THESIS

EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON OXIDATIVE STRESS IN YOUNG EXERCISING HORSES

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ABSTRACT

EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON OXIDATIVE STRESS IN YOUNG EXERCISING HORSES

The production of reactive oxygen species (ROS) increases with exercise. An excess of ROS compared to antioxidants, known as oxidative stress (OS), can contribute to inflammation and diseases detrimental to horse performance. Our objective was to evaluate the effect of an antioxidant supplement on OS in young horses beginning training. 18 horses (10 geldings, 8 mares; age 29.0±6.5 months; BW 457.6±45.4 kg; BCS 5.5±0.6) were blocked by age and sex into 2 treatments groups; supplement (SUPP) or placebo (CON). After treatment began, horses underwent 30 days at maintenance followed by 30 days at moderate work. On days 30 and 60, horses completed a standardized exercise test (SET). Blood samples were collected once on day 0, before each SET (PRE), and 0 (POST), 1 (1h), and 24 hours (24h) after. Plasma was analyzed for glutathione peroxidase activity (GPX), superoxide dismutase activity (SOD), and thiobarbituric acid reactive substances concentration (TBARS). Horses were recorded at the walk (W) and trot (T) to determine stride duration (SD), and knee and hock range of motion (KRM, HRM) using motion analysis software. Data were analyzed using mixed linear models with type II ANOVA and estimated marginal means. There was a time effect for GPX (p<0.001), SOD (p=0.041), and TBARS (p<0.001). Compared to d30-PRE, GPX was still elevated at d30-1h (p=0.026) and TBARS did not recover until d30-24h (p=1.00). On d60, GPX (p=0.771) was unchanged and TBARS recovered by d60-1h (p=1.00), suggesting conditioning led to greater antioxidant capacity.

Over all time points, TBARS tended to be lower for SUPP than CON (p=0.071). Treatment x day effect was significant for T-KRM (p=0.014) and a trend was found for W-DUR (p=0.099) and W-HRM (p=0.061), suggesting SUPP mitigated some decreases in mobility. Day had an effect on T-SD (p<0.001), W-KRM (p<0.001), and T-HRM (p<0.001), indicating reconditioning may have led to restricted movement. In conclusion, though the most significant changes to antioxidant status were due to reconditioning, SUPP mitigated aspects of exercise-induced OS and related changes in movement in young horses undergoing training.

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CHAPTER I

LITERATURE REVIEW

1. Introduction

Oxidative stress (OS) can be defined as a state in which the concentration of free radical compounds exceeds the capacity of natural antioxidant mechanisms, leading to damage to cellular components, tissues, and overall health [1].

1.1. Free Radicals

Free radicals are small chemical compounds characterized by an unpaired electron, causing them to be highly reactive [1,2]. In biological systems, these free radicals primarily fall within the subclass of reactive oxygen species (ROS) including compounds such as hydrogen peroxide, superoxide anion, nitric oxide, or hydroxyl radicals [1,2]. Some have estimated that 90% ROS are produced as a result of aerobic respiration [3], during which 1-2% of molecular oxygen is incompletely reduced [4]. This leads to the formation of superoxide and other ROS [4]. Other sources of ROS within the body include the immune response, regulation of vascular tone, and other essential metabolic processes such as the oxidation of catecholamines [1]. Additionally, environmental agents including UV radiation, air pollution, and various chemical agents can all instigate the production of free radicals [1,5].

As previously mentioned, ROS are highly unstable and will often react with nearby molecules in effort to fill the incomplete electron pair. Within cells, biological

macromolecules are the most commonly affected molecule [1]. ROS will react with lipids, proteins, or nucleotides to donate or receive a single electron, resulting in the ROS now having an even number of electrons, and the previously stable biomolecule becoming radicalized. The new radical will react similarly with nearby biomolecules, creating a chain reaction of oxidative damage [1,6].

These reactions can lead to severe damage, dysfunction, and disease on the cellular and systemic levels. Oxidative damage to mitochondrial components such as cytochrome C and CPT-1 transporter have been observed, which limit the capacity for ATP production [1,6]. ROS can also react with nucleotide bases found in DNA and RNA, leading to single and double strand breaks, protein/DNA crosslinks, and genetic mutations [1]. Lipid molecules, particularly polyunsaturated fatty acids (PUFAs) found in cell membranes, are a common target of oxidative damage. This causes changes in membrane fluidity, increased permeability, and even cell membrane rupture [1].

Moderate levels of ROS are essential for normal body functions. These “physiological radicals” play critical roles in many cellular processes. An example of this is nitric oxide, which is the primary regulator of vascular tone [1]. In addition, phagocytic cells will produce and use superoxide and hypochlorous acid to kill invading pathogens [7]. The presence of ROS also triggers the elimination of xenobiotics and pro-carcinogenic species [7]. Another common ROS, hydrogen peroxide, has been shown to be an essential part of regulating pro-apoptotic pathways and triggering mitosis, allowing for normal cell turnover and elimination of diseased cells [7,8]. However, the positive applications of ROS on metabolic processes are perhaps best demonstrated during exercise. As discussed above, exercise is known to lead to increased production of ROS.

Research has also shown that these ROS allow for maximum muscle force production [7,9], training-induced adaptations in endurance [7], adaptation of endogenous antioxidant defense systems [10], and initiation of repair of damaged muscle [11] in vitro and in humans. When extracellular calcium increases to trigger muscle contractions, it also leads to an increase in nitric oxide (NO). NO then reacts with superoxide ions, generating peroxynitrite, a potent ROS. This scavenging of superoxide by NO, as well as endogenous NO itself, modulate muscle contraction and result in increased muscle force production [7]. Studies conducted in vitro by adding exogenous radicals to muscle tissue saw an increase in peak twitch stress and extended time to peak tension, indicating increased contractility, while the addition of the antioxidant catalase decreased muscle contractility [12]. Others have found that in humans, when training is consistent, the mild increase in ROS encourages muscle remodeling that allows for increased endurance through the NF-κB pathway [13,14]. Consistent training also promotes the expression of genes in myotubes that allow for greater antioxidative capacity, lending to the adaptation of antioxidant defenses to exercise [13]. Human studies have shown that even five consecutive days of moderate exercise led to an increase in antioxidants in skeletal muscle [14]. Then during recovery, ROS stimulate inflammatory pathways that facilitate the regeneration of damaged muscle tissues [11].

1.2. Antioxidants

In order to maintain the levels of ROS to support these vital processes without allowing for excessive oxidative damage, the body utilizes antioxidants. These antioxidants, defined as a compound that neutralizes free radicals and thereby protects the cell from damage caused by oxidative stress [5], include a wide range of molecules that act through a variety of mechanisms. By in large, the mechanisms of antioxidants fall within the categories

of scavenging ROS, inactivating oxidative compounds, repair of oxidative damage, or promoting the activity of other antioxidants [4]. Under normal conditions, these antioxidants provide “checks and balances” for physiological radicals, allowing them to perform essential functions while protecting biomolecules from oxidation. Under stress conditions when high levels of ROS are present, they will sequester radicals and reduce concentrations back to normal levels

Some examples include glutathione peroxidase (GPx) and superoxide dismutase (SOD). While both are endogenous enzymatic antioxidants, they function through very different mechanisms from one another. SOD has very high specificity for superoxide, reacting with it directly and converting it to hydrogen peroxide and molecular oxygen [1]. While hydrogen peroxide is still cytotoxic and considered a ROS, it is a far weaker oxidant than superoxide. Other antioxidant enzymes will then degrade the hydrogen peroxide, primarily catalase and GPx. GPx does not react directly with hydrogen peroxide but instead catalyzes reactions with non-enzymatic antioxidant glutathione [1]. In this process, a glutathione molecule will react with typically either hydrogen peroxide or a lipid peroxide and donate an electron to stabilize the radical [1]. Consequently, the glutathione molecule itself becomes a radical, however binding with another glutathione radical will form a single oxidized glutathione molecule [1].

In addition, the body utilizes a number of exogenous and non-enzymatic antioxidants such as vitamins A, C, and E, selenium, folic acid, and α-lipoic acid [2,5,8]. Typically, these compounds are characterized by the ability to stabilize ROS while not causing oxidative damage themselves. For example, vitamin E will scavenge peroxyl radicals within the cell membrane and donate an electron, forming a stable lipid and a tocopherol radical. The

tocopherol radical is much more stable than the previous peroxyl radical and can be converted back to active α-tocopherol by ascorbic acid [1].

Both endogenous and exogenous antioxidants require nutrients that must be provided in the diet for proper synthesis and function [2]. For example, SOD uses copper and zinc or manganese as cofactors [2,7], and GPx is selenium-dependent [2]. Additionally, vitamins A and E cannot be synthesized and must be obtained through feed [2]. Energy intake is also a critical factor in antioxidant function. NADH, an electron carrier produced during energy metabolism, is required for the recycling of enzymes such as GPx [15]. Additionally, glucose is required for biosynthesis of vitamin C, another antioxidative compound [5]. Without proper intake of these nutrients, the body is unable to regulate even normal levels of ROS, resulting in widespread oxidative damage.

1.3. Consequences of Oxidative Stress

Oxidative damage inflicted by ROS at the cellular level results in profound changes at larger scales. Immune cells are particularly susceptible to OS due to the high concentration of PUFAs in their membrane, with excessive damage leading to a loss of function [3,16]. Oxidative damage, particularly as it relates to exercise, is thought to contribute to low-grade muscle tissue damage which manifests as fatigue, decreased exercise tolerance, reduced muscle force production, delayed-onset muscle soreness (DOMS) and overall poor athletic performance [17,18]. In horses, OS has been associated with performance-limiting conditions such as exertional rhabdomyolysis, degenerative myeloencephalopathy, and recurrent airway obstruction [2,5,9].

OS is particularly problematic when it is long-term, which has been linked to chronic inflammatory conditions such as carcinogenesis, osteoarthritis, cardiovascular disease, and

other degenerative age-related diseases [5,19]. OS is also thought to be a major driver of the aging process; progressive declines in cellular function result in an inability to maintain homeostasis and thereby contribute to many age-related diseases [2,7,8]. Others have shown that mitochondrial ROS production across a species is inversely correlated with average lifespan [20], further supporting the role of OS in aging and disease.

1.4. Measuring Oxidative Stress

Within the context of research, measuring oxidative stress can be approached in a few ways. One method would be to directly measure ROS and track the presence of radical species. In practice, many ROS such as hydroxyl radicals have half-lives of less than a nanosecond, and even more stable compounds like hydrogen peroxide or peroxyl radicals have half-lives of seconds to minutes [8]. Therefore, this method is difficult, impractical, and seldom used outside of highly controlled environments.

Another potential method is indirectly measuring ROS through markers of oxidative damage. When ROS react with biomolecules they will often create end-products more stable than the radical themselves, which can be measured more easily [8]. Protein, lipid, and nucleotide oxidation can all be measured using their respective end products. For example, malondialdehyde (MDA) is a well-established measure of lipid peroxidation [8,21]. It is produced as a result of reactions between ROS and PUFAs, which initially create highly unstable hydroperoxides that degrade into more stable aldehydes such as MDA [21].

Besides measuring ROS or oxidative damage, we can also evaluate OS by measuring antioxidant status. This method addresses the capacity to quench free radicals as opposed to generation of ROS [1,8]. By measuring the concentrations or activities of antioxidants, we can evaluate if certain compounds are being depleted or upregulated in response to OS [1,8].

Beyond that, assays for total antioxidant capacity can be used to measure the overall ability for a sample to resist oxidation, allowing for a global view of antioxidant status [8].

Lastly, we can evaluate measures of health and performance known to be associated with OS. Incidence of disease, athletic performance, and biomarkers of tissue damage or inflammation can all be used as supporting measures of OS. Particularly through the lens of exercise-induced OS, DOMS is a common result of ROS overproduction in skeletal muscle. This can be measured through biomarkers of inflammation or tissue damage, subjective score of muscle soreness, changes in force production, and differences in movement can all indicate DOMS. These indicators are often not exclusive to OS and alone cannot be used to demonstrate oxidative damage. However, in combination with the methods discussed above, they can be helpful in evaluating the eventual consequences of oxidative stress on the body.

2. Oxidative Stress in the Performance Horse

2.1. Typical Performance Horse Management and Oxidative Stress

2.1.1. Exercise

Research has shown that many common practices in the performance horse industry can contribute to both acute and chronic OS. Exercise itself, even at low- to medium-intensities, triggers the creation of free radicals through increased oxygen consumption and cellular respiration [22]. Increased cellular respiration also increases the occurrence of incomplete respiration, which is thought to be the source of 90% of generated ROS [3]. Additionally, muscle stress due to intense muscular contractions decrease blood flow to endothelial cells, promoting generation of ROS through aerobic respiration [7]. Simultaneously, damaged muscle cells result in autooxidation of hemoglobin and myoglobin

releases free iron, producing hydroxyl radicals [5]. It has also been shown that microtears in muscle fibers and disruption of cell membranes allow calcium influx, activating enzymes and enhancing ROS production [7]. Then during recovery, immune cells activate to repair muscle damage, releasing ROS such as superoxide and hypochlorous acid [5]. Over time, repeated bouts of exercise without sufficient recovery or antioxidant support may lead to cumulative oxidative damage.

2.1.2. Nutrition

Typical diets fed to domestic horses can contribute to nutrient deficiencies that impair antioxidant defense systems [23]. Selenium is a mineral and essential nutrient that plays a significant role in antioxidant systems [5]. However, one study found that 3.3% of horses were deficient in selenium and 13.6% had a marginally deficient status, as determined by plasma concentrations [23]. Vitamin E is another essential nutrient contributing to antioxidant systems, as discussed prior, however the same study showed that according to plasma concentrations were only considered sufficient in 64.7% of horses, while 15.4% of horses were considered deficient, and another 19.9% were marginal [23]. The study also found that access to green pasture for over 6 hours per day was strongly associated with plasma alpha tocopherol and beta carotene concentrations [23]. As performance horses often lack access to green grass and turnout, this may contribute to an inability to effectively combat chronic OS.

2.1.3. Transport

Performance horse management also often includes hauling, whether to competitions or for other reasons. Long-distance transport in particular is known to increase OS in the

horse, as research shows that horses undergoing 12-hour transport had increased plasma MDA and decreased plasma SOD activity [24]. This can have significant effects; another study in horses undergoing 51 hours of transit over 4 days of transportation demonstrated an impaired cell-mediated immune response which is particularly important when horses may be traveling to novel environments such as a competition [24].

2.1.4. Stereotypic Behaviors

Typical performance horse management such as stalling also increases the occurrence of stereotypic behaviors [5]. Research suggests that horses who engage in cribbing behaviors had significantly decreased total antioxidant status, as well as SOD, GPx, and catalase concentrations [25]. Though a causative relationship has yet to be established, the brain is known to be a significant target of oxidative damage due to its high oxygen consumption [25]. Cribbing behaviors have been tied to increased sensitivity to stress, decreased physiologically and psychologically flexibility, and a reduced ability for operant learning [25]. In addition, it can cause physical harm in the form of increased risk of epiploic foramen entrapment colic and altered neuromuscular function [25].

2.1.5. Age

Age is also a relevant consideration in performance horse management, as advancements in veterinary science allow horses to have longer performance careers [26]. ROS production is known to contribute to aging; in humans, the free radical theory of aging suggests that the aging process is associated with an accumulation of ROS, and therefore cellular damage including apoptosis [27]. Immune cells are uniquely sensitive to oxidative damage as they contain high concentrations of PUFAs in their cell membranes [3,16]. This

possibly explains why in horses aging has been associated with increased risk of infections [28–30] and decreased overall immune response [31–33]. In the context of exercise, senior horses had significantly increased white blood cell apoptosis after exercise than younger yet mature horses, indicating that senior horses may suffer from being additionally immunocompromised during and after exercise [27].

2.1.6. Non-Steroidal Anti-Inflammatory Medication Use

The use of non-steroidal anti-inflammatory medications (NSAIDs) is known to cause oxidative injury [34]. One such NSAID, phenylbutazone, causes the accumulation of ROS in colonocytes, leading to oxidative injury [34]. Additionally, as weak organic acids, NSAIDs are known to damage the mitochondria [34]. NSAID administration in horses also affects the intestinal microbiome, particularly leading to a decrease in *Pseudobutyrivibrio*, a bacterium which typically produces butyrate in large quantities [34]. Butyrate has important functions for maintaining intestinal homeostasis and is a potent antioxidant [34].

2.2. Oxidative Stress and Issues of Concern in the Equine Industry

In addition to experiencing greater levels of OS, performance horses may suffer greater ramifications from acute and chronic OS. Decreased performance and greater health issues may contribute to loss of use, reduced welfare, and economic losses for owners [35].

2.2.1. Athletic Performance

Exercise in and of itself can trigger OS. However, OS also leads to decreased athletic performance in the horse [22]. In endurance horses, performance was best in horses with greater glutathione reductase capacity and decreased TBARS post-race [36], suggesting a

relationship between competition results and antioxidant status. Additionally, exercise-induced OS can contribute to musculoskeletal injury. Exercise increases the permeability of muscle membranes and creates tears in muscle fibers, releasing biomarkers of muscle damage such as CK, myoglobin, and troponin. Other effects of exercise include the tearing of muscles' connective tissue, vasculature damage, changes in calcium regulation in myocytes which affects muscular contractions, and increased cortisol which can trigger protein breakdown and tissue wasting [7,37]. This is highly significant, as research has shown that in racehorses the greatest reason for days lost to training was lameness, and of those 18% were lamenesses due to muscle injury [38].

2.2.2. Gait Kinematics

OS plays a recognized role in the development of musculoskeletal discomfort in horses, directly affecting gait kinematics. One proposed mechanism suggests ROS target hyaluronan in synovial fluid, leading to reduced joint viscosity and, consequently, increased friction and inflammation [22]. Clinically, both acute and chronic lameness are associated with elevated ROS levels [39], and inflamed joints typically contain higher numbers of superoxide-producing phagocytes [40]. Additionally, reductions in ROM have also been linked to elevated concentrations of the pro-inflammatory cytokine IL-1β [41]. These changes often manifest as restricted ROM and, to a lesser extent, decreased stride duration in affected horses [41].

Exercise-induced muscle damage (EIMD) is considered both a contributor to and a consequence of oxidative stress [5,18]. ROS generated during exercise and the recovery period contribute to muscle tissue damage, which can result in DOMS and systemic inflammation [18,19]. In horses, stride duration has been used as an indicator of DOMS [42],

with important implications for health and welfare. Related kinematic parameters, such as stride length, have been associated with increased risk of musculoskeletal injury in racehorses [43] and greater asymmetry of movement [44].

Beyond the clinical implications, gait kinematics have significant relevance for equine performance and the broader industry. Increased stride duration and hock ROM have been positively correlated with judges' subjective assessments of gait quality in young warmbloods at the trot [45], while greater knee ROM has been interpreted as indicative of a more supple and expressive forelimb action [40]. Furthermore, various kinematic measures have been effectively used to predict jumping faults in eventing horses [46].

2.2.3. Recurrent Airway Obstruction

Recurrent Airway Obstruction (RAO) is another condition in horses known to be associated with OS [22]. Symptoms of RAO include dyspnea at rest, chronic coughing and nasal discharge, and exercise intolerance [22,47]. RAO is often triggered by hay dust antigens in the absence of infection and severe RAO is connected to airway neutrophilia and remodeling [47]. In addition, RAO primarily affects adult and geriatric horses, so "inflamm-aging" is thought to be implicated in the pathogenesis of the disease [47]. Even with treatment such as bronchodilators [47], RAO is often performance-limiting and requires lifelong therapy [22].

The causative relationship between OS and RAO is unclear, but research suggests that oxidative damage is an effect of airway inflammation and neutrophil activation [22]. RAO horses in acute crisis, triggered by exposure to bedding and hay allergens, displayed increased glutathione and glutathione redox ratio in their pulmonary epithelial lining fluid [48,49]. These horses in RAO crisis also displayed decreased pulmonary epithelial lining

fluid vitamin C concentrations as compared to horses with RAO but without active airway inflammation [50]. Additionally, for horses in RAO crisis a compromised antioxidant status was associated with increased airway inflammation and decreased pulmonary function [48,49].

When exercised, horses in RAO crisis displayed decreased respiratory capacity causing decreased blood oxygenation, thereby favoring anaerobic glycolysis resulting in decreased ATP formation [22]. These horses also displayed increased hemolysate glutathione and changes in the glutathione redox ratio during exercise than horses in RAO remission, suggesting OS was greater for horses in RAO crisis [22].

2.2.4. Osteoarthritis

Another condition of great concern in the equine industry is osteoarthritis (OA), a degenerative joint disease characterized by cartilage degeneration resulting in pain, decreased range of motion, and lameness [35]. OA is generally accepted to be one sequelae of chronic OS, as research has shown that horses with OA have increased concentrations of ROS, particularly those who were acutely lame [39]. OA is primarily immune-mediated, and studies suggest inflamed joints have greater concentrations of phagocytic cells [22], and the blood of horses with OA had increased neutrophils and markers of neutrophil activity [39], with both types of cells producing ROS as part of their normal functions. In addition, neutrophil activity was significantly correlated with clinical lameness grade in both acutely and chronically lame horses [39]. Additionally, one study found joints of horses with OA had greater concentrations of apoptotic chondrocytes, a driver of cartilage degeneration, with the areas of apoptotic chondrocytes coinciding with areas with greater concentrations of ROS.

This evidence suggests a relationship between oxidative stress and cartilage matrix degeneration [22].

The proposed mechanism is that ROS break down hyaluronan into smaller chains, decreasing the viscosity of synovial fluid and causing friction. It also eliminates hyaluronan's ability to inhibit phagocytosis, which causes more free radicals to be produced. This can directly damage the compounds and biological factors that contribute to cartilage maintenance [22].

OA is highly significant in the equine industry, as a common cause of decreased performance and loss of use [22]. It is the single most common cause of lameness; about 60% of lamenesses are related to OA [35]. Currently there are various treatments for OA but no cure, making it a lifelong condition [35]. It is also of great economic impact, as some estimate the direct cost of OA to a horse owner to be $3,000 a year, with indirect costs mounting to $10,000-15,000 [35].

3. Astaxanthin

3.1. Overview

Astaxanthin is a xanthophyll carotenoid compound that has long been known as a red pigment and animal feed additive [16]. In production animals, supplementation has been shown to result in improved meat color, richer yolk color, and prevention of discoloration of meat products [3,16]. Additionally, it has also been shown to provide a number of beneficial health effects. In fish, astaxanthin supplementation was shown to improve survival rates, stress tolerance, disease resistance, growth rates, and reproductive success [16]. Similarly, poultry supplemented with astaxanthin had increased weight gain, muscle mass, fertility and

salmonella resistance, while swine demonstrated increased muscle mass and decreased backfat thickness [16]. These findings have contributed to interest in astaxanthin as a supplement for other species, including humans and horses.

However, in recent years it has been recognized as a potent antioxidant [3,16]. Despite being a carotenoid, it does not share any of the functions of vitamin A in the body [16]. Instead, interest in astaxanthin as an antioxidant stems from research demonstrating its potential in combating oxidative stress. Research has shown that the oxidant-quenching capacity of astaxanthin is 6000x greater than vitamin C, 10x greater than beta-carotene, and 100x greater than alpha-tocopherol [51]. As a lipid-soluble compound, it is particularly suited to prevent lipid peroxidation and was found to be twice as effective as beta-carotene in preventing liposomal peroxidation [16].

3.1.1. Sources of Astaxanthin

Astaxanthin is most often sourced from the microalgae *Haematococcus pluvialis (H. pluvialis)*, though it can also be found in other algae species [16]. These algae appear to use astaxanthin as a protection mechanism against high salinity, nitrogen deficiency, high temperatures, and light exposure [16]. Not only does *H. pluvialis* contain greater concentrations of astaxanthin than other algae [52], but it also preferentially produces the 3S, 3S' stereoisomer which has increased bioavailability in humans compared to other stereoisomers [53].

3.1.2. Chemical Structure of Astaxanthin

Astaxanthin is made up of two terminal β-ionone rings, connected with a polyene chain [3,16,53]. This allows the molecule to interact with cell membranes, as the hydrophilic

β-ionone rings can lie at the surface while the lipophilic polyene chain resides within the membrane [3,16,53]. Specifically, the terminal rings placed at each end of the polyene chain contain a hydroxyl group, helping to orient molecule at surface of cell membrane [53]. The hydroxyl group, as well as the keto group of the ionone rings are responsible for antioxidant activity of the moiety [16]. The terminal rings scavenge radicals at the outer and inner surfaces of the membrane [16], allowing it to act preferentially to the polyene chain [53]. Conversely, the polyene chain connects the two terminal rings and scavenges radicals within the cell membrane [3,16,53]. It includes conjugated double bonds in the center, allowing the molecule to donate electrons to free radicals, giving the molecule it's antioxidant functionality [16,51]. Comparison to other carotenoid compounds has shown that each moiety of the molecule acts independently, as demonstrated by the fact that the groups attached to the terminal rings had no effect on the action of the polyene chain [53].

3.2. Absorption, Bioavailability, and Dosing of Astaxanthin

3.2.1. Absorption and Bioavailability of Astaxanthin

Like other carotenoids, astaxanthin is digested and absorbