THESIS

COMPARISON OF AN ANTIOXIDANT SOURCE AND ANTIOXIDANT PLUS BCAA ON ATHLETIC PERFORMANCE AND POST EXERCISE RECOVERY OF HORSES

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ABSTRACT

COMPARISON OF AN ANTIOXIDANT SOURCE AND ANTIOXIDANT PLUS BCAA ON ATHLETIC PERFORMANCE AND POST EXERCISE RECOVERY OF HORSES

Antioxidant supplementation has been shown to decrease post-exercise oxidative stress but can lead to decreased post-exercise muscle protein synthesis. The objective of this study was to compare the effects of the supplementation with a control feed with low antioxidant content (CON) to a high antioxidant feed (AO), versus a high antioxidant and branched-chain amino acid feed (BCAO) on post-exercise protein synthesis and oxidative stress. Our hypothesis is that supplementing AO with BCAA will reduce oxidative stress without hindering muscle protein synthesis. Eighteen mixed breed conditioned polo horses were assigned to one of the three treatments. All horses consumed the CON diet for 30 days and were then assessed using a lactate threshold test (LT). One hour later and following subsequential LT, horses were assigned to the experimental groups and given their treatments. Follow-up LTs were conducted on days 15 and 30 of supplementation. Blood was collected before, two and four hours after LT, and oxidative stress was assessed by determining glutathione peroxidase, superoxide dismutase and malondialdehyde concentrations by ELISA. Muscle biopsies were taken before and 4 hours after LT and analyzed for the expression of protein synthesis by RT-PCR. Results were analyzed in a mixed model by ANOVA and compared by LSM. A reduction of oxidative stress was found over time ($P<0.050$) with no treatment effect ($P>0.050$) when using the measured oxidative stress parameters mentioned above. An upregulation in the production of mRNA transcripts related to muscle protein synthesis after exercise was found for muscle primers CD36, CPT1, PDK4, MyF5, and Myogenin ($P<0.050$). There was a treatment by exercise effect for MyoD

(P=0.0041), where AO was upregulated the most after exercise compared to BCAO and CON. MRF4 had a time by treatment effect (p=0.045) where AO was upregulated from day 0 to day 15 and 30 compared to BCAO and CON. This study demonstrated post-exercise muscle synthesis with no advantage of AO plus BCAA compared to AO.

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INTRODUCTION

Oxidative stress is a general term encompassing the degradation or loss of function of important molecules, and the production of reactive oxygen species (Ching Kuang Chow, 1991). Oxidative stress can vary based on the amount of antioxidants present or the accumulation of reactive oxygen species found in the body (Williams, 2016). Although oxidative stress can lead to permanent damage and is a prominent cause of health issues such as aging and cancer, reactive oxygen species formed are beneficial at minimal levels (Williams, 2016). Understanding how oxidative stress affects muscle protein synthesis is the first step in analyzing the role antioxidants play in endurance, muscle growth and enzyme function. It was once thought that any level of reactive oxygen species should be eliminated from the body to prevent health issues. Still, over the years we have found that reactive oxygen species have many benefits including protection against invading bacteria, regulation of cellular pathways (Williams, 2016) and muscle training to increase performance as seen in rats (Reid et al., 1993).

There has been conflicting research on this topic; some would argue that antioxidant presence in rats does not affect muscle fatigue (Coombes et al., 2001). Others claim that antioxidant supplementation in mice would benefit muscle training and endurance (Novelli et al., 1990). Would the benefits of antioxidant supplementation come at a cost? Would muscle protein synthesis decrease due to a reduction in availability of branch-chain amino acids or would there be no significant effect of muscle growth with antioxidant supplementation?

To shed more light on these questions, we proposed an experiment that compared antioxidant supplementation to antioxidant and branched-chain amino acid supplementation. We hoped that the amino acids could be used for muscle growth and antioxidants would allow for an increase in performance without hindering the positive effects from ROS found in the body. We

observed both muscle protein synthesis and antioxidant status post-exercise to understand about the function of reactive oxygen species in the body. Currently, the science remains inconclusive, with results varying between numerous studies. There are beneficial effects of acute administration of antioxidants, but there are differing opinions on the effects of chronic administration based on a review from 2015 (Gomez-Cabrera et al., 2015)**.** With this uncertainty, we hoped that our research would provide more insight into the effects of chronic administration of antioxidant and branched-chain amino acids.

Although there are vast amounts of research on antioxidants and their relation to oxidative stress in exercise, there is a lack of research on this topic in the equine industry. Several studies have looked at each aspect of this topic separately, but none have evaluated all components in one design experiment: oxidative stress, amino acid availability, antioxidant influence, muscle protein synthesis and exercise performance. I will present research from different angles, including human and animal studies. Through analysis of the current research, an illustration of where we stand on these ideas in research and what more is needed to make informed conclusions will become evident.

CHAPTER I: LITERATURE REVIEW

1. Muscle Metabolism

To understand how muscle protein synthesis is affected by antioxidants, we must first understand muscle metabolism and how it functions during exercise. We must also investigate oxidative stress and how we can appropriately avoid it during training.

Exercise intensity and duration are determined by the amount of ATP available in the body. ATP is regenerated by three main pathways: phosphagen system (anaerobic), glycolytic system (anaerobic) and mitochondrial respiration (aerobic) (Baker et al., 2010). These pathways are influenced by diet, environment, and training regimen. ATP stores in muscle are relatively small and can only provide energy for several seconds (Hargreaves and Spriet, 2020). The aerobic and anaerobic mechanisms mentioned above are necessary for harvesting more energy during exercise. There is a constant ebb and flow of ATP into ADP, AMP and Pi allowing for a release of free energy used for muscle contraction (Baker et al., 2010).

These three pathways are used to meet ATP demands in the body during intense exercise. The phosphagen system consists of three reactions involving creatine kinase, adenylate kinase and AMP deaminase (Baker et al., 2010). The creatine kinase reaction produces ATP and consumes a large amount of H^+, usually at the beginning of exercise, often burning through energy stored in phosphocreatine within the first 10 seconds (Bogdanis et al., 1996). This can cause a slight alkalization within the muscles but doesn't seem to cause problems with muscle metabolism during exercise or recovery. The production of ATP from phosphocreatine has the fastest turnover rate, does not require oxygen and dominates as the energy source for short, intense muscle contractions (5 seconds in duration) (Greenhaff and Timmons, 1998). Also,

during exercise when the phosphagen system is activated, acid accumulation in the blood causes metabolic acidosis, activating AMP deaminase to convert AMP and excess H^+ into IMP and NH_4^+. This conversion allows for high phosphate transfer potential in the muscle during exercise and the production and elimination of ammonia (Scrutton, 1979). Both amino acid oxidation and the phosphagen system produce toxic ammonia which is removed from the body by the urea cycle. The production of AMP from adenylate kinase in the phosphagen system is a prominent influencer on the rate of ATP production within the glycolytic system. AMP activates phosphorylase and phosphofructokinase, both of which increase the flow of glucose-6-phosphate through glycolysis (Baker et al., 2010). If exercise continues past the initial few seconds of the energy burst provided by the phosphagen system, blood glucose and glycogen stores are used to produce ATP through an 8-step reaction called glycolysis (Baker et al., 2010). Although the max net gain of ATP is not immediate in the glycolytic system, a human study in 1988 has suggested an immediate activation of this system during exercise that can eventually produce double the ATP that PCr can produce in a 30 second period (Serresse et al., 1988). Both systems are anaerobic, with glycolysis producing lactic acid as a byproduct. Over the years there has been controversy when discussing the benefits and detriments of lactic acid production. Still, recently there has been strong support for its importance in the production of NAD^+ needed for glycolysis and in the retardation of metabolic acidosis due to metabolic proton buffering from the lactate dehydrogenase reaction (Baker et al., 2010).

The last pathway used to produce ATP is mitochondrial respiration which consumes different fuels in the presence of oxygen to produce energy (Baker et al., 2010). Joe Pagan details the amount of energy fuel in grams found in a horse based on location and type. Energy storage is found most in triacylglycerols stored in adipose tissue, weighing 40,000 grams in a

450kg horse. Glycogen stores found in the muscle and liver seem small in comparison ranging from 3150-4095g and 90-220g respectively (Pagan, 1998). Storage use depends on muscle fiber type and exercise intensity/duration. In 1984, David Barlow analyzed different muscle fiber types in thoroughbred yearlings. He took muscle biopsies and found type I (slow oxidative) and II (glycolytic) muscle fiber types were easily distinguishable, finding type II to predominate in horses used for sprinting events (Barlow et al., 1984) and type I to be found in endurance horses. Type II muscle fibers can be broken down further into IIA (fast oxidative glycolytic) and IIX (fast glycolytic) depending on the source of fuel (Rivero, 2007). Since these muscle fibers are easily distinguishable and found in varying amounts depending on the performance specialty of the horse, it would make sense that each type utilizes a different energy source. As a horse increases its exercise intensity or duration, the muscle fiber type used changes from type I to type IIA and then IIX. Type IIX muscle fibers are usually stimulated during maximal exercise (sprint or prolonged exercise), requiring quick energy sources and ample energy supply (Rivero, 2007).

Type I fibers can utilize triacylglycerols found in adipose tissue. Aerobic metabolism is slower and long endurance races give the horses enough time to use this storage type. However, as a horse speeds up and goes from a walk to a canter or gallop, type II fibers are utilized and are fueled by glycogen and fat metabolism. Once the speed increases enough that the type I muscle fibers don't have ample time to provide energy, type IIA fibers become the primary source of movement. Eventually, the ATP demand is too great to be adequately supplied by aerobic pathways. Type IIX fibers are then activated and utilize anaerobic metabolism from glycogen, the fastest energy production source (Rivero, 2007). In a human study performed in 1994, type II fibers demonstrated higher activities of the phosphagen and glycolytic systems and thrived in anaerobic environments (Greenhaff et al., 1994). Studies in mice and humans showed these type

II fibers often produce higher power output and increase ATP turnover (Crow and Kushmerick, 1982; Faulkner et al., 1986). However, this quick fuel comes at a detriment because lactic acid accumulation occurs through the conversion of glucose to lactic acid in the absence of oxygen (Pagan, 1998). This lactic acid accumulation is beneficial based on Baker's research but can also lead to muscle fatigue. Based on Greenhaff's study, sprinting exercise in humans resulted in a significant decrease in plasma pH due to increased blood lactate. This decline was the most significant at the end of the 30-second treadmill sprint (Greenhaff et al., 1994).

2. Amino Acid Metabolism

Amino acids contain acid and amine groups in a specific formation, each indicative of a particular function. 20 of some 300 amino acids in nature have the ability to build protein. Essentially, amino acid metabolism involves the breaking down the amino acid components, carboxylic acid and the amine group. The carboxylic group gets converted to acetyl-CoA which can be oxidized into carbon dioxide and water using the Kreb's cycle, a component of aerobic metabolism, providing a minimal amount of ATP (Wu, 2009). Depending on the specific amino acid, each can enter the Krebs cycle at different points, providing varying levels of ATP production. More specifically, branched-chain amino acids include leucine, isoleucine and valine. Leucine is converted to acetoacetyl-CoA and enters the beginning of the Kreb's cycle while isoleucine and valine are converted to succinyl-CoA and enter at the end of the cycle.

During exercise, protein turnover is ongoing. Muscle protein breakdown will still occur in the presence of branched-chain amino acids to provide the body with the essential amino acids needed for muscle protein synthesis. According to a review done in 2017, only two studies involving human subjects tested intravenous branched-chain amino acids and their effects. In

both studies, muscle protein synthesis decreased, leading Wolfe to conclude that consuming branched-chain amino acid supplements is unnecessary. However, some research in his review article found that branched-chain amino acid supplementation increases the recycling efficiency of essential amino acids to use for muscle protein synthesis by 50% (Wolfe, 2017). Some human studies have shown other branched-chain amino acid supplementation benefits. Matsumoto and colleagues found that supplementation reduced muscle soreness and fatigue during exercise (Matsumoto et al., 2009). Kumar found that supplementation was directly correlated with upregulation of the mammalian target of rapamycin which is responsible for muscle protein synthesis (Kumar et al., 2009)**.**

3. Oxidative Stress

An increase in lactic acid concentrations and fatigue s correlated with an increase in reactive oxygen species and oxidative stress. Reactive oxygen species are free radicals derived from oxygen and include non-radicals such as reactive nitrogen species (Halliwell and Gutteridge, 2015)**.** The most common free radicals that form during exercise include superoxide and nitrogen monoxide, both easily converted to dangerous compounds such as hydrogen peroxide**.** However, these compounds can combine to form peroxynitrite which can be 3x more efficient than an antioxidant's scavenging ability such as superoxide dismutase (Halliwell and Gutteridge, 1981). It's essential to keep a balance of both nitric oxide and superoxide in cells so that removal of both species is easily attained without extra help from antioxidants.

