacturer. The recommended daily intake for the study horses was based on a moderate workload [19]. Forage and grain intake equaled approximately 2.0 % BW and 0.4% BW on dry matter intake, respectively . The daily estimated intake of grain by horse for each treatment is presented in Table 2. The treatment grains were supplied individually in feed bags divided in two feedings given at 7:30 AM and 4:30 PM, except for LT days, when half the morning allotted grain was given before the LT test and the other half one hour after finishing the LT test. Nutrient intake for grain with treatments was calculated based on the ingested grain (Table 2). The nutrient composition of hay for all diets is presented in Table 3 as a percentage of DM, based on estimated hay consumption. Individual hay intake per day was estimated by subtracting the daily offered hay minus leftovers per pen, divided by the number of horses in the pen. Horses were blocked by age, BW, and BCS and assigned to one of the three treatment groups (n = 6 per treatment). Horses were transitioned to their respective treatments following treadmill session and LT test. Horses remained on their treatment diet for the duration of the study.

Table 3
Nutritional analysis of hay.

	Hay
DM, %	91.9
DE, Mcal/kg	2.15
CP, %	15.6
Estimated Lysine, %	0.54
Lignin, %	5.7
ADF, %	37.3
aNDF, %	51.3
WSC, %	7.6
ESC, %	6.1
Starch, %	0.6
NFC, %	20.2
Crude Fat, %	2.6
Ash, %	10.3
Amino Acids	
Leucine %	0.86
Isoleucine %	0.48
Valine %	0.64

Hay supplied was analyzed for nutritional content by proximate analysis (Equi analyticl.com, Ithaca, NY) and aminoacid content following Association of Official Analytical Chemists guidelines (Cumberland Valley International Services, Rockville, MD). Hay was not analyzed for vitamin E content. The BCAA amounts supplied were based on a previous study [9].

2.4. Lactate Threshold Treadmill Test

For the duration of the treatment period, nine horses were taken to the treadmill each week (three horses per day for 3 days) with a 15-day period in between each LT test (i.e. H#1-9 LT test week 1, H#10-18 LT test week 2, H#1-9 LT test week 3, etc.), when horses followed the same training protocol as described before the LT. All LT tests were done in the morning between 9 AM and 11 AM. On testing day, horses running on the treadmill were given half their morning allotment of grain 3–6 hours before the LT test and the remaining half one hour after (¼ total daily grain allotment given before exercise and ¼ total daily grain allotment given after exercise). The first treadmill session and LT test were used to establish baseline measurements for all horses. On this day, horses received the control diet in before the test and the treatment diet following the LT on day 0. Recently a study demonstrated that a maximal degree of phosphorylation of mTOR proteins occurs at approximately 90 minutes post-feeding [17]. Therefore, we supplemented horses with ¼ of their allotted treatment grain 90 minutes before each LT and ¼ of their morning treatment grain 1 hour after the LT at day 15 and 30 [17,22]. Study horses had access to and consumed hay until they were put in the trailer and transported to the treadmill barn. The horses were trailered for 15 minutes (5 km) and were accustomed to it before the study period.

Blood lactate levels were measured by aseptically placing a 14 g, 13 cm catheter (Mila, International), in the jugular vein with an 50 cm extension set, 30 minutes before the LT tests. The first blood sample was collected to record the baseline lactate levels of each horse at rest. The horse was then led onto the treadmill. The LT test involved a 3-minute walk and a 2-minute trot at 0° incline. The incline was increased to 3°, and the treadmill speed increased until the horse began to gallop, which varied by horse based on the stride length. In the LT test, during the last 10 seconds of each 2-minute interval, 5 mL of blood were drawn after discharging the

left over blood in the extension set and catheter. After the blood collection, the catheter was flushed with saline (0.9%). Lactate was analyzed with a portable lactate analyzer (Lactate Scout, https://www.ekfdiagnostics.com, Elkhart, IN,USA). If the horse had not yet reached their lactate threshold (blood lactate level > 4 mmol/L), the speed of the treadmill was increased in 1m/s increments. This speed was increased every 2 minutes until the horse reached the threshold value or was no longer able to maintain position on the treadmill. Following the test procedure, the treadmill incline was returned to 0° and the speed was reduced to a trot for 5 minutes and a walk for 2-minute for a cooldown period. A blood sample was collected 10-minute post-exercise to determine the recovering lactate values of each horse for comparison to their baseline levels.

2.5. Blood Collection

Pre-exercise blood samples were obtained from the jugular vein through vacutainer needles, by filling two 9 mL heparinized vacutainer tubes (Thermo Fischer Scientific,USA) for each horse participating in the LT test that day, five minutes before muscle biopsies were taken. The heparin blood tubes were placed in ice water at 4°C, taken to the laboratory, and centrifuged for 5 minutes at 2,500 g and 4°C within 30 minutes of sampling. Plasma was separated and aliquoted with half the volume frozen at -20°C until used to measure oxidative stress. An aliquote of plasma was sent to the pathology lab to assess muscle enzymes, resting vitamin E levels, and blood urea nitrogen (BUN). Post-exercise blood samples were collected 2 and 4 hours following the LT test through vacutainer needles into heparinized vacutainer tubes (two 9 mL tubes at 2 hours and 1 tube at 4 hours after LT).

2.6. Muscle Tissue collection

Horses were placed in stocks and the biopsy site was prepared by shaving the hair and scrubbing the site with betadine and alcohol, before local anesthesia of the area through subcutaneous administration of 1mL of 2% lidocaine (VetOne, Boise, ID). Tissue biopsies were collected aseptically, after incision with a scalpel, by inserting a Bergstrom biopsy needle (Popper and Sons, New York, NY, 6 mm external diameter, 9 cm length) midway between the tuber coxae and ischiatic tuberosity to a depth of 6 cm. Muscle tissue was immediately placed in 500 µL of RNAlater (Thermo Fischer Scientific, USA), and processed according to the manufacturer's recommendations to preserve messenger RNA for gene expression analysis. Samples were biopsied from alternating gluteal muscles for each LT test and from the same area in each of the 3 LT tests for the study.

2.7. Blood Plasma Analysis

Pre-exercise blood plasma samples collected at each of the three LT tests were thawed and used to determine oxidative stress-related muscle enzyme activity, resting vitamin E levels, and blood urea nitrogen (BUN). The samples represented days 0, 15, and 30 of the study.

2.7.1. Lipid Peroxidase, Glutathioine Peroxidase, and Superoxide Dismutase

Lipid peroxidation in the collected plasma was calculated using the MDA-TBA concentrations determined by measuring absorbance at 535 nm with a plate reader (SynergyHT, Biotek; Cayman Chemical TBARS Assay kit - Item No. 700870). Using standard fluorometric preparation, three plasma samples per horse were tested for each LT test (pre-exercise, 2 hours post-exercise, 4 hours post-exercise). Glutathione peroxidase (GPX) activity was measured using the Cayman Chemical GPx kit (Item No. 703102) to observe the production of oxidized glutathione (GSSG) from the reduction hydroperoxide into GSSG, water, and alcohol (R-OH). This assay measures the change in concentration of GSSG over time by determining the absorbance at 340 nm for five time points with the plate reader. Superoxide dismutate (SOD) activity was measured in the plasma samples using the Cayman Chemical SOD Assay kit (Item No. 706002). This kit measures the activity of three SOD enzymes. The assay is measured at an absorbance between 440 and 460 nm on the plate reader.

2.7.2. Creatine Kinase, Aspartate Aminotransferase, and Blood Urea Nitrogen

The concentrations of creatine kinase (CK), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were determined by photometric analysis at Clinical Pathology Lab at Colorado State University using a Cobas 501c Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN).

2.7.3. Vitamin E Analysis

Pre-exercise plasma samples were submitted to the Michigan State University Veterinary Diagnostic Laboratory for vitamin E testing. Vitamin E concentrations were determined by gas chromatography after extraction [23].

2.8. Muscle Biopsy Analysis

2.8.1. RNA Isolation

Total RNA was isolated from the muscle biopsy samples using TRIzol [24]. Briefly, once thawed, ~50 mg of the tissue sample was pulverized in 1 mL of TRIzol (Invitrogen, Inc.) using a BeadBug (Benchmark Scientific, Inc.) and incubated at room temperature for 5 minutes. Chloroform (200 µL per 1mL of TRIzol reagent) was added to each sample and mixed by vortexing. The samples were incubated at room temperature for 10 minutes and then centrifuged for 15 minutes at 4°C and 12,000g. The upper aqueous phase was transferred to a new 1.5mL tube. RNA was precipitated by adding 500 µL of isopropanol per sample, vortexing, and incubating on the benchtop for 10 minutes. The precipitated RNA was collected by centrifugation for 8 minutes at 4°C and 12,000 g. The supernatant was removed and the RNA was washed twice with 75% ethanol to remove impurities. Residual ethanol was removed and the pellet air-dried for 3–5 minutes. The RNA was solubilized by incubating the pellet at 55°C–60°C for 15 minutes in 30 µL of nuclease-free water. Samples were treated using TURBO DNase (Invitorgen AM2238) to reduce potential genomic DNA contamination. RNA concentration and purity were determined using a NanoDrop 1C spectrophotometer (Thermo Fisher), labeled appropriately, and stored for the analysis of gene expression.

2.8.2. Reverse Transcription

Total RNA from each sample was reverse transcribed into cDNA using the ImProm-II Reverse Transcription Kit (Promega). One microgram (1,000 ng) of RNA isolated from each sample was combined with 1 µL of primer mix (1:1 ratio of random hexamers and oligo dT) and brought to a total volume of 10 µL with water. This mixture was incubated in a temperature-controlled heat block at 70°C for 5 minutes and then immediately placed on ice to hybridize the primers to the RNA template. Following incubation on ice, 10 µL of RT master mix (4 µL of 5x RT Mix, 2.4 µL $MgCl_2$, 1 µL 10 µM dNTP mix, 1 µL RNasin, 1 µL RT, and 0.6 µL of water) was added to each sample. The reactions were then incubated at 42°C for 60 minutes in a temperature-controlled heat block. Once removed from the heat block, the resulting cDNA was diluted by half (20 µL of water was added to the 20 µL RT reaction) and placed at -20°C for further analysis.

2.8.3. qRT-PCR Protocol

Quantitative reverse-transcriptase PCR (qRT-PCR) was performed to determine transcript abundances for genes associated with metabolic functions in muscle. Targets included myogenin (MYOG), myogenic factor 5 (MYF5), myogenic factor 6 (MYF6), myogenic differentiation 1 (MYOD1), CD36 molecule (CD36), carnitine palmitoyltransferase 1A (CPT1A), hexokinase 2 (HK2), and immunoglobulin heavy diversity 5–5 (IGHD5-5). Briefly, for each target gene, 10 µL of SYBR Green Mastermix (Applied Biosystems No. A46109), 0.6 µL of primer (10 uM each of the forward and reverse primers), 2 µL of template, and 7.4 µL of water were combined in a 96-well PCR plate then aliquoted in triplicate to a 384 well-plate (3 wells x 6µL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included as an endogenous control. A no-template control was also included on each plate. Data was generated using an Applied Biosystems QuantStudio 5 real-time PCR system. Primer set efficiencies were calculated using LinRegPCR version 2020.2.0.1 [25]. Relative quantification (RQ) values were determined using the method described by [26] in order to incorporate primer efficiency and correct for differences between samples. RQ presents a change in abundance based on the endogenous control gene, and a baseline for each horse set as pre-exercise on day 0. A RQ value >1 represents an increase in mRNA expression in the gene of interest compared to GAPDH. An RQ value <1 represents a decreased expression compared to GAPDH.The primer sequences and efficiencies are presented in Table 3. Primers sequences were chosen based on a previous study [27] and modified and designed using the NCBI Primer-Blast design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Maximum product length was limited to 300 base pairs and the forward and reverse primers needed to be separated by an exon-exon junction to reduce the likelihood that genomic DNA contamination would confound the interpretation of the amplification results. Primer specificity for the intended target was confirmed by BLAST analysis against available equine Refseq mRNA sequences.

2.9. Statistical Analysis

Dependent variables were analyzed for normal distribution by the Shapiro-Wilk W test. The effects of day (day 0, day 15, and day 30), exercise (LT: pre-exercise, 2 hours and 4 hours post-exercise), and treatment (trt: CON, AO, and BCAO) were analyzed by ANOVA in a mixed model with SAS (9.2) software (Cary, NC). The statistical model used for the analysis was $\gamma = \mu + trti + dayj + LTk + trti*dayj + trti*LT\ k + dayj*LTk + trti*dayj*LTk + \varepsilon$, with the fixed effect of treatment (trt, I = CON, AO, BCAO), the fixed effect of day (day, j = 0,15, 30), the fixed effect of LT(LT; k = pre-, 2hr post, 4hr post), and all interactions. Dependent variables were relative expression of protein synthesis enzyme activity measurements, oxidative stress data, and vitamin E, BW, BCS, and BUN values. Least-square means analysis was used to determine significance with a threshold of $P \leq .05$. The muscle enzyme activity data are log-transformed due to abnormal distribution. Data are presented as least square means and SE. Data not normally distributed were log-transformed for statistical analysis.

3. Results

3.1. General Horse Health

BW and BCS were recorded every 2 weeks during the treatment period. Average BW and BCS were 530 ± 17.3 kg and 6/9 respectively for horses. No significant treatment, day or exercise effects were found, data not shown.

3.2. Lactate Threshold test

Blood lactate levels were measured every 2 minutes on the treadmill during the exercise test. Baseline and lactate levels at threshold with associated treadmill speed are presented in Table 1 of the the supplementary material**.** Significant increases in lactate levels during the LT were found for all treatment groups ($P = .0025$). Heart rate was also measured during the LT test to ensure the horses' safety and provide evidence of the exercise intensity and recovery after exercise (data not shown).

Average lactate levels at threshold showed a time effect where it decreased from day 0 (6.54 ± 0.29 mmol/L) to day 15 (5.61 ± 0.29 mml/L) remaining stable until day 30 (5.02 ± 0.29 mmol/L) with not treatment effect. The speed required to reach the lactate threshold did not have a treatment or time interaction (Table 4).

3.3. Plasma Antioxidant and Oxidation Product Expression

Superoxide dismutase activity had a day ($P < .0001$) and day*LT effect ($P = .0015$). Average SOD decreased ($P < .001$) from 0.029 ± 0.001 (U/mL) day 0, to0.018 ± 0.001 (U/mL) on day 15, and further decreased ($P < .001$) to 0.014 ± 0.001 (U/mL) day 30 compared to day 0. When analyzing Day*LT as an exercise effect ($P = .005$), there no significant change in SOD activity from pre to 2 and 4 hous post LT at day 0. There was a significant decrease ($P < .0132$) in SOD activity on day 15, from pre LT from 0.0027 ± 0.003 (U/mL) to 0.016 ±0.003 (U/mL) at 2 hours after the LT, and no further change to 4 hours after the LT. At day 30, SOD, did not change significantly after exercise. (Table 5). No treatment effect was detected.

GPx activity had LT effect ($P < .0014$). (Table 5). GPx increased from rest 37.33 ± 3.23 (nmol/min/ml) to 51.53 ± 3.23 (nmol/min/ml) at 2 hours after LT, remaining at 49.84 ± 3.23 (nmol/min/ml) at 4 hours after LT. No treatment effect was observed.

MDA activity demonstrated a day ($P < .0001$) and day*LT ($P < .0001$) effect (Table 6). MDA production decreased ($P < .001$) from 1.97 ± 0.069 (µM) at day 0 to 1.63 ± 0.007 (µM) on day 15, and further decreased to 1.00 ± 0.007 (µM) on day 30. There was a significant increase in MDA levels on day 0 at pre LT from 1.64 ± 0.12 (µM) to 1.95 ± 0.12 (µM) at 2 hours post LT, and a further increase to 2.31 ± 0.12 (µM) at 4 hours post LT. On day 15, there was a significant decrease in MDA from 2.19 ± 0.012 (µM) to 1.55 ± 0.12 (µM) at 2 hours post LT, and then a further decrease to 1.15 ± 0.15 (µM) at 4 hours post LT. No changes in MDA activity were seen after the LT at 30 days of supplementation. No treatment effects were observed.

3.4. Vitamin E Analysis

Pre-exercise Vitamin E levels were not significantly different among treatment groups, and did not have a day effect (Table 7).

3.5. Plasma Muscle Enzymes

CK and AST were measured in IU/L and log-transformed for statistical analysis due to non-normal distribution. lnCK increased significantly ($P < .0001$) from pre- to post-LT. AST demonstrated an exercise (LT) effect as well, with lnAST increasing significantly ($P = .010$) from pre- to post-exercise (supplementary materials Table 2). CK and AST enzyme concentrations are shown in (supplementary materials Table 3) and had no significant treatment effects.

Table 4
Qrt-PCR primers and efficiencies.

Symbol	Gene Name	Forward Primer	Reverse Primer	Eff
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	CATCAAATGGGGGCATGCTG	GGTTCACGCCCATCACAAAC	1.88
MYF6	Myogenic factor 6	GCTCGTGATAACGGCTAAGGAA	CGATGGAAGAAAGGCATCGA	1.67
MYOD1	Myogenic Differentiation 1	TGCCACAACGGACGACTTC	CGGGTCCAGGTCTTCGAA	1.76
MYOG	Myogenin	GCCTCCTGCAGTCCAGAGT	AGTGCAGGTTGTGGGCATCT	1.8
CD36	CD36 molecule	GGCAGTCAACAGCAGCAAAA	CCGCTACAGCCAGATTGAGA	1.81
CPT1A	Carnitine palmitoyl-transferase 1A	GAAGCAGATCTGTCTGTCCG	ACGATGAGCCAACTGGAAGG	1.87
MYF5	Myogenic factor 5	TCAGGAATGCCATCCGCTAC	CCAGACGGGGCTGTTACATT	1.84
HK2	Hexokinase 2	TTGTCCGTAACATTCTCATCGATT	TGTCTTGAGCCGCTCTGAGAT	1.89
PDK4	Pyruvate dehydrogenase kinase 4	TCCACTGCACCAACGCCT	TGGCAAGCCGTAACCAAAA	1.76

Table 5
Effects of AO, BCAA compared to control supplementation on plasma SOD and GPx.

		SOD Activity (U/mL)			GPx Activity (nmol/min/mL)			*P*-Value	SOD	GPx
	Time	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	TRT	0.97	0.31
CON	Pre-LT	0.025	0.022	0.014	33.2	40.6	38.4	DAY	<0.0001	0.38
	2 hr Post-LT	0.025	0.025	0.009	87.3	51.8	49.2	LT	0.54	0.0014
	4 hr Post-LT	0.032	0.001	0.022	57.3	54.1	47.9	DAY*TRT	0.82	0.41
	SEM	0.0056	0.0056	0.0056	9.9	9.9	9.9	DAY*LT	0.0015	0.24
AO	Pre-LT	0.023	0.038	0.013	37.7	49.86	32.71	TRT* LT	0.81	0.41
	2 hrs Post-LT	0.029	0.011	0.014	49.3	46.6	44.4	DAY*TRT* LT	0.25	0.83
	4 hrs Post-LT	0.035	0.011	0.014	42.3	54.1	46.8			
	SEM	0.0047	0.005	0.0047	8.3	8.3	8.3			
BCAO	Pre-LT	0.028	0.021	0.011	30.4	37.5	34.7			
	2 hrs Post-LT	0.030	0.013	0.016	45.2	45.7	44.4			
	4 hrs Post-LT	0.033	0.013	0.018	45.7	53.8	46.5			
	SEM	0.0051	0.0051	0.0056	9	9	9.9			

LT effect: Pre-LT refers to pre-exercise; 2hrs Post-LT refers to 2 hours post-exercise; 4hours Post-LT refers to 4 hours post-exercise.

Table 6
Effect of AO, BCAA supplementation compared to control on plasma MDA.

	MDA Production (μM)						
CON	Time	Day 0	Day 15	Day 30		TRT	0.36
	Pre-LT	1.8	2.3	1.0		DAY	<0.0001
	2 hrs Post-LT	2.5	1.5	0.99		LT	0.27
	4 hrs Post-LT	2.4	1.0	1.0	P-Value	DAY*TRT	0.19
	SEM	0.22	0.22	0.22		DAY*LT	<0.0001
AO	Pre-LT	1.4	2.2	0.99		TRT*LT	0.57
	2 hrs Post-LT	1.7	1.7	1.1		DAY*TRT*LT	0.71
	4 hrs Post-LT	2.3	1.3	1.0			
	SEM	0.19	0.19	0.19			
BCAO	Pre-E	1.7	2.1	1.1			
	2 hrs Post-LT	1.6	1.5	0.95			
	4 hrs Post-LT	2.3	1.1	0.85			
	SEM	0.2	0.2	0.22			

Table 7
Effects of antioxidant and branched-chain amino acid supplementation compared to control on resting plasma Vitamin E.

		Vit E ug/mL	Day 0	Day 15	Day 30	P-value	TRT	0.27
Treatments	CON	3.5	3.6	3.3	3.6		DAY	0.17
	AO	4.1	4.3	3.9	4		TRT*DAY	0.34
	BCAO	3.8	3.7	3.8	3.9			
	SEM	0.24	0.26	0.26	0.26			

3.6. Plasma BUN

BUN values demonstrated an LT ($P = .0017$) and treatment effect ($P = .042$), along with a LT*trt trend ($P = .061$). BUN increased from 18.22 ± 0.63 mg/dL pre LT, to 19.68 ± 0.68 mg/dL after LT, for all horses. Horses on CON, had lower BUN levels 16.56 ± 1.19 mg/dL, compared to BCAO 21.08 ± 1.01 mg/dL; CON and AO (19.20 ± 1.01 mg/dL) BUN levels were similar ($P = .11$), and AO and BCAO did not differ ($P = .22$). BUN showed a day by treatment effect trend ($P = .0611$). At day 0, CON (17.38 ± 1.35 mg/dL) was had similar values to AO (19.99 ± 1.13 mg/dL) and BCAO (20.88 ± 1.25 mg/dL); $P < .058$). At day 15, CON (15.00 ± 1.32 mg/dL) had lower BUN values compared to AO (19.43 ± 1.11 mg/dL) and BCAO (20.88 ± 1.25 mg/dL). Additionally, BCAO and AO BUN levels were similar. At day 30, CON (17.30 ± 1.32 mg/dL) had similar BUN levels, compared with AO (18.19 ± 1.13 mg/dL) BUN levels, however BCAO plasma BUN (21.50 ± 1.30 mg/dL) was higher than CON and AO levels ($P <= .049$) (Table 8).

3.7. Gene Expression in Muscle

Metabolic target genes included CD36, CPT1A, HK2, and PDK4. All targets but HK2 showed an LT effect ($P < .05$) with increase ex-

Table 8
Effects of AO, BCAA supplementation compared to control on plasma BUN Values.

	BUN mg/dL						
	DAY	Day 0	Day 15	Day 30	*P*-Value	TRT	0.042
CON	Pre-LT	17.2	12.6	16.8		DAY	0.2188
	4 hrs Post-LT	17.6	17.4	17.8		LT	0.0017
	SEM	1.54	1.49	1.49		DAY*TRT	0.061
AO	Pre-LT	19.4	18.9	17.7		DAY*LT	0.41
	4 hrs Post-LT	20.6	20	18.7		TRT*LT	0.64
	SEM	1.29	1.26	1.29		DAY*TRT*LT	0.40
BCAO	Pre-LT	20.3	20.4	20.8			
	4 hrs Post-LT	21.5	21.4	22.2			
	SEM	1.4	1.43	1.36			

LT: Pre-LT refers to pre-LT; 2 hrs Post-LT refers to 2 hours post-LT; 4 hours Post-LT refers to 4 hours post-LT.
LT effect.
Trt.

Table 9
Effects of antioxidant and branched-chain amino acid supplementation compared to control on expression of Myogenic and Metabolic Genes.

Target		CON			AO			BCAO		
	DAY	Pre-LT	4 hrs Post-LT	SEM	Pre-LT	4 hrs Post-LT	SEM	Pre-LT	4 hrs Post-LT	SEM
CD36 RQ	Day 0	1.00	2.71	0.70	1.00	2.13	0.59	1.00	2.28	0.64
	Day 15	0.77	1.91	0.70	1.09	2.34	0.59	1.46	1.62	0.68
	Day 30	1.43	3.45	0.70	1.28	2.45	0.59	1.02	1.69	0.64
CPT1 RQ	Day 0	1.00	2.69	0.99	1.00	3.32	0.84	1.00	3.99	0.91
	Day 15	0.71	2.18	0.99	0.66	3.33	0.84	1.85	1.92	0.97
	Day 30	2.22	3.72	0.99	0.94	2.26	0.84	2.05	3.50	0.91
PDK4 RQ	Day 0	1.00	18.56	12.92	1.00	18.37	10.92	1.00	12.60	11.79
	Day 15	0.32	7.0	12.92	0.86	54.30	10.92	2.14	5.01	12.87
	Day 30	15.35	18.38	12.92	1.11	15.08	10.92	1.49	8.99	11.79
MYF5 RQ	Day 0	1.00	3.10	0.78	1.00	1.86	0.62	1.00	2.68	0.67
	Day 15	0.75	2.24	0.74	1.00	3.47	0.63	0.94	1.41	0.74
	Day 30	0.96	3.18	0.74	1.10	1.58	0.62	1.00	1.28	0.67
MYOG RQ	Day 0	1.10	2.85	0.72	1.00	2.38	0.58	1.00	1.54	0.62
	Day 15	0.70	0.90	0.72	0.84	1.83	0.58	0.87	1.12	0.67
	Day 30	2.23	2.01	0.68	2.13	2.39	0.58	0.94	0.70	0.62

P-Value

	TRT	DAY	LT	DAY*TRT	DAY*LT	TRT*LT	DAY* TRT*LT
CD36 RQ	0.86	0.48	<0.0001	0.46	0.64	0.33	0.89
CPT1 RQ	0.87	0.27	<0.0001	0.33	0.43	0.70	0.47
PDK4 RQ	0.51	0.91	0.0066	0.28	0.62	0.18	0.46
MYF5 RQ	0.60	0.80	<0.0001	0.36	0.73	0.38	0.44
MYOG RQ	0.41	0.070	0.037	0.32	0.12	0.51	0.92

LT: Pre-LT refers to pre-exercise, 4 hours Post-LT refers to 4 hours post-exercise.

pression (RQ value) from pre-LT to post-LT. HK2 had no significant treatment, time or exercise effects.

Myogenic target genes included MYOG, MYF5, MYF6, and MYOD1. MYF5 and MYOG demonstrated an LT effect ($P < .0001$ and $P = .037$ respectively). MYF5 had significant increases in relative expression from pre- to post-exercise for all three days. MYOG expression increased from pre- to post-LT on day 0 and day 15 in the CON and BCAO treatment groups, and in the post-samples exercise on days 0, 15, and 30 in the AO treatment.

MYF6 expression had a day*treatment effect ($P = .045$). CON gene expression of MYF6 was 1.00 ± 0.23 at day 0, decreasing ($P = .008$) to 0.41 ± 0.23 at day 15 and remaining stable at 0.58 ± 0.23 at day 30 ($P > .05$). AO gene expression for MYF6 was 1.05 ± 0.19 at day 0, and remained stable ($P = .32$) at 1.24 ± 0.20 at day 15, and increasing ($P = .054$) to 1.25 ± 0.20 at day 30. BCAO gene expression of MYF6 was 0.96 ± 0.21 at day 0, staying at ($P = .21$) to 0.97 ± 0.21 at day 15, decreasing (0.054) to 0.70 ± 0.21 at day 30. All treatments were similar at day 0. At day 15, gene expression of MYF6 was higher in AO ($P = .0085$), compared to CON, but similar to BCAO ($P = .089$). At day 30, CON was similar to BCAO, and AO had a higher gene expression of MYF6 ($P < .054$).

Gene expression of MYOD1 had a Trt*LT effect (Table 9) with a significant increase in expression ($P = .0041$) pre- to post-LT for the AO treatment compared to the BCAO and CON treatments. In the CON group, gene expression of MyoD1 was 0.65 ± 0.57 pre LT, and 0.90 ± 0.59 after the LTs with no significant changes ($P = .65$). In the AO group, gene expression of MYOD1 increased ($P = .003$) from 1.02 ± 0.49 pre-LT to 2.76 ± 0.49, 4 hours after the LT. In the BCAO group, gene expression of MYOD1 remained stable ($P = .23$) from 1.55 ± 0.53 at pre-LT to 1.00 ± 0.53 after the LT (Tables 9 and 10).

Table 10
Effects of antioxidant and branched-chain amino acid supplementation on MYOD1 and MYF6 Gene Expression in Muscle.

Target	MYF6 RQ				MYOD1 RQ					
	DAY	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30			
CON	Pre-LT	1.00	0.45	0.46	1.00	0.39	0.57			
	4 hrs Post-LT	1.01	0.37	0.70	1.92	0.41	0.39			
	SEM	0.28	0.28	0.28	0.82	0.79	0.79	*P*-Value		
AO	Pre-E	1.00	1.05	1.17	1.00	0.73	1.33			
	4 hrs Post-LT	1.10	1.43	1.35	1.80	3.92	2.58			
	SEM	0.24	0.24	0.24	0.66	0.66	0.66			
BCAO	Pre-LT	1.00	0.97	0.84	1.00	2.14	1.51			
	4 hrs Post-LT	0.92	0.96	0.55	1.49	1.05	0.48			
	SEM	0.25	0.27	0.25	0.72	0.77	0.72			

	MYF6 RQ	MYOD1 RQ
TRT	0.15	0.27
DAY	0.35	0.69
LT	0.6	0.10
DAY* TRT	0.045	0.22
DAY* LT	0.93	0.52
TRT*LT	0.31	0.0041
DAY* TRT*LT	0.82	0.17

LT: Pre-LT refers to pre-exercise, 4 hrs Post-LT refers to 4 hours post-LT.

4. Discussion

The main findings for the current study are that higher AO supplementation, stimulated muscle protein synthesis after lactate threshold tests, characterized by an increase in the gene expression of MYF6 a day 30 of treatment, compared to BCAO and CON and an increase in the gene expression of MYOD1 in the AO group after LTs compared to BCAO and CON. Our initial hypothesis that AO supplementation would inhibit muscle adaptation was rejected. Furthermore, the addition of BCAA in the BCAO treatment mitigated the gene expression for the miogenic genes. Higher supply of vitamin E in AO had a positive effects of muscle gene expression of muscle hypertrophy, when comparing to CON and AO.

4.1. Myogenic and Metabolic Gene Expression

To assess potential changes in the regulation of post-exercise muscle growth and cell proliferation, the expression of myogenic target genes MYOG, MYF5, MYF6, and MYOD1 was assessed. MYOG is a muscle-specific transcription factor which initiates myogenesis and suppresses apoptosis [28]. MYOD1 and MYF5 are both involved in the activation of target genes related to muscle cell proliferation [29], both are necessary for skeletal muscle formation, and it is therefore critical that their expression is upregulated post-exercise to support muscle growth and recovery [30]. MYF6 (formerly MRF4 or muscle regulatory factor 4), is responsible for expressing the end phenotype during muscle growth and cell differentiation [31]. MYOG,MYF5, MYF6 and MYOD1 were upregulated in the post-exercise period for all treatment groups, demonstrating that the baseline nutrient values of all diets were sufficient to stimulate muscle cell growth and differentiation after the LTs. Furthermore, exercise was the main driver for the increase in these myogenic factors.

The expression of metabolic gene targets CD36, CPT1A, PDK4, and HK2 was assessed. These genes and associated protein products play a critical role in energy metabolism during exercise. Although HK2 showed no significant changes in expression at 4 hours post-exercise, it is important to note its role in energy metabolism. As a rate-limiting enzyme of glucose metabolism in skeletal muscle, HK2 function is necessary to convert glucose to glucose-6-phosphate [32] and the current exercises were mainly aerobic, since exercise was stopped at the lactate threshold. Stimulation of glycolytic enzymes occur during exercise hypoxia [33] when horses have to exercise above their maximal oxygen consumption. CD36, fatty acid translocase, is involved in fatty acid uptake and regulates its oxidation. This upregulation of CD36 expression increases fatty acid availability for energy production through beta-oxidation and the electron transport chain while also participating in fatty acid storage when in excess [34]. CPT1A, palmitoyltransferase 1, is the rate-limiting step involved in fatty acid oxidation in muscle by transporting long-chain fatty acids into the mitochondria [35]. Lastly, PDK4, pyruvate dehydrogenase 4, is involved in regulating blood glucose levels by monitoring to convert pyruvate to acetyl-CoA in the mitochondria. This key regulator of glycolysis is necessary for energy metabolism, especially during aerobic exercise. This enzyme is inhibited by insulin, which is released after a meal but is upregulated during times of exercise or fasting [36]. The upregulation in both fatty acid metabolism and aerobic glucose metabolism enzymes suggested by the increased expression of CD36, CPT1A, and PDK4 from pre- to post-exercise indicates the impact of the exercise on muscle metabolism. This upregulation was independent of day and treatment, showing a direct correlation between exercise and increased energy demands, followed by muscle adaptations. Monitoring the expression of metabolic and myogenic enzymes could be used to assess the benefits of conditioning on muscle adaptation to exercise.

The hypothesis that branched-chain amino acid supplementation would improve protein synthesis in muscles was not supported. There was no additional benefit from the supplementation of BCAA beyond what was observed with high levels of antioxidants. Hauss performed a study to investigate the supplementation of N-acetylcysteine (NAC), a precursor to glutathione, to investigate its possible detrimental effect on the mTOR pathway post-exercise [37]. Our study and theirs showed that, regardless of antioxidant supplementation, protein muscle synthesis was stimulated.

4.2. Oxidative Stress and Tissue Damage

Oxidative stress levels were not affected by the dosage of the anti-oxidant supplementation in our study. Vitamin E plasma concentrations indicated that the antioxidants levels supplied by all treatments may have been sufficient to mitigate oxidative stress after exercise. We observed a decrease in SOD and MDA activity from day 0 to day 15 and further decrease to day 30, compared to day 0 across all treatments, confirming the antioxidant activity of the supplementation with vitamin E. A decrease in muscle SOD and MDA activity was observed with the supplementation with vitamin E and lipoic acid in trained or untrained rats [38,39]. The supplementation of N-acetyl cysteine and coenzyme 10 did not change the muscle activity of SOD in fit Thoroughbred horses submitted to submaximal exercise [5]. In the current study, the decrease in SOD and MDA was likely attributed to reduced oxidative stress after aerobic exercise tests due to the administration of the vitamin E but also due to some conditioning since the lactate levels at threshold decreased from day 0 to day 15 [40].

Glutathione peroxidase (GPx) reduces hydrogen peroxide and produces NADP+ used in other energy metabolism reactions [41]. GPx acts as a protective enzyme against oxidative damage and removes reactive oxygen species (ROS) formed during exercise. GPx increased with exercise in our study; however, this increase was independent of the amount of vitamin E supplementation in our

treatments. A human study involving aerobic, anaerobic, and combined training found increased plasma GPx concentrations independent of exercise type [42]. In horses participating in endurance races, MDA activity was increased [43,44].

Protein degradation is balanced with protein synthesis at rest, allowing for constant protein turnover. During exercise, protein degradation increases in the liver and contractile muscles and is often associated with the break-down and mobilization of other cellular components during exercise [45]. The addition of BCAA in the BCAO treatment mitigated the protein expression of MYF6 and MYOD1 responsible for muscle protein synthesis. BUN was highest in horses receiving the BCAO at day 30. A possible reason for that mitogation is that the BCAA in the treatment unbalanced the ideal aminoacid profile of the rations, and the excess protein was eliminated. Leucine is a potent stimulator of the m-TOR pathway [46], this pathway however is saturable [16], and extra leucine will not lead to extra simulation of the protein synthesis, and instead could be metabolized and eliminated. Furthermore ideal stimulation of protein synthesis in resting horses is between 0.25 and 0.5 mg /kg BW. The rations supplied in the current study in AO and CON averaged 1,836 g of CP. The BCAO ration supplied 1,870 g of CP, including the 34 g of BCAA. The BCAA added in that treatment could have unbalanced the ideal protein profile of the rations, however more studies are necessary to confirm this concept.

5. Conclusion

Our objective was to determine if supplementing horses with antioxidants and branched-chain amino acids might help to improve protein synthesis in muscle while minimizing oxidative stress levels. Our hypothesis that branched-chain amino acids improve muscle protein synthesis was not supported in this study. There was no benefit associated with the addition of branched-chain amino acids to antioxidant supplementation compared to antioxidant supplementation alone. Overall, our results failed to reject the null hypothesis of the experiment. Branched-chain amino acid supplementation did not play a major role in upregulating gene expression in skeletal muscle tissues following exercisein the current study. Additionally, antioxidant supplementation did not interfere with metabolic and myogenic gene expression in muscle tissue following exercise as previously suggested. This is the first study in horses to characterize muscle protein synthesis by analyzing the gene expression of specific targets related to muscle hypertrophy and energy metabolism. Future studies should characterize the muscle synthesis by measuring the specific proteins stimulated by the gene expressions analyzed.

Acknowledgment

The authors thank the Colorado State University Equine Sciences Polo Club for the use of the polo horses and the students involved riding the horses and collecting of data. The authors thank the Equine Orthopedic Center crew, mainly Jennifer Damiels and Ryan Shelton, for the acclimatization of horses to the treadmill, and helping running the LT on the treadmill.

Financial disclosure

The present study was partially funded by Cooperative Research Farms, 1919 Huguenot RoaddSuite 200, Richmond, Virginia 23235-4321.

The student was funded by the Y-cross fellowship.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jevs.2022.104200.

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