Sources of these radicals directly related to exercise were first explored in the late 1970s in both humans and rats (Brady et al., 1979; Dillard et al., 1978) and then again in 1982 by Davies et al. when testing rats who were fed either a control or a vitamin E deficient feed

(Davies et al., 1982). He demonstrated that exercise promotes the production of reactive oxygen species, specifically in skeletal muscle and the liver. Others have found that exercise increases lipid, protein and DNA oxidation. In the illustration below (*Figure 1)* presented by Powers et al., there are several sites within human skeletal muscle where reactive oxygen species production is prominent during contraction (Powers et al., 2011).

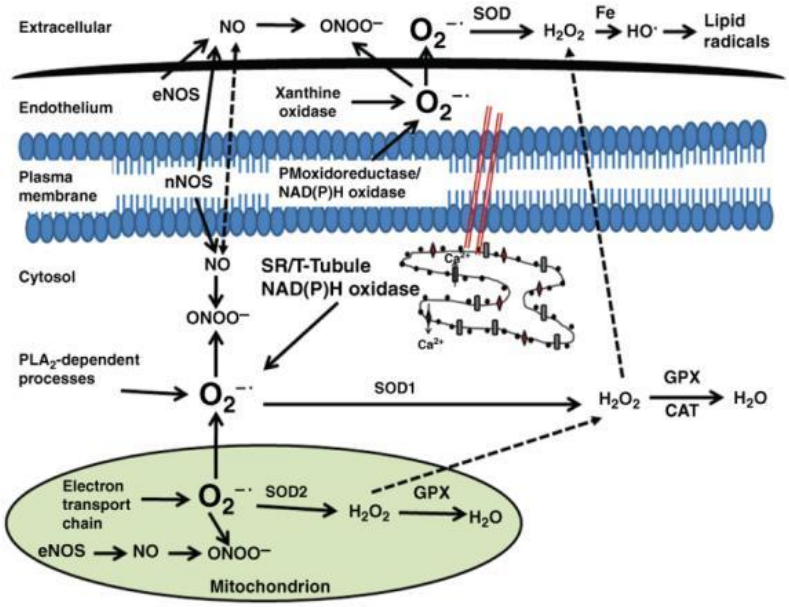


Figure 1: Potential sites for superoxide and nitric oxide (NO) production in human skeletal muscle. Key to abbreviations: CAT = catalase; SOD1 = superoxide dismutase 1; SOD2 = superoxide dismutase 2; GPX = glutathione peroxidase. Original Source: Powers et al., 2011

3.1 Sources of Reactive Oxygen Species Generation in Skeletal Muscle

Reactive oxygen species generation in various mammalian and bird species is thought to predominantly occur in the mitochondria of muscle fibers with superoxide production found in complexes I and III of the electron transport chain (Barja, 1999). This electron leakage producing significant amounts of reactive oxygen species is thought to be caused by the iron-sulfur clusters

in complex I and the Qo semiquinone of complex III (Muller et al., 2004). However, more recently (Jackson et al., 2007), findings suggest that mitochondria may not be the sole source of reactive oxygen species production during exercise. Mitochondrial oxygen used to from superoxide during exercise was once thought to be 2-5% but is now approximately 0.15% based on St-Pierre's findings in rats (St-Pierre et al., 2002). Other sources of ROS generation currently being investigated include the sarcoplasmic reticulum and plasma membrane. Studies have found a correlation between NADH oxidase enzymes and the sarcoplasmic reticulum. These enzymes found within the membrane of the sarcoplasmic reticulum have been linked to superoxide production in cardiomyocytes (Griendling et al., 2000). However, Xia et al. found that NADH oxidase in rabbits doesn't contribute towards extracellular reactive oxygen species concentrations because of its inability to leave the sarcoplasmic reticulum (Xia et al., 2003). Plasma membrane redox systems also play a crucial role in extracellular reactive oxygen species. These redox systems allow electrons to cross through the plasma membrane and release superoxide into the extracellular space. For example, the NADH-dependent oxidoreductase system found within the plasma membrane of humans can be easily influenced by an increase in NADH levels from exercise (Sahlin, 1985). Further research is needed in determining the many sources of reactive oxygen species, but those mentioned are important involving skeletal muscle during exercise.

A study done in 2006 by Anderson and Neufer looked at rat mitochondrial dysfunction due to atrophy of type II muscle fibers. Through a novel approach for measuring hydrogen peroxide production, they found that the ratio of hydrogen peroxide produced/oxygen consumed is 2-3x greater in type II muscle fibers compared to type I. They also found that the scavenging capability of type II muscle fibers is lower than type I (Jackson et al., 2007), possibly explaining

a mechanism for mitochondrial dysfunction due to increased reactive oxygen species concentration in those fibers (Anderson and Neufer, 2006)**.** Horses with a measurable decrease in antioxidants within the plasma indicate an oxidative stress situation. The more oxidative stress placed upon tissue, the more antioxidants are used to combat the radical nature found in the body (Powers and Jackson, 2008)**.** Although it's not a fool-proof method for measuring oxidative stress, it could help explain exercise influences and is a good source for analyzing data.

Another source of reactive oxygen species production includes reactions that occur post-exercise. Muscle damage typically causes a release of macrophages and phagocytic cells that invade the damaged tissue. Through muscle regeneration, reactive oxygen species are produced through respiratory bursts by phagocytic cells which can cause damage to other muscle cells and cause injury as seen in a mouse study (Zerba et al., 1990). Overall, exercise (muscle contraction) is the main driving force for increased reactive oxygen species production and has many areas of research ahead including the location of reactive oxygen species release, the amount produced based on exercise and the type of muscle fiber influencing production.

3.2 Measuring Oxidative Stress

Reactive oxygen species have beneficial effects involving cellular signaling, but they can also lead to irreversible oxidative damage in tissues found in essential organs such as the liver, kidneys and heart (Halliwell and Gutteridge, 2015; Morrow et al., 1992)**.** A buildup of reactive oxygen species causes oxidative stress due to an inhibition of cellular signaling and the occurrence of molecular damage (Sies and Cadenas, 1985)**.** Oxidative stress has been shown to cause different forms of apoptosis such as lipid degradation and an imbalance of the intracellular homeostatic nature of calcium (Clutton, 1997). This oxidative stress can be measured in several

ways, including measurements of oxidants in the body, antioxidants in the body, oxidation products, and measurements of a redox balance (Powers and Jackson, 2008). There are techniques to measure antioxidants such as superoxide dismutase and glutathione peroxidase to determine oxidative stress in the body. Levels of malondialdehyde are also measured to determine oxidative stress.

Malondialdehyde is a product of lipid peroxidation that is measured using a TBARS assay kit through a colorimetric or fluorometric detection method. In the fluorometric detection protocol, malondialdehyde reacts with thiobarbituric acid to produce a malondialdehyde-thiobarbituric acid product that turns pink/red in color that can be detected between 530-540nm. However, there are some concerns when using malondialdehyde to measure for oxidative stress in the body. These include low stability of malondialdehyde (Khoubnasabjafari et al., 2015; Shin et al., 1972) and measuring malondialdehyde produced from reactions other than lipid peroxidation (Janero, 1990). For these reasons and others, such as the specificity of TBARS towards other molecules explained in the TBARS Assay Kit from Cayman Chemical, malondialdehyde activity should not be the only data taken during a study to measure oxidative stress. With that being said, Armstrong and Browne state that the TBARS kit measuring lipid peroxidation is the most widely used assay (Armstrong and Browne, 1994). Based on this cautionary advice, malondialdehyde data should be paired with other measurements of oxidative stress such as superoxide dismutase and glutathione peroxidase which is very common in human exercise studies (Ammar et al., 2020; Azizbeigi et al., 2014). An analysis performed in 2005 by Bloomer and colleagues found that malondialdehyde had no significant interaction or effects when comparing aerobic and anaerobic exercise on a group of men. They looked at plasma malondialdehyde pre-exercise, immediate, 1 hour, 6 hours and 24 hours post-exercise (Bloomer

et al., 2005). Another study performed on parrots found that malondialdehyde levels post-exercise decreased after 9 weeks of training compared to a single session of exercise. This study involved a diet aspect, with a control group and a group fed antioxidants for 12 months. However, they found that diet did not affect malondialdehyde levels only by exercise training (Larcombe et al., 2010). As previously mentioned, there are varying results of malondialdehyde activity in exercise studies, but exercise training plays a prominent role in oxidative stress management in the body.

Superoxide dismutases are metalloenzymes that form hydrogen peroxide and oxygen from a superoxide anion (O2-) to protect against cellular oxidation (Malmström et al., 1975). Superoxide dismutases are characterized based on their metal content: copper/zinc (CuZnSOD), manganese (MnSOD), and iron (FeSOD). There are three types of superoxide dismutases in humans: extracellular, mitochondrial and cytosolic superoxide dismutase (Weydert and Cullen, 2010). The superoxide dismutase assay kit through Cayman Chemical measures all three superoxide dismutase forms by measuring the dismutation of O_2- formed from a xanthine oxidase reaction. After dismutation, hydrogen peroxide can be reduced to water by glutathione peroxidase and thus reduce oxidative stress in the body through several simple steps. A study done by Oh-ishi et al. was interested in changes of all three superoxide dismutases in rat skeletal muscle during acute and chronic exercise. Their results suggest that exercise training increases superoxide dismutase activity and resting concentration in trained rats while untrained rats seem susceptible to oxidative stress based on low superoxide dismutase levels (Oh-ishi et al., 1997). There have been varying results on superoxide dismutase activity after exercise, some claiming that exercise training promotes an increase in superoxide dismutase activity. In contrast, others claim that exercise training has no effect on activity (Powers et al., 2011). A study examining the

effects of vitamin E supplementation on untrained horses after acute exercise found no significant changes across the treatment groups over time (Nemec Svete et al., 2021). Superoxide dismutase activity is an important indicator of oxidative stress and is the first defense mechanism against it (Finaud et al., 2006).

Glutathione peroxidase is an antioxidant enzyme involved in reducing hydrogen peroxide to reduce its detrimental effects on the body. Selenium is required for this enzymatic reaction and first reacts with the peroxide to form an intermediate, selenic acid, at the selenol active site. One glutathione molecule reduces the selenic acid intermediate and forms glutathiolated selenol, another intermediate. Lastly, a second glutathione molecule reduces the glutathiolated selenol further and forms oxidized glutathione. This product is converted back into two molecules of glutathione and used again to reduce hydrogen peroxide by reacting with NADPH and H^+ under the glutathione reductase enzyme. $NADP^+$ produced from this reaction is used in the pentose-phosphate pathway, demonstrating several benefits of the glutathione peroxidase and glutathione reductase reactions (Lubos et al., 2011). This decrease in oxidative stress must be appropriately balanced with an avoidance of reductive stress. Although a relatively new concept, the accumulation of reducing equivalents can cause several dysfunctions in the body such as decreased cellular metabolism and reduced mitochondrial activity (Lubos et al., 2011). Reductive stress has been explained in almost every article previously mentioned but without any literal term tied to it: a deficiency of cellular oxidants (reductive stress) leading to diminished cell growth. Like malondialdehyde activity, glutathione peroxidase is measured in most studies involving oxidative stress biomarkers during exercise. A study performed by Ammar et al. observing the effects of aerobic and anaerobic exercise on oxidative stress found a significant increase in glutathione peroxidase levels in young adults immediately following both types of

exercise. Both glutathione peroxidase and glutathione reductase levels were most elevated 20 minutes post-exercise for aerobic and acute exercise compared with four other time points (Ammar et al., 2020). Another human study on blood oxidative biomarkers during aerobic and anaerobic exercise found no correlation between exercise type and oxidized glutathione blood levels. However, they found that blood oxidized glutathione increased and glutathione decreased immediately following exercise indicating an upregulation of glutathione peroxidase (Bloomer et al., 2005). *Figure 2* below illustrates both antioxidant enzymes working together to form water to reduce oxidative stress and NADP- for glycolysis (Weydert and Cullen, 2010).

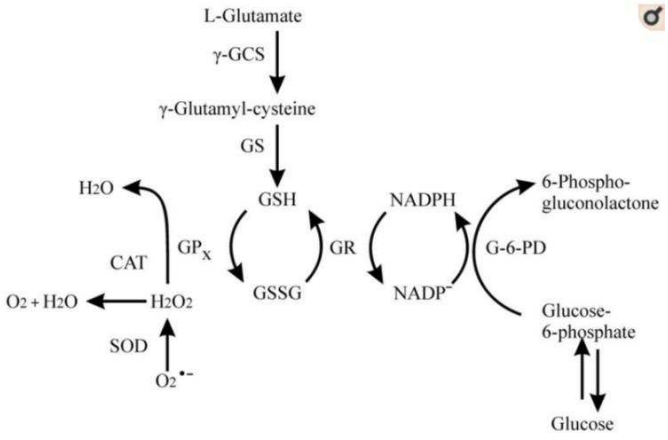


Figure 2. Antioxidant Enzymes and the Reduction of Oxidative Stress
Original Source: Weydert and Cullen, 2010

Exercise intensity/duration can determine whether antioxidant supplementation is needed. There are different effects on the body depending on whether someone lives a sedentary lifestyle or participates in the acute or chronic exercise. Takuji Kawamura presents a review on the effects of acute exercise on oxidative stress and concludes that the effects of antioxidants on oxidative stress are predicated on varying basal levels of antioxidants found in the subjects (Kawamura and Muraoka, 2018). These levels of antioxidants found in the body depend on muscle stress, diet and the environment. Vassilis Paschalis et al. performed a study with 10 untrained female participants and observed their blood oxidative stress before and after a strenuous leg extensor

exercise. They found that the body will adapt to muscle stress and can alter antioxidant capacity depending on the situation (Paschalis et al., 2007).

Research has demonstrated varying effects of supplementation on oxidative stress and how to reduce such for improved performance. Yfanti et al. claim that supplementing male participants with antioxidants such as Vitamin E and C has no effect on lactate threshold workload and maximal power output (Yfanti et al., 2010). On the other hand, Novelli and his colleagues studied muscle endurance in mice. They found that vitamin E extended the time to muscle fatigue and other antioxidants combat ROS's detrimental effects (Novelli et al., 1990)**.** Fagan found that natural vitamin E supplementation compared to synthetic vitamin E demonstrated a reduction in oxidative stress during exercise in horses noted through various markers of muscle damage, tissue inflammation and antioxidant levels (Fagan et al., 2020). To argue with these studies, Coombes found that supplementation with Vitamin E and lipoic acid had no effect on muscle fatigue in rats (Coombes et al., 2001). Williams also found that supplementing Standardbreds with vitamin E did not decrease oxidative stress compared to the control group (Williams and Carlucci, 2006).

4. Muscle Protein Synthesis

Muscle protein synthesis is thought to be regulated by the mammalian target of rapamycin signaling pathway (Bodine et al., 2001; Glass, 2005; Miyazaki and Esser, 2009). The mammalian target of rapamycin pathway can be broken down into multi-protein complexes (complex 1 and 2), and has other functions including apoptosis, autophagy and cell proliferation. In *figure 4* below, Miyazaki shows regulators of the mammalian target of rapamycin, with the IGF-1 pathway being thoroughly studied and well understood. Although there are unknowns

involving amino acid availability in relation to the mammalian target of rapamycin signaling pathway, there is a consensus on the direct relationship of an increase in amino acids leading to an increase in the mammalian target of rapamycin signaling (Hara et al., 1998; Long et al., 2005) and in turn, an increase in skeletal muscle protein synthesis. Supplementation with the branched-chain amino acids leucine, isoleucine and valine can be linked to an increased rate of protein synthesis through phosphorylation of the mammalian target of rapamycin signaling pathway and activation of the p70 S6 kinase (Blomstrand et al., 2006). Branched-chain amino acids provide anabolic effects during muscle protein synthesis by increasing the rate of synthesis and inhibiting degradation (Buse, 1981) as well as increasing the efficiency of recycling essential amino acids for muscle protein synthesis (Wolfe, 2017). *Figure 3* provides detail for the effects of amino acids and their relation to muscle protein synthesis. Time of feeding has also been correlated with the regulation of protein synthesis. One human study in 2001 found that muscle synthesis was stimulated following protein ingestion immediately after exercise, but not stimulated after protein ingestion 3 hours post-exercise (Levenhagen et al., 2001).

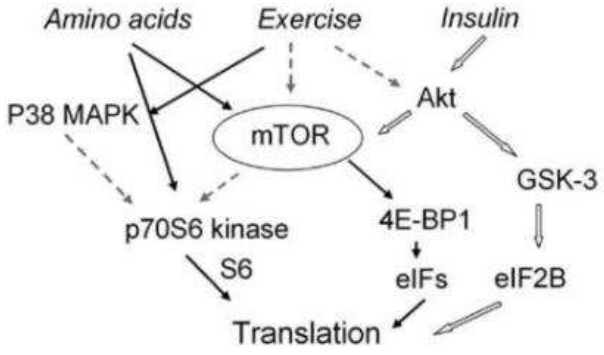


Figure 3. Original Source: (Blomstrand et al., 2006)
Proposed scheme for the activation of signaling pathways in protein synthesis by amino acids/BCAAs and resistance exercise in human muscle. Solid arrows demonstrated effect; dashed arrows demonstrated possible effect; open arrows demonstrated the effect of insulin.

Based on various research, the mammalian target of rapamycin pathway can still be activated without amino acids and growth factors through mechanical stretch or overload (Miyazaki and Esser, 2009)**.** This notion was supported by a study in 2018 where Latham and colleagues looked at amino acid supplementation on muscle protein synthesis in aged horses. They found that the control group's whole-body protein metabolism was not significantly different from those fed amino acids (Latham et al., 2019). Latham's study did not include exercise which could be an important factor in amino acids availability for muscle protein synthesis. Although more research is needed in horses related to antioxidant and branched-chain amino acid supplementation, if amino acids supplementation is correlated to increased mammalian target of rapamycin signaling which could increase skeletal muscle metabolism, what is the harm in supplementation? Amino acids are relatively inexpensive and any excess amino acids in the body can be stored as ketones with nitrogenous waste excreted as urea. In fact, with continued muscle contraction during strenuous exercise or an inadequate supply of carbohydrates from the diet, amino acids oxidation can be used for energy fuel. Various human research shows that without easy access to free amino acids, protein degradation can occur during bouts of exercise such as our treadmill tests (Baker et al., 2010).

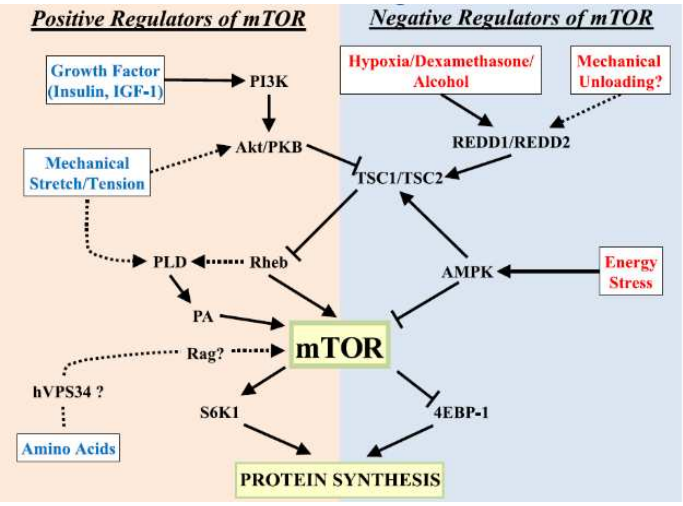


Figure 4. Positive and Negative Regulators of mTOR
Original Source: Miyazaki and Esser, 2009

The mammalian target of rapamycin pathway can also be regulated by energy status such as the scenarios described in the previous section about energy availability and muscle fibers triggered. Using the AMP-activated protein kinase pathway, the mammalian target of rapamycin signal is adjusted based on cellular energy storage levels (AMP:ATP in the body) (Inoki et al., 2003). The AMP-activated protein kinase pathway becomes activated when cellular energy levels are low. It phosphorylates several different residues (TSC2, Ser1345, etc.) which work to inhibit mammalian target of rapamycin signaling (Inoki et al., 2003). The mammalian target of rapamycin pathway has many influences on muscle protein synthesis, but this thesis focuses on amino acid availability and exercise.

4.1 Primers for Measuring Muscle Protein Expression

Muscle protein synthesis can be measured analyzing of 8 different gene targets. These include pyruvate dehydrogenase kinase 4, hexokinase II, muscle regulatory factor 4, myogenic factor 5, fatty acid translocase, palmitoyltransferase 1, myogenic differentiation and myogenin. These markers for muscle protein synthesis vary based on function and involvement within the biological system. Muscle regulatory factor 4, myogenic factor 5, myogenic differentiation and myogenin genes are involved in muscle growth/ cell differentiation. Pyruvate dehydrogenase kinase 4, hexokinase II, fatty acid translocase and palmitoyltransferase 1 are involved in carbohydrate and fat metabolism. These myogenic and metabolic genes have been studied in relation to acute bouts of exercise in humans, but not in a run exercise (Yang et al., 2005). There is no change in these genes beyond 8 hours post-exercise for pyruvate dehydrogenase kinase 4, hexokinase II (Nordsborg et al., 2003), muscle regulatory factor 4 and myogenic differentiation

12 hours after exercise (Bickel et al., 2005; Yang et al., 2005) in humans. Haddad and Adams found that myogenin mRNA in rats remained elevated 24 hours post-exercise (Haddad and Adams, 2002). Yang et al. claimed to perform the first study involving measurements of both myogenic and metabolic genes in a run exercise design. He found that peak expression of mRNA in humans when looking at the genes above occurred between 4-8 hours (Yang et al., 2005). Raue et al. tested myogenic gene expression in old and young women after exercise and took samples 4 hours post-exercise with significant results (Raue et al., 2006).

4.2 *Metabolic Genes*

Metabolic genes encode the enzymes and other components of metabolic processes and environmental factors, specifically the diet, influencing their expression. Regulation of metabolic gene expression can occur at any step going from DNA to proteins in a cell, but regulation of expression during transcriptional initiation is the most common (Towle et al., 1996). The metabolic genes of interest in this study are listed above and involve either carbohydrate or fat metabolism.

Fatty acid translocase is an integral membrane protein that functions to uptake long-chain fatty acids. When dietary fat is in excess, it often participates in lipid storage in muscle and adipose tissue (Pepino et al., 2014). It is found in many different cells including adipocytes, myocytes, enterocytes, etc. Palmitoyltransferase 1 is a family of 3 enzymes involved in lipid oxidation in the liver, brain or muscle. Palmitoyltransferase 1B is the rate-limiting enzyme involved in fatty acid oxidation in muscle mitochondria (Maples et al., 2015). This enzyme is responsible for transporting long-chain fatty acids into the mitochondria from the cytoplasm for oxidation. Pyruvate dehydrogenase kinase 4 is an enzyme involving the conversion of pyruvate

to acetyl-CoA in mitochondria and can be classified as a key regulator of glucose (carbohydrate) oxidation (Connaughton et al., 2010). Pyruvate dehydrogenase kinase 4 is increased by environmental factors such as exercise or fasting and is inhibited by insulin released after consuming diets high in glucose to ensure that blood glucose levels are maintained (Connaughton et al., 2010). Lastly, hexokinase II is 1 of the 4 possible isoforms of hexokinases involved in glucose metabolism. It is the dominant isoform residing in insulin-dependent tissues such as skeletal muscle and is a rate-limiting enzyme involved converting of glucose to glucose-6-phosphate. Hexokinase II has also been shown to act as a protective molecule against stress and can exhibit antioxidant effects (Roberts and Miyamoto, 2015).

4.3 *Myogenic Genes*

Myogenic regulatory factors are transcription factors that involve regulation at specific points in the muscle. These genes contain a basic helix-loop-helix domain and are required to direct cell differentiation helping to determine skeletal muscle phenotype (Hernández-Hernández et al., 2017). Myogenic differentiation was the first protein discovered within the muscle regulatory factor family by converting cultured fibroblasts into skeletal myocytes. Conerly et al. found that myogenic differentiation in embryonic mouse fibroblasts is more effective at activating differentiation target genes compared to myogenic factor 5 even though they control similar genes related to cell proliferation (Conerly et al., 2016). Myogenic factor 5 is thought to head the ancestral family with muscle regulatory factor 4 arising from gene duplication and myogenin arising from muscle regulatory factor 4 due to another gene-duplication incident to a second chromosome. Lastly, myogenic differentiation came from myogenic factor 5 in a third chromosome occurrence from gene-duplication. Studies have concluded that myogenic differentiation and myogenic factor 5 are involved in establishing a myogenic lineage whereas

myogenin and muscle regulatory factor 4 are involved in determining the terminal phenotype (Rudnicki and Jaenisch, 1995). Based on this information and research done by Megeney et al., it's been noted that myogenin and muscle regulatory factor 4 are more similar to one another and myogenic factor 5 and myogenic differentiation are more similar to one another (Megeney and Rudnicki, 1995). To determine the roles of these genes in myogenic reactions, a study was done on newborn knockout mice which lacked myogenic differentiation and myogenic factor 5. These mice showed no evidence of myoblasts and myofibers and lacked any sort of skeletal muscle formation (Rudnicki et al., 1993). In contrast, mice without myogenin expression had normal myoblast formation but incomplete skeletal muscle growth with little differentiation (Hasty et al., 1993). This data shows the importance of all 4 myogenic genes for the proper growth and development of skeletal muscle.

5. Measurements for Muscle Tissue Damage

Along with muscle protein synthesis and oxidative stress measurements done in this study, there are two blood indicators of muscle tissue damage: creatine kinase and aspartate aminotransferase. Both muscle enzymes have been studied to find a possible explanation for the rise in enzyme activity in the blood post-exercise (SICILIANO et al., 1995). Anderson claimed that rather than skeletal muscle damage, causing leakage of these enzymes into the bloodstream, exercise changes cell permeability of creatine kinase and aspartate aminotransferase in the horse (Anderson, 1975). Volfinger and colleagues concluded that very high values of creatine kinase in horses (>10,000u/L) could reflect muscle damage (Volfinger et al., 1994). Van der Muelen et al. also concluded that there was an immense overestimation of tissue damage based solely on measurements of serum muscle enzymes in rats (van der Meulen et al., 1991). Either way,

studies that have found muscle pain and fatigue in humans with elevated serum muscle enzymes post-exercise (Clarkson et al., 1992).

A study performed by Siciliano on 5 untrained horses observed exercise effects on creatine kinase and aspartate aminotransferase activity. They performed a submaximal exercise test on a treadmill before and after an 8-week conditioning period. For each treadmill test, they took blood samples prior to and at 0, 1, 3, 6, 8, and 24 hours post-exercise to test for muscle enzyme levels. Exercise caused an elevation in muscle enzyme levels that lasted 8 hours post-exercise for creatine kinase and 24 hours post-exercise for aspartate amino transferase. However, they also found that elevated creatine kinase and aspartate aminotransferase values after exercise were attenuated when comparing pre-conditioning and post 8-week conditioning periods (SICILIANO et al., 1995). There are several possible reasons for this attenuation of rising muscle enzyme values during repeated exercise. One possibility is an increase in antioxidant defenses against oxidative stress seen in rats proposing a possible link between free radical production and the upregulation of the antioxidant output. This study observed glutathione peroxidase and superoxide dismutase levels in 3 groups: control, high intensity, and moderate-intensity exercise. They found that average glutathione peroxidase levels in the soleus muscle increased significantly with the high exercise group and superoxide dismutase activity in the same muscle increased in both exercise groups compared to control. This upregulation of antioxidant enzymes supports the idea that an increase in free radical production seen after exercise causes an increase in enzyme production to combat it (Criswell et al., 1993). Also, Fridén and Lieber claim that a conversion from type IIb muscle fibers to type IIa through repeated submaximal exercise could decrease the susceptibility of skeletal muscle to tissue damage (Fridén and Lieber, 1992). There is still much investigation needed, but creatine kinase

and aspartate aminotransferase serum activity can indicate exercise stress and should be closely monitored, especially for conditions such as recurrent exertional rhabdomyolysis. Measuring muscle enzyme activity can catch recurrent exertional rhabdomyolysis before it causes irreversible muscle necrosis, possibly saving horses from muscle pain, stiffness and injury (SICILIANO et al., 1995). Normally, levels should be a 4-35-fold increase for serum creatine kinase and a 2-6-fold increase for serum aspartate aminotransferase from pre- to post-exercise. However, horses with recurrent exertional rhabdomyolysis often experience serum creatine kinase levels at a 10-900-fold increase and serum aspartate aminotransferase at a 5-100-fold increase (SICILIANO et al., 1995).

6. Hypothesis and Objectives

Feeding a grain diet with supplementation of antioxidants and branched-chain amino acids will decrease the cell death caused by the buildup of reactive oxygen species and stop the hindrance of protein synthesis post-exercise caused by the use of antioxidants. To address this hypothesis, the following targets were presented: study both the oxidative stress and the performance level of each horse before and after supplementation, perform a lactate analysis using blood samples during the treadmill test, and perform muscle biopsies to determine the efficiency of muscle protein synthesis before and after exercise. The research and protocols previously mentioned are the basis for the approach used in this thesis and include time of sampling, type of samples taken, supplementation given, conditioning and treadmill test protocol.

7. Conclusion

Exercise has dozens of influences on the body based on type, duration, and intensity. Someone's prior lifestyle habits also indicate of their metabolic response to exercise training. Our study used horses on pasture for 3 months before our conditioning began. Most were overweight, with a body condition score of 7 making training difficult. As time went on, the horses lost weight, gained strength and endurance, and were at an acceptable performance level before testing began. Although exercise was a significant factor in reactive oxygen species production and muscle recovery, other factors such as diet and the environment played a key role. Our horses were given a specific diet of grain and hay based on treatment group and endured various weather conditions during the study period. We were able to rule out certain environmental influences using a control group and did our best to ensure the health and safety of the horses during training.

The topics explained in the previous sections are all linked, each affecting the outcome of the other. For example, the amount of energy available affects muscle metabolism, which can be influenced by diet. Diet can also control the regulate antioxidants available to reduce oxidative stress caused by exertion during exercise. This reduction in oxidative stress allows muscle regeneration to occur, allowing the horses to gain strength and improve endurance over 12 weeks. Each part of this topic plays an integral role in ensuring homeostasis in the body, even in the presence of exercise. Although many aspects of these topics are covered, our focus was on chronic antioxidant and branched-chain amino acids supplementation in polo horses. We looked at muscle enzyme expression before and after exercise, glutathione peroxidase activity, superoxide dismutase activity, malondialdehyde activity, creatine kinase and aspartate aminotransferase activity and the influence of lactate concentrations during exercise training.

These biomarkers may uncover more information that is lacking in the equine industry regarding muscle protein synthesis and its relation to diet and exercise.

CHAPTER II: COMPARISON OF AN ANTIOXIDANT SOURCE AND ANTIOXIDANT PLUS BCAA ON ATHLETIC PERFORMANCE AND POST EXERCISE RECOVERY OF HORSES

1. Introduction

Exercise has been shown to cause oxidative stress, which induces cellular apoptosis in the body. Natural oxidative reactions needed for physiological activities can cause damage if in excess. The reactions producing an overabundance of reactive oxygen species (ROS) are 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 (Williams, 2016). In exercising horses, oxidative stress may cause muscle damage, increased lipid peroxidation levels (Kinnunen et al., 2005) and is related to recurrent rhabdomyolysis (RER) indicated by increased plasma levels of creatine kinase (CK) and aspartate aminotransferase (AST) (SICILIANO et al., 1995). Previous studies demonstrate that oxidative stress can be moderated by antioxidant (AO) supplementation (Clarkson and Thompson, 2000; Williams, 2016). However, recent evidence suggests that AO supplementation may interfere with the beneficial effects of ROS mechanisms such as cellular signaling and skeletal muscle sensitivity during exercise (Merry and Ristow, 2016). For instance, it was found that rats given Vitamin C had attenuated skeletal muscle hypertrophy indicative of a possible issue with supplementation during exercise (Makanae et al., 2013).

In contrast, branched-chain amino acids (BCAA) have shown reduced muscle soreness and fatigue during intense exercise in humans (Matsumoto et al., 2009). Several findings suggest a direct correlation between branched-chain amino acid (BCAA) supplementation and an upregulation of the mammalian target of rapamycin (mTOR) pathway responsible for muscle protein synthesis in humans and cell culture respectively (Kumar et al., 2009; Long et al., 2005).

As a serine/threonine kinase, mTOR plays a role in regulating cellular growth and metabolism. This large protein is made up of two complexes: mTORC1 and mTORC2. These are regulated by phosphatidylinositol 3-kinase (P13K), Akt and tuberous sclerosis complex 1 and 2 (TSC1-2), initiating protein translation in complex 1 and protein degradation in complex 2. Stimulation of mTORC1 is done by signaling two substrates: 70kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E- binding protein 1 (4E-BP1), both initiating protein translation. Rapamycin is an inhibitor of gene activity for cell growth first discovered in *Saccharomyces cerevisiae* yeasts. This compound is seen as a direct inhibitor of mTORC1, preventing its effects on protein translation and cellular growth (Anjum and Blenis, 2008; Hay and Sonenberg, 2004; Sandri, 2008).

The mTOR pathway responsible for muscle protein synthesis and hypertrophy can be stimulated by exercise, amino acid, glucose presence and activation of the neuronal nitric oxide synthase (nNOS enzyme). nNOS is responsible for producing free radical nitric oxide (NO). NO is upregulated during exercise and through several stepwise reactions in mice, a direct relationship between nitric oxide production and muscle hypertrophy is evident (Ito et al., 2013).

•NO + O2•- → ONOO-

•NO + ONOO- → activation of Trpv-1 → increase in intracellular calcium levels → activation of mTOR pathway → upregulation of muscle hypertrophy

There are several key mechanisms involved in exercise physiology and muscle hypertrophy, but our focus was on AO supplementation and the effect on muscle protein synthesis. To prevent a possible reduction in muscle growth associated with AO supplementation, we hypothesized that supplementing horses with AO plus BCAA would inhibit excessive ROS production while

simultaneously stimulating muscle protein synthesis post-exercise. Our objective involved supplementation with AO and BCAA to improve muscle protein synthesis while keeping oxidative stress levels at a minimum. We compared the treatment previously mentioned (BCAO) to low AO supplementation as the control (CON) and high AO supplementation without BCAA (AO).

2. Materials and Methods

2.1 Subjects

IACUC has approved all procedures used in this study (ID: 20-9958A). A total of 18 mixed thoroughbred polo mares (11) and geldings (7) were used for this study. These horses were from the CSU polo team but were on pasture for 3 months before the study start date due to COVID. CSU polo horses are often donated and are either retired polo horses or were used for other specialties. During normal circumstances, the horses are used 9 months out of the year and conditioned at varying intensity anywhere from 2-5x per week with a collegiate game once a month. One game consists of 2 teams with 6 total players on the field each riding horses and using mallets to pass a ball down the field to land between goalposts. The game involves 4, 6 ½ minute rounds called chukkas and horses cannot participate in consecutive chukkas. This high-intensity game involves sprinting and a quick response time for stopping, turning, or backing up (Inness and Morgan, 2015). The home team provides the horses for the visiting team's use during the game, so a team of 12 horses is often needed.

The horses were not moved to pens and fed grain until conditioning began in July. Up until that point, they were given free choice hay. Once transferred to the pens, they were provided approximately 0.1% BW of grain/day to acclimate them to the research feed. This allotment was slowly increased until approximately 0.4% BW in grain/day was consumed using

feed bags. The grain intake met working horses' recommended daily dry matter intake (Lewis et al., 2007). Horses were fed to meet the requirement for light working horses. Over 2-3 weeks, these horses were trained aerobically at low intensity to increase their fitness level back to their previous conditioning before training anaerobically at high intensity. In mid-August, these polo horses practiced with experienced riders on the team approximately 2x per week working on drills and practicing field movements. They also participated in our weekly conditioning schedule described in the *Training Design.* Beginning August 5th, we acclimated the horses to the treadmill and assigned them to treatment groups blocked by age, body condition score (BCS) and body weight (BW) (*Table 1).*

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

* $p<0.05$ indicates time effect when measurements were taken

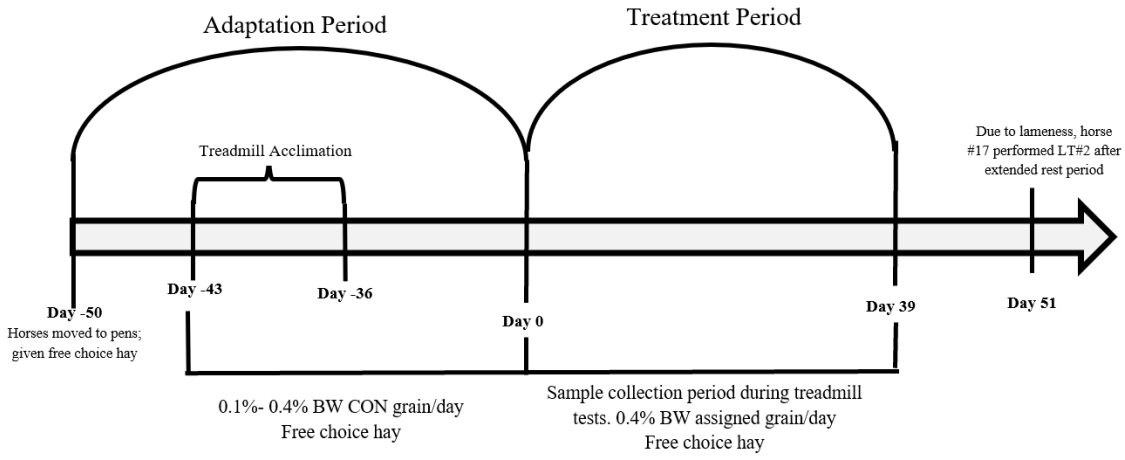


Figure 5: Timeline of Study beginning July 27, 2020

2.2 Training Design

Prior to the treatment period, 19 horses were conditioned with low- and high-intensity training that continued through the adaptation and treatment period (*Figure 5).* Two horses were deemed unable to participate due to lameness, one of which was replaced 2 weeks into training. Training sessions lasted 30-45 minutes with varying intensity of exercise. Each horse was exercised twice a week at low intensity and once a week at high intensity performing interval training. Horses were fitted with *Polar* heart rate monitors for high-intensity exercise. The low-intensity exercise involved walk and trot intervals each 7 minutes in duration (walk both directions, trot both directions, walk both directions). The high-intensity exercise involved a warmup for 7 minutes at a walk and trot, followed by interval training consisting of 3 repetitions of 3 to 5-minute gallop at heart rates above 160 bpm alternating with 3-4- minute recovery periods. This high-intensity conditioning concluded with a 10-minute cooldown period at a trot and a walk in a sand arena (Kang et al., 2012). Later in the study period, the horses were also conditioned during polo practice 2x a week that involved straight-line sprints technique exercises, substituting the interval training day.

Forty-three days before the treatment period began, horses were acclimated to the treadmill (EquiGym, KY, USA) for 2-3 occasions. Acclimation involved walking them into the treadmill room and allowing them to stand on the treadmill and then exiting without having to walk or trot. Once the horses could stand on the treadmill comfortably, we let them slowly walk and then trot at 0% incline. When horses were able to gallop at two increasing steps/speeds on the treadmill, they were deemed ready to perform the lactate threshold (LT) tests.

2.3 Feeding Protocol

During the adaptation period (*Figure 5),* horses were given CON feed 2x daily and 20 lb of hay was dropped in a feed bunk with enough space for each horse, equating to about 2.4% BW in dry matter intake per day. After blocking for age, BW and BCS, horses were assigned into 3 feed groups: BCAO, AO and CON (*Table 2*). The CON feed represents a commercial feed containing a small amount of AO. BCAO is the same feed as CON, only with added antioxidants and branched-chain amino acids. The AO group has the same amount of AO added as the BCAO group, but without branched-chain amino acids.

The daily estimated intake for each horse is found in *Table 2* gives nutrient estimations based on total average feed intake**.** The nutrient composition of hay is found in *Table 3* lists % of nutrient content based on hay consumption. The recommended daily intake was based on a moderate workload with a forage intake of approximately 2% BW and grain intake of roughly 0.4% BW (Lewis et al., 2007).

Table 2

Daily Average Intake of Nutrients 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

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

Each horse consumed approximately 1.9kg of grain/day split into 2 feedings
[a] CON, control diet; AO, vitamin E supplement; BCAO, vitamin E and branched-chain amino acid supplement
[b]Lys, Lysine; Met, Methionine; Cys, Cysteine; The, Threonine
*Branched chain amino acids: Leu, Leucine; Ile, Isoleucine; Val, Valine
**DE calculations based on 1-7b equation taken from *Nutrient Requirement of Horses:* Sixth Edition (Lewis et al., 2007)

After the first lactate threshold (LT) test, each horse was given feed based on their assigned group (i.e. Horse #1 assigned to the AO group transitioned from CON feed to AO feed after the first treadmill test on Sept. 14, 2020.) After each horse transitioned from CON to a particular feed based on group assignment, they remained on that feed until the end of the treatment period (*Figure 5)*.

2.4 Lactate Threshold Treadmill Test

For the duration of the treatment period, 9 horses were taken to the treadmill each week (3 per day for 3 days) with an approximate 15-day rest 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.). On testing day, horses running on the treadmill were given ½ their usual allotment of grain in the morning (3-6 hours before the LT test) and the other ½ approximately 1 hour after the LT test (¼ of total daily grain intake given before and after exercise). The first LT test was calculated as a baseline for all 18 horses, with ½ the usual allotment of CON feed given before the test and ½ the assigned group feed given after the test. Pre-exercise nutrient intake is a well-researched topic, providing evidence on the importance of consuming carbohydrates between 15-75 minutes pre-exercise for fuel availability (Hawley and Burke, 1997). Horses consumed hay until they were put in the trailer and taken to the treadmill, approximately 1hr before the LT test. One team in 1998 found that a carbohydrate bolus given 30 minutes post-exercise proved to be more effective at restoring muscle glycogen than 2hr post-exercise in humans (Ivy, 1998). A review done in 2010 looked at the similarities and differences between horse and human glycogen synthesis finding that horses have significantly slower glycogen synthesis compared to man. Either way, horses receiving a high carbohydrate diet had 150% increase in glycogen synthesis compared to low or mixed carbohydrate feed (Waller and Lindinger, 2010). An analysis of the effects of BCAA

supplementation demonstrated a benefit both before and after exercise in promoting muscle protein synthesis and reducing tissue damage in humans (Shimomura et al., 2004). Due to the strenuous level of the treadmill test, our horses weren't inclined to eat directly after exercise, so we adjusted the feeding time to 1hr post-exercise to allow for an adequate cooldown period.

Prior to the LT test on the treadmill, a catheter was placed in the jugular vein and a baseline blood sample was taken to record the lactate levels of each horse at rest (~1mmol/L). The horse was then led onto the treadmill before starting the exercise. The LT test involved a 3-minute walk and a 2-minute trot at 0° before increasing the incline to 3° and speeding up the treadmill until each horse began to gallop, which varied based on stride length. During the last 20 seconds of each 2-minute interval, blood was drawn using a catheter for the lactate test. If the horse had not yet reached threshold (blood lactate level >4mmol/L), the speed was increased by 1m/s. This speed increase continued every 2 minutes until the horse reached threshold or was no longer able to maintain position, at which point the horse was taken down to a trot for 5 minutes and a walk for 2-minute at 0° incline for a cooldown period. A 10-minute post-exercise blood test was taken to determine recovering lactate values of each horse for comparison to baseline lactate levels. *Table 4* demonstrates the threshold lactate levels between horses based on fitness level.

2.5 Blood Collection

Pre-exercise blood samples were obtained from the jugular vein filling two 9 mL heparinized vacutainer tubes (Thermo Fischer Scientific) and 1 Tempus tube (Thermo Fischer Scientific) for each horse participating in the LT test that day. The Tempus tube was placed in the freezer after vigorously shaking for 10 sec. The heparin blood tubes were centrifuged for 5 minutes at 2,500g and 4°C. Plasma was separated aliquoted, and frozen at -20°C for measuring oxidative stress

muscle enzymes, resting vitamin E, and blood urea nitrogen (BUN). Half the plasma for each horse was put in the fridge at 4°C and sent off to the Clinical Pathology Lab at the end of the week. The other half was placed in the freezer at -20°C for future analysis of antioxidant levels. Approximately 2 hours after the LT test, blood samples were taken from the jugular vein filling 2 heparin tubes with the protocol previously mentioned. All plasma extracted from the heparin tubes 2 hours post-exercise were placed directly in the freezer. 4 hours after completion of the LT test, blood samples were again taken from the jugular vein, filling 1 heparin tube and 1 Tempus tube with the same protocol done pre-exercise.

2.6 Muscle Tissue collection

A muscle biopsy was taken before and 4 hours after each LT test from the gluteal muscle at 6 cm depth in an area in the first third of the line between the tuber coxae and the tuber ischii. The tissue extraction was performed on alternating sides for each LT test and near one another between the 3 LT tests (i.e. LT1: AM left biopsy sample, PM right biopsy sample, LT2: AM left biopsy sample, PM right biopsy sample, etc.). The extraction site was shaved, topically disinfected, and anesthetized subcutaneously using 1-2cc lidocaine. Muscle tissue was removed using a biopsy needle and immediately placed in 500µL of RNAlater (Thermo Fischer Scientific) and stored at -20°C for future analysis.

2.7 Blood Plasma Analysis

TBARS Assay Analysis

Using a Cayman Chemical TBARS Assay kit (Item No. 700870), lipid peroxidation in plasma was calculated based on MDA-TBA concentrations found at an absorbance of 535 nm with a plate reader. Three plasma samples were tested for each horse at each LT test (pre-exercise, 2 hours post-exercise, 4 hours post-exercise) using standard fluorometric preparation.

Glutathione Peroxidase Analysis

The Cayman Chemical GPx kit (Item No. 703102) measures GPX activity indirectly by observing the production of oxidized glutathione (GSSG) produced in reducing hydroperoxide into GSSG, water and R-OH. This assay looks at the change in concentration over time by reading the absorbance of GPX at 340 nm for 5 time points with the plate reader.

Superoxide Dismutase Analysis

The Cayman Chemical SOD Assay kit (Item No. 706002) measures SOD activity in the plasma necessary to alter superoxide anions produced during exercise enzymatically. This kit measures three SODs found in our bodies to counteract superoxide radicals. The assay is measured at an absorbance between 440-460 nm on the plate reader.

Creatine Kinase and Aspartate Aminotransferase Analysis and Blood Urea Nitrogen

The photometric analysis used at Clinical Pathology Lab at Colorado State University involved running plasma through the Cobas 501c Chemistry Analyzer to produce concentrations of creatine kinase (CK) and aspartate aminotransferase (AST) and blood urea nitrogen (BUN) run through a Cobas 501c Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN) through photometric analysis.

Vitamin E Analysis

Pre-exercise plasma samples were shipped to Michigan State University Veterinary Diagnostic Laboratory for testing. Results were presented as Vitamin E concentrations in ug/mL. To extract fat-soluble vitamins from serum or plasma samples, all samples and solutions were brought to room temperature. In-house bovine and equine serum pools were used as controls. A 1 mL aliquot of each sample and control was placed in a glass test tube for extraction. If 1 mL of

sample was not available, the volume of available serum/plasma was recorded and the total volume was brought to 1mL volume with phosphate-buffered saline. An internal standard (trans-b-APO-8"-carotenal, Fluka 10829, one drop in 100 mL chromatographic mobile phase, 20 mL), ethanol with butylated hydroxytoluene (1 mL) and hexane (1 mL) were added. The mixture was agitated by vortexing for 5 minutes. Tubes were then centrifuged at 3,000 rpm for 10 minutes. An aliquot of the hexane layer was removed using a positive-displacement pipette, placed in a clean test tube, and evaporated under reduced pressure in a vortexing chamber. The extracts were dissolved in a chromatographic mobile phase and placed in autosampler vials. For standards, apocarotenal was dissolved in methylene chloride to an absorbance of 0.10e0.15 at 450 nm to make the working internal standard solution. A five-point calibration curve was constructed using the working standards described below. Stock alphatocopherol (Sigma T3251) solution was prepared by adding the alphatocopherol standard to the chromatographic mobile phase. Working solutions were made by diluting the stock in the chromatographic mobile phase to achieve an absorbance of 0.09e0.11 at 292 nm. The concentration of the working solution is calculated as alpha-tocopherol (ug/mL) ¼ Abs292/75.8*10000*0.98. Samples were analyzed chromatographically using a Waters ACQUITY system (Waters Corporation, Milford, MA 01757) and Waters Empower Pro Chromatography Manager software. Elution is made isocratic using a mobile phase of acetonitrile: methylene chloride: methanol (70:20:10, v/v/v) and a Symmetry C185, 3.5 mm, 2.1 _ 50 mm analytical column. The system also contains a Sentry guard column, C18, 3.5 mm. The flow rate is 0.5 mL/min and detection is by UV absorption at 292 nm for alpha-tocopherol and 450 nm for apocarotenal. Peak integration is by the ApexTrack method of Empower Pro. All peaks are reviewed manually after initial autointegration.

Otherwise questionable peaks are reviewed for purity using spectral data at the peak wavelength for that analyte.

2.8 Muscle Biopsy Analysis

RNA Isolation

RNA was isolated from the muscle biopsy samples following a standard protocol. Once thawed, 50-100mg of tissue sample was pulverized in 1mL of TRI reagent using a BeadBug (3x30 seconds with 1-minute intervals in ice to avoid overheating) and incubated at room temperature for 5 minutes. Chloroform (200µL per 1mL TRI 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 500µL per sample, vortexing, and incubation on the benchtop for 10 minutes at room temperature. The precipitated RNA was collected by centrifugation for 8 minutes at 4°C and 12,000g. The supernatant was removed and the RNA was washed twice to remove impurities (wash steps: add 1mL of 75% ethanol, vortex, centrifuge at 7,500g for 5 minutes at 4°C, and remove the supernatant). Residual ethanol was removed and the pellet air dried for 3-5 minutes. The RNA was solubilized with 30µL of nuclease-free water to dissolve the pellet before incubating the sample between 55-60°C for 15 minutes. RNA concentration was determined using a NanoDrop 1C spectrophotometer (Thermo Fisher). Samples were DNAse treated to removed genomic DNA by mixing with 2µL of 10X DNAse I buffer and 1µL rDNAse I and incubating for 20 minutes at 37°C. Following incubation, 2µL of DNAse Inactivation Reagent was added and mixed by vortexing then incubated at room temperature for 2 minutes to stop the reaction. The inactivated enzyme was removed from the

samples by centrifuging at 10,000g for 90 seconds. The supernatant was transferred to a new tube, RNA concentration and purity checked again, and labeled appropriately.

Reverse Transcription Protocol

The isolated RNA was reverse transcribed into cDNA in two steps using ImProm-II Reverse Transcription System Kit (Promega). Depending on the RNA concentrations of each sample, up to 9µL of total RNA extracted from each sample was used. (equation used: 1000ng/ [ng/µl concentration of total RNA] = number of µL needed). The RNA from each sample was combined with 1ml of RT primer (1:1 ratio of random hexamers and oligo dT) and brought to 10µl 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, 10µl of RT master mix (4µL of 5x RT Mix, 2.4µL $MgCl_2$, 1µL 10µM dNTP mix, 1µL RNasin, 1µL RT and 0.6µL of water per sample) was added to each sample. The reactions were 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 (20µL of water was added to the 20µL RT reaction) and placed at -20°C for further analysis.

qPCR Protocol

Quantitative reverse transcriptase PCR (qRT-PCR) was performed using the cDNA produced from the muscle biopsies (procedures described above) to determine transcript abundances for metabolic genes (reference lit review). Briefly for each target gene, 10µL of SYBR Green Mastermix (Applied Biosystems No. A46109), 0.6µL of primer (10uM each of the forward and reverse primers), 2µL of template, and 7.4µL of water was combined in a 96-well PCR plate then aliquoted in triplicate to a 384 well-plate (3 wells x 6µL). GAPDH was included

on each plate as an endogenous positive control. A no-template water control was also included on each plate to ensure that any observed expression resulted from the combination of primer and cDNA. Each primer set was tested using both negative and positive controls listed above, along with the samples taken from all horses.

Using equation 5 from Pfaffl's esearch on *Relative Quantification,* normalized Q values were calculated from the data collected on the LineRegPCR 2020.2.0.1 version. The primers and their efficiencies for accurate analysis are listed below based on a 2-point scale (i.e 1.9= 95% efficiency).

GAPDH: 1.88	MRF4: 1.67
MyoGenin: 1.80	HKII: 1.89
DK4: 1.76	MyoD: 1.76
CPT1: 1.87	MyF5: 1.84
CD36: 1.81	

2.9 Statistical Analysis

All data was analyzed using SAS software with ANOVA in a mixed model while horse by treatment was the subject. The effects of treatment time (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 in SAS with a mixed model. Dependent variables were muscle gene expression, muscle enzyme activity, oxidative stress data and vitamin E, BW, BCS, and BUN. Results were analyzed by least-square means analysis with a significance at a P-value <0.05. Relative Quantification (RQ) values were used to show significance of muscle protein synthesis and muscle tissue damage. RQ presents a change in mRNA expression levels based on a housekeeping gene, GAPDH, and a baseline for each horse set as pre-exercise on day 0 (Pfaffl, 2007). 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.

Muscle enzymes are log-transformed to provide more symmetrical data and allow for easier visualization of the significant effects. Data is presented as means and SE. Data not normally distributed were log transformed for statistical analysis

3 Results

3.1 General Horse Health

Bodyweight and BCS were recorded every two weeks during the treatment period. Bodyweight average was 530 ± 17.3kg and BCS was 6/9 for horses over all measurement days. The horses received enough grain and hay for maintenance based on a light conditioning schedule (Lewis et al., 2007). Horse #5 showed signs of recurrent rhabdomyolysis after the 1st LT test indicated by high levels of CK and AST (SICILIANO et al., 1995)and was switched from CON to AO feed. Horse #11 needed corn syrup added to her feed for palatability during the adaptation period (*Figure 5)*. Horses #14 and 16 were periodically lame during the study but had unaffected LT tests. Horse #15 was lame for a short time due to a soft tissue injury that didn't affect her LT schedule. Horse #17 was lame several days after the 1st LT test and was rested until his last LT test on day 51 of the treatment period, setting his 2 LT tests 40 days apart. Overall, only minor injuries occurred during the study. These injuries were monitored and treated appropriately to ensure the safety of all participants.

3.2 Blood Lactate Levels

Blood lactate levels were measured every 2 minutes on the treadmill during the exercise test. Baseline and peak lactate threshold levels with associated speeds are presented in *Table 4***.** Significant increases in lactate levels during the LT were found for all treatment groups ($p = 0.0025$). 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).

Table 4: Blood lactate levels based on pre-exercise and peak lactate threshold

Blood lactate Concentrations	LT1			LT2			LT3		
Horse ID	Base line	Peak Lactate threshold	Speed m/s	Base line	Peak Lactate Threshold	Speed m/s	Base line	Peak Lactate Threshold	Speed m/s
1	1.2	4.8	9	1.2	5.5	9	1.0	5.5	8
2	1.2	4.1	8	0.9	4.6	9	0.9	5.0	9
3	1.3	4.8	8	1.1	5.0	7	0.8	5.0	8
4	1.4	6.2	9	1.2	5.5	9	1.0	4.4	9
5	1.6	4.6	10	1.2	5.8	11	0.8	4.6	11
6	1.1	6.2	10	1.1	4.7	9	0.8	4.7	9
7	0.8	5.9	9.5	0.9	5.5	9	0.7	4.4	9
8	0.9	6.3	9	1.0	5.0	9	0.9	4.3	9
9	1.2	5.5	9.5	0.9	5.6	9	1.0	6.0	10
10	1.1	4.7	9	1.0	6.1	9	0.6	5.1	10
11	0.9	6.7	7.5	0.9	4.5	6	1.3	5.6	7
12	1.5	4.1	10	0.9	4.5	8.5	0.7	4.6	10
13	1.0	6.3	10	0.9	4.4	10	0.7	4.0	10
14	0.9	4.1	9.5	1.0	6.0	9	0.6	5.9	10
15	1.1	4.5	9	0.8	5.1	9	0.7	4.1	9
16	0.8	8.4	8	1.3	4.8	8	0.6	3.9	9
17	0.9	5.2	8	N/A	N/A	N/A	0.9	6.4	9
18	1.2	4.5	9	1.3	4.3	7	1.0	5.4	9

*Blood Lactate Concentrations listed in mmol/L
N/A: Horse #17 participated in 2 LT tests approx. one month apart.

3.3 Plasma AO and Oxidation Product Expression

Superoxide dismutase activity had a time ($p<0.0001$) and time*LT effect ($p=0.0015$). When analyzing time*LT, at 2 hours and 4 hours post-exercise, there was a significant decrease ($p<0.05$) in SOD activity from day 0 to day 15 and day 30 for all treatment groups. This decrease was also seen from day 0 to day 30 for pre-exercise (*Table 5*). There was an increase in expression from pre-exercise to 4 hours post exercise at day 0 and day 30. No treatment effects were seen.

GPx activity had an exercise (LT) effect ($p<0.0014$). There was a large increase in GPx activity from pre-exercise to 2 hours and 4 hours post-exercise. However, no changes were found from 2 to 4 hours post-exercise (*Table 5*). No treatment effect was seen.

MDA activity demonstrated a time ($p<0.0001$) and time*LT ($p<0.0001$) effect (*Table 6)*. There was a significant decrease in MDA production on day 15 from before to after exercise and a significant increase from pre to 4 hours post-exercise for day 0 (*Figure 6*). All treatment groups had a significant decrease in MDA production for 4 hours post-exercise from day 0 to day 15 and day 30. At 2 hours and 4 hours post exercise, all treatment groups experienced a significant decrease in oxidative stress from day 0 to day 30 (*Figure 6).* No treatment effects were seen.

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

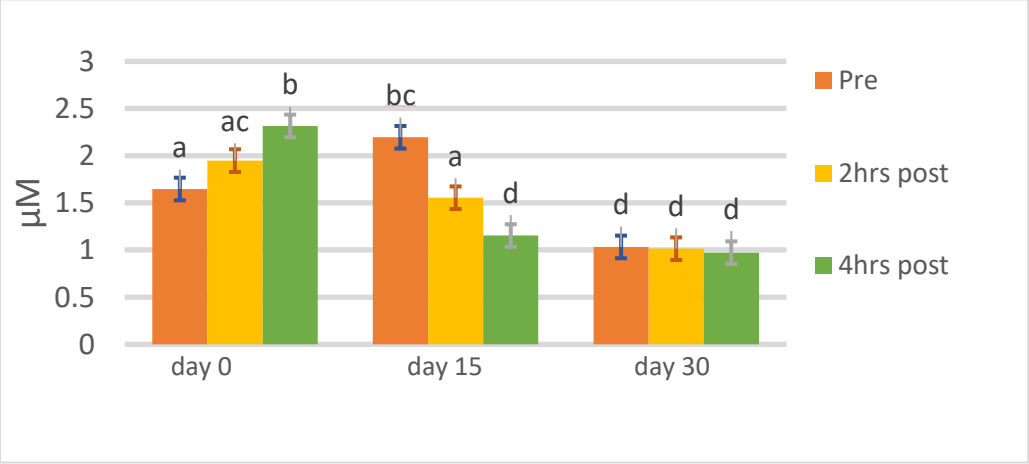
		SOD Activity (U/mL)			GPx Activity (nmol/min/ml)			P-value	SOD	GPx
	Time	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	TRT	0.97	0.31
CON	Pre-E[a]	0.025†°*	0.022†**	0.014‡**	33.2^{A}	40.6^{A}	38.4^{A}	TIME	<0.0001	0.38
	2 hr Post-E[b]	0.025†*	0.025‡**	0.009‡**	87.3^{B}	51.8^{B}	49.2^{B}	LT	0.54	0.0014
	4 hr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

Exercise/:T: Pre-E refers to pre-exercise; 2hrs Post-E refers to 2 hours post-exercise; 4hrs Post-E refers to 4 hours post-exercise
LT effect: different superscript capital letters ($p<0.05$)
Time*LT effect: † vs ‡ vs ° ($p<0.05$)
Time effect: * vs ** indicate $p<0.05$
NOTE: Don't compare letters between different AO activities. Only compare data from each AO activity to itself

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

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

Exercise/LT: Pre-E refers to pre-exercise; 2 hrs Post-E refers to 2 hours post-exercise; 4 hrs Post-E refers to 4 hours post-exercise
Time*LT effect: Different superscript lowercase letters ($p<0.05$)
Time effect: * vs ** indicant $p< 0.05$



Different superscript letters differ $P < 0.05$

Figure 6: Exercise effects and combined treatment time on plasma MDA

3.4 Vitamin E Analysis

Vitamin E levels measured pre-exercise were not different among treatment groups (*Table 7).*

Table 7: Effects of AO, BCAA supplementation compared to control on resting plasma Vitamin

		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		TIME	0.17
	AO	4.1	4.3	3.9	4		TRT*TIME	0.34
	BCAO	3.8	3.7	3.8	3.9			
	SEM	0.24	0.26	0.26	0.26			

3.5 Plasma Muscle Enzyme

CK and AST were measured in IU/L and log-transformed for statistical analysis before and 4 hours post exercise due to abnormal distribution. lnCK had a significant increase ($p<0.0001$) from pre- to post-exercise. AST demonstrated an exercise (LT) effect as well, with lnAST increasing significantly ($p = 0.01$) from pre- to post-exercise (*Table 8*). CK and AST enzyme concentrations are shown in (*Table 9)* and had no significant effects.

3.6 Plasma BUN

BUN values demonstrated an LT ($p=0.0017$) and trt effect ($p=0.042$), along with a time*trt trend ($p=0.061$). Values increased significantly from pre- to post-exercise for all trt groups with CON having the lowest BUN values with higher values that are similar between BCAO and AO (*Table 10)*. The time*trt trend increases from CON to AO and BCAO at each time point (day 0, 15 and 30).

Table 8: Effects of AO, BCAA supplementation compared to control and exercise on Muscle Enzymes

Muscle Enzymes	lnCK				lnAST			P-Value		lnCK	lnAST
	Time	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30		TRT	0.41	0.5
CON	Pre-E	5.7^{A}	5.7^{A}	6.0^{A}	5.9^{A}	5.8^{A}	5.9^{A}		TIME	0.12	0.079
	4 hrs Post-E	6.4^{B}	6.0^{B}	6.2^{B}	5.9^{B}	5.9^{B}	5.9^{B}		LT	<0.0001	0.01
	SEM	0.28	0.28	0.28	0.15	0.15	0.15		TIME*TRT	0.31	0.26
AO	Pre-E	5.7^{A}	5.6^{A}	5.8^{A}	6.0^{A}	5.9^{A}	6.0^{A}		TIME*LT	0.14	0.57
	4 hrs Post-E	7.1^{B}	6.2^{B}	6.2^{B}	6.3^{B}	6.0^{B}	6.0^{B}		TRT*LT	0.26	0.28
	SEM	0.24	0.24	0.24	0.12	0.12	0.12		TIME*TRT *LT	0.23	0.34
BCAO	Pre-E	5.6^{A}	5.5^{A}	5.4^{A}	5.8^{A}	5.8^{A}	5.8^{A}				
	4 hrs Post-E	6.1^{B}	6.0^{B}	5.9^{B}	6.0^{B}	5.9^{B}	5.8^{B}				
	SEM	0.25	0.27	0.25	0.13	0.14	0.13				

Exercise/LT: Pre-E refers to pre-exercise, 4 hrs Post-E refers to 4 hours post-exercise
LT effect: Different superscript capital letters ($p<0.05$)
NOTE: Don't compare letters between different enzymes. Only compare data from each muscle enzyme to itself

Table 9: Muscle Enzyme Raw Data

Muscle Enzymes	CK				AST			P-Value		CK	AST
	Time	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30		TRT	0.47	0.45
CON	Pre-E	296.8	300.08	517.2	393.2	344.39	349.8		TIME	0.46	0.12
	4 hrs Post-E	658.2	450.2	695.8	349	355	369.8		LT	0.22	0.066
	SEM	1488.92	1576.41	1488.92	91.13	92.94	91.13		TIME *TRT	0.51	0.37
AO	Pre-E	299.14	286.57	400.43	417.57	395.71	459		TIME *LT	0.43	0.43
	4 hrs Post-E	5701.1	596.86	597	647.86	420.57	482.29		TRT* LT	0.41	0.15
	SEM	1258.37	1258.37	1258.37	77.02	77.02	77.02		TIME *TRT *LT	0.5	0.17
BCAO	Pre-E	293.5	245.48	224.83	347.67	346.49	327.17				
	4 hrs Post-E	467.5	521.88	396.17	395.67	365.49	349.33				
	SEM	1359.19	1488.45	1359.19	83.2	85.93	83.19				

LT/Exercise: Pre-E refers to pre-exercise, 4hrs Post-E refers to 4 hours post-exercise

Table 10: Effects of AO, BCAA supplementation compared to control on plasma BUN Values

	BUN mg/dL						
	Time	Day 0	Day 15	Day 30		TRT	0.042
CON	Pre-E	17.2^{A+}	12.6^{A+}	16.8^{A+}		TIME	0.2188
	4 hrs Post-E	17.6^{B+}	17.4^{B+}	17.8^{B+}		LT	0.0017
	SEM	1.54	1.49	1.49	P-Value	TIME*TRT	0.061
AO	Pre-E	19.4^{A+}†	18.9^{A+}†	17.7^{A+}†		TIME*LT	0.41
	4 hrs Post-E	20.6^{B+}†	20^{B+}†	18.7^{B+}†		TRT*LT	0.64
	SEM	1.29	1.26	1.29		TIME*TRT*LT	0.4
BCAO	Pre-E	20.3^{A}†	20.4^{A}†	20.8^{A}†			
	4 hrs Post-E	21.5^{B}†	21.4^{B}†	22.2^{B}†			
	SEM	1.4	1.43	1.36			

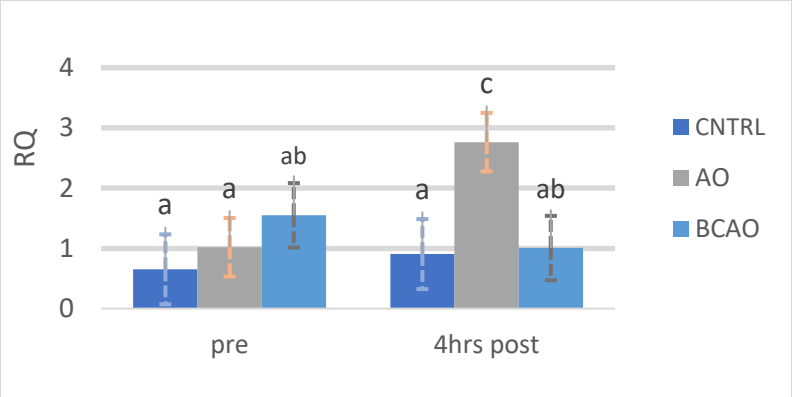
Exercise/LT: Pre-E refers to pre-exercise; 2 hrs Post-E refers to 2 hours post-exercise; 4 hrs Post-E refers to 4 hours post-exercise
LT effect: Different superscript capital letters ($p<0.05$)
Trt effect: + vs † ($p<0.05$)

3.7 Gene Expression in Muscle

Metabolic genes include CD36, CPT1, HKII and PDK4. When looking at RQ data, all but HKII had an exercise (LT) effect ($p<0.05$) showing a significant increase in expression from pre-exercise to post-exercise. HKII had no significant treatment, time or exercise effect.

Myogenic genes include Myf5, Myogenin, MRF4 and MyoD. Myf5 and Myogenin presented an exercise (LT) effect ($p<0.0001$ and $p = 0.037$ respectively). Myf5 had significant increases in expression from pre- to post-exercise for all 3 time points. Myogenin experienced increased expression from pre- to post-exercise on day 0 and day 15 in the CON and the BCAO group, and all after exercise at day 0, 15 and 30 for the AO group. In BCAO and CON groups there was a decreased expression for myogenin on day 30 after exercise (*Table 10).*

MRF4 had a time*trt effect (p = 0.045) and MyoD had a Trt*LT effect (p = 0.0041) (*Table 11)*. MFR4 had a decrease in expression for the CON group from day 0 to 15 days at 4 hours post-exercise. The AO group had increased MRF4 expression from day 0 to day 15 and day 0 to day 30 at 4 hours post-exercise and day 0 and 15 to day 30 pre-exercise. The BCAO group had no change in MRF4 expression over the 30 days of treatment. The MyoD trt*LT effect significantly increases in MyoD expression for AO compared to BCAO and CON when looking from pre- to post-exercise (*Figure 7)*.



Different superscript letters differ p < 0.05

Figure 7: Effects of AO, BCAA supplementation compared to control on and exercise on protein MyoD expression (Treatment by Exercise/LT test effect p=0.004)

Table 11: Effects of AO, BCAA compared to control supplementation on of Myogenic and Metabolic Gene

Primer		CON			AO			BCAO		
	Time	Pre-E	4 hrs Post-E	SEM	Pre-E	4 hrs Post-E	SEM	Pre-E	4 hrs Post-E	SEM
CD36 RQ	Day 0	1.00^{A}	2.71^{B}	0.70	1.00^{A}	2.13^{B}	0.59	1.00^{A}	2.28^{B}	0.64
	Day 15	0.77^{A}	1.91^{B}	0.70	1.09^{A}	2.34^{B}	0.59	1.46^{A}	1.62^{B}	0.68
	Day 30	1.43^{A}	3.45^{B}	0.70	1.28^{A}	2.45^{B}	0.59	1.02^{A}	1.69^{B}	0.64
CPT1 RQ	Day 0	1.00^{C}	2.69^{D}	0.99	1.00^{C}	3.32^{D}	0.84	1.00^{C}	3.99^{D}	0.91
	Day 15	0.71^{C}	2.18^{D}	0.99	0.66^{C}	3.33^{D}	0.84	1.85^{C}	1.92^{D}	0.97
	Day 30	2.22^{C}	3.72^{D}	0.99	0.94^{C}	2.26^{D}	0.84	2.05^{C}	3.50^{D}	0.91
PDK4 RQ	Day 0	1.00^{E}	18.56^{F}	12.92	1.00^{E}	18.37^{F}	10.92	1.00^{E}	12.60^{F}	11.79
	Day 15	0.32^{E}	7.0^{F}	12.92	0.86^{E}	54.30^{F}	10.92	2.14^{E}	5.01^{F}	12.87
	Day 30	15.35^{E}	18.38^{F}	12.92	1.11^{E}	15.08^{F}	10.92	1.49^{E}	8.99^{F}	11.79
MyF5 RQ	Day 0	1.00^{G}	3.10^{H}	0.78	1.00^{G}	1.86^{H}	0.62	1.00^{G}	2.68^{H}	0.67
	Day 15	0.75^{G}	2.24^{H}	0.74	1.00^{G}	3.47^{H}	0.63	0.94^{G}	1.41^{H}	0.74
	Day 30	0.96^{G}	3.18^{H}	0.74	1.10^{G}	1.58^{H}	0.623	1.00^{G}	1.28^{H}	0.67
Myogenin RQ	Day 0	1.10^{I}	2.85^{J}	0.72	1.00^{I}	2.38^{J}	0.58	1.00^{I}	1.54^{J}	0.62
	Day 15	0.70^{I}	0.90^{J}	0.72	0.84^{I}	1.83^{J}	0.58	0.87^{I}	1.12^{J}	0.67
	Day 30	2.23^{I}	2.01^{J}	0.68	2.13^{I}	2.39^{J}	0.58	0.94^{I}	0.70^{J}	0.62

P-Value							
	TRT	TIME	LT	TIME* TRT	TIME* LT	TRT*LT	TIME* 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
Myogenin RQ	0.41	0.070	0.037	0.32	0.12	0.51	0.92

Exercise/LT: Pre-E refers to pre-exercise, 4 hrs Post-E refers to 4 hours post-exercise
LT effect: Different superscript capital letters ($p<0.05$)
NOTE: Don't compare letters between different primers. Only compare data from each primer to itself

Table 12: Effects of AO, BCAA compared to control supplementation on MyoD and MRF4 Muscle Gene Expression

Primers	MRF4 RQ				MyoD RQ			P-Value
	Time	Day 0	Day 15	Day 30	Day 0	Day 15	Day 30	
CON	Pre-E	1.00[a]	0.45[a]	0.46[a]	1.00[+]	0.39[+]	0.57[+]	
	4 hrs Post-E	1.01[a]	0.37[b]	0.70[a]	1.92[+]	0.41[+]	0.39[+]	
	SEM	0.28	0.28	0.28	0.82	0.79	0.79	
AO	Pre-E	1.00[a]	1.05[a]	1.17[ac]	1.00[+]	0.73[+]	1.33[+]	
	4 hrs Post-E	1.10[a]	1.43[ac]	1.35[ac]	1.80[+]	3.92[++]	2.58[++]	
	SEM	0.24	0.24	0.24	0.66	0.66	0.66	
BCAO	Pre-E	1.00[a]	0.97[a]	0.84[a]	1.00[+]	2.14[+]	1.51[+]	
	4 hrs Post-E	0.92[a]	0.96[a]	0.55[a]	1.49[+]	1.05[+]	0.48[+]	
	SEM	0.25	0.27	0.25	0.72	0.77	0.72	

	MRF4 RQ	MyoD RQ
TRT	0.15	0.27
TIME	0.35	0.69
LT	0.6	0.1
TIME* TRT	0.045	0.22
TIME* LT	0.93	0.52
TRT*LT	0.31	0.0041
TIME* TRT*LT	0.82	0.17

Exercise/LT: Pre-E refers to pre-exercise, 4 hrs Post-E refers to 4 hours post-exercise
Trt*LT effect: + vs ++ ($p<0.05$)
Time*trt effect: Different lowercase superscript letters ($p<0.05$)

4 Discussion

Our objective involved supplementing feed with AO and BCAA to improve muscle protein synthesis while keeping oxidative stress levels minimum. The main finding is that neither high nor low antioxidant supplementation hindered post-exercise muscle synthesis measured by the gene expression of enzymes involved in muscle hypertrophy and energy metabolism. Furthermore, high versus low antioxidant treatments did not influence oxidative stress parameters.

4.1 General Observations

All 18 horses maintained their body weight and consumed the allotted grain during the study period. Although horse #5 demonstrated signs of recurrent exertional rhabdomyolysis (RER)

after the first LT test, moving her to the AO feed group decreased her CK and AST plasma concentrations. Based on her previous episodes of "tying-up" we changed her feed to prevent other signs of RER from manifesting (i.e. muscle tremors, dark urine and significant stiffness post-exercise). Horses #14 and #16 were lame during the study for several weeks. The lameness affected the training schedule and we adjusted accordingly without treating either horse with NSAIDs. The lameness didn't affect the horses on the LT test days as they were not lame on those dates. For several weeks, horse #15 was treated for her soft tissue injury and wore a bandage on her right front limb. This injury did not cause lameness, so she was able to complete the study. It was discovered that Horse #17's lameness was due to osteoarthritis in the coffin joint which was closely monitored for the duration of the study. The unequal treatment group distribution resulted from Horse #5's feed adjustment. There were 5 horses in the CON group, 6 in the BCAO group and 7 in the AO group.

4.2 Muscle Enzyme Expression

Protein degradation is balanced with protein synthesis at rest, allowing for constant protein turnover. During exercise, protein degradation in the liver and contractile muscle is upregulated. This increased degradation can be associated with other cellular components that are broken down or mobilized during exercise (Lynis Dohm et al., 1987). When this upregulated protein degradation occurs causing increased serum levels of cellular components, protein synthesis begins shortly after exercise to rebuild muscle. Creatine kinase (CK) and aspartate aminotransferase (AST) are blood indicators of muscle tissue damage and protein degradation linked to oxidative stress (SICILIANO et al., 1995). These components of muscle tissue damage are significantly increased after exercising due to the upregulation of protein degradation in muscle. CK and AST values can vary based on type of exercise, horse health, and data collection

time. In one study, barrel racing horses were exercised and CK and AST values were measured at 4 time points pre- and post-exercise (before, immediately after, 30 minutes after and 2 hours after exercise). CK values between 268 and 317 ± 112 IU/L and AST values between 310 and 342 ± 125 IU/L were recorded (Binda et al., 2016). Jumping horses were also tested for their CK and AST levels, finding their average CK and AST levels to be significantly less based on exercise type. Rather than sprinting around barrels, their test involved a 10-minute warmup and then 12 obstacles varying in design to be completed over 600m. Both CK and AST were less than 150 IU/L at the same 4 time points in the previous study (Macedo et al., 2017). A study involving polo horses during a game was more similar to our findings than the other two exercise types based on length of exercise and level of effort. Blood was drawn from polo horses before a game, in between chukkas and 30 minutes post-exercise had very high levels of CK and AST. Their AST values ranged between 463 and 533 ±5 IU/L and CK values were between 144 and 208 ± 19 IU/L. The normal ranges of AST and CK for this exercise type were 100-600 IU/L and 10-350 IU/L respectively (Zobba et al., 2011). Our raw data had similar findings, with several samples overshooting the upper limit of the ranges for both AST and CK (*Table 9*). For these high CK and AST values, pre-exercise was also increased, indicating the baseline for some horses was already outside normal parameters. This could be due to breed of horse, level of conditioning or amount of exercise or stress leading up to the treadmill test.

Our results showed a significant increase in plasma concentration from pre- to post-exercise for CK and AST independent of treatment and time. Conclusions from this data could indicate a variety of circumstances, including the change in cell permeability to these enzymes due to membrane disruption or damage and subsequent leakage (Anderson, 1975), a reflection of muscle damage (Volfinger et al., 1994), or an overestimation of tissue damage (van der Meulen

et al., 1991). Using these indicators of tissue damage by themselves is not substantial in providing accurate information about the status of the horse post-exercise.

4.3 Plasma AO and Oxidation Product Expression

SOD, GPx and TBARs assay kits were used to measure oxidative stress. Superoxide dismutases (SODs) are metalloenzymes that form hydrogen peroxide and oxygen to reduce the amount of superoxide anions present in the body (Malmström et al., 1975). The 3 types of SODs measured using the assay kit were based on their metal content: copper/zinc, manganese and iron (Weydert and Cullen, 2010). The hydrogen peroxide produced can be converted into water through the action of glutathione peroxidase (GPx). Like Oh-ishi's findings, on day 0 and day 30, there was an increase in SOD from pre-exercise to 4 hours post-exercise in all trt groups (Oh-ishi et al., 1997) but on day 15, there was a significant decrease in SOD activity. Similar to Power's findings, after 15 days of treatment no significant changes in SOD were seen before and after exercise (Powers et al., 2011). This could be attributed to less oxidative stress during exercise after continual conditioning or a muscle adaptation to oxidative stress allowing for better management of oxidant buildup in the body. Either way, SOD is the first defense mechanism to combat oxidative stress (Finaud et al., 2006). The antioxidants present in the control feed and the two other treatments were enough to help mitigate oxidative stress because there was not a treatment effect in this case.

Glutathione peroxidase (GPx) reduces hydrogen peroxide and produces NADP+ used in other energy metabolism reactions (Lubos et al., 2011). GPx acts as a protective enzyme against oxidative damage and removes reactive oxygen species (ROS) formed during exercise. A human study involving aerobic, anaerobic, and combined exercise found increased plasma GPx concentrations independent of exercise type (Ammar et al., 2020). Our study demonstrated an

increase in GPx activity from pre-exercise to 2 hours post-exercise independent of treatment group. GPx activity remained elevated 4 hours post-exercise, but the significant spike in GPx activity occurred 2 hours post-exercise.

MDA or malondialdehyde is a lipid peroxidation product that reacts with thiobarbituric acid (TBA) in the assay kit to produce MDA-TBA that can be measured using fluorometric detection. The results were similar to SOD activity, with an increase pre vs post-exercise at day 0, a decrease at day 15 and no change at day 30 in all treatment groups. It's important to note that MDA is insatiable and can be produced from other reactions not involving oxidative stress (Janero, 1990; Khoubnasabjafari et al., 2015; Shin et al., 1972). Pairing this data with other measurements of oxidative stress provides a more accurate explanation of the oxidative stress levels seen amongst the horses.

Lastly, vitamin E concentrations (*Table 7*) indicated that the AO supplied in all treatments may have been sufficient to reduce oxidative stress after exercise, as seen with similar enzyme activity across all treatment groups.

4.4 Gene Expression in Muscle

This study was the first to assess muscle gene expression during exercise with both AO and BCAA supplementation in horses. Exercise is an important stimulus of the mTOR pathway, allowing for muscle hypertrophy post-exercise, especially with available fuel such as BCAA. However, our hypothesis that BCAA alters protein synthesis in muscles was not demonstrated. There was no additional benefit from supplementing with BCAA compared to high AO because the AO treatment groups showed increased gene expression in muscle without additional BCAA.

There was still an upregulation of gene expression for 2 selected gene targets in the treatment groups with high AO supplementation.

In 2021, a study was performed to investigate the supplementation of N-acetylcysteine (NAC), a precursor to glutathione. This antioxidant precursor was observed for possible detrimental effects on the mTOR pathway post-exercise. Using a protocol resembling our own, 8 Thoroughbreds were conditioned on a treadmill to measure levels of BCAA, NAC, Akt and rpS6 (Hauss et al., 2021). Our study and theirs showed that anaerobic exercise was the driving component of muscle protein synthesis, regardless of treatment.

MRF4 and MyoD showed a treatment effect where the group with higher AO supplementation indicates increased muscle gene expression for muscle hypertrophy. The significant increase in MyoD expression for the AO group is correlated with high antioxidant supplementation. MRF4, or muscle regulatory factor 4, is a myogenic gene responsible for expressing the end phenotype during muscle growth and cell differentiation (Megeney and Rudnicki, 1995). MRF4 showed a significant increase in expression for both pre- and 4 hours post exercise for the AO group, indicating findings like MyoD. Increased MRF4 expression was noted for the CON group post-exercise, indicating that the minimal AO supplement in the feed compounded with exercise was enough to increase muscle gene expression. The other 5 genes of interest provided exercise (LT) or time effects, none of which were affected by treatment.

It's important to note that day 0 values are considered baseline because the horses received CON feed leading up to the first LT test. They received their treatment feed 1 hour after the first LT test in which they only received ¼ of their allotted grain for the day, approximately 1lb.

The myogenic genes with significant expression changes include MyF5, MyoD, MRF4 and Myogenin. MyoD, myogenic differentiation, is more effective than MyF5, myogenic factor 5, in activation of target genes related to muscle cell proliferation (Conerly et al., 2016). However, both MyF5 and MyoD are necessary for skeletal muscle formation and therefore important that expression is upregulated post-exercise for muscle growth (Rudnicki et al., 1993). Myogenin and MyF5 were upregulated in all treatment groups from pre- to post-exercise, demonstrating that the basal nutrient values found in the CON group were enough to stimulate muscle cell growth and differentiation.

Metabolic genes play a significant role in energy metabolism during exercise and include CD36, CPT1, PDK4 and HKII. Although HKII, hexokinase II, showed no significant increases in expression at 4 hours after exercise, it's important to note its role in energy metabolism. As a rate-limiting enzyme of glucose metabolism in skeletal muscle, its function is necessary to convert glucose to glucose-6-phosphate (Roberts and Miyamoto, 2015). 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 (Manio et al., 2017). CPT1, palmitoyltransferase 1, is the rate-limiting step involved in fatty acid oxidation in muscle by transporting long-chain fatty acids into the mitochondria (Maples et al., 2015). 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 exercise. This enzyme is inhibited by insulin, which is released after a meal, but is upregulated during times of exercise or fasting (Connaughton et al., 2010). The upregulation in both fatty acid metabolism and aerobic

glucose metabolism enzymes indicates the low-intensity effect of the aerobic component of the LT. CD36, CPT1 and PDK4 demonstrated an exercise (LT) effect, showing increased gene expression from pre- to post-exercise. This upregulation was independent of time and trt, showing a direct correlation between exercise and increased energy demands. The measurement of the gene expression of those enzymes could be used to assess the benefits of aerobic exercise on muscle adaptation to exercise.

5 Conclusion

Our hypothesis that BCAA improves muscle protein synthesis was not shown in this study. There was no additional benefit of supplementing with BCAA compared to high AO because the AO treatment groups showed increased muscle gene expression without additional BCAA. This was seen with increased MRF4 and MyoD expression in the high antioxidant treatment group, both important for muscle cell differentiation and proliferation. Further research needs to be conducted with similar factors, but one where the CON group receives little to no AO in the feed. This study showed exercise effects in horses with variables not previously studied. Overall, our data showed results contrary to our hypothesis. BCAA supplementation did not play a major role in upregulating muscle gene expression. However, BCAA increased blood urea, potentially indicating excess protein. Antioxidant supplementation did not interfere with metabolic and myogenic muscle responses after exercise as hypothesized.

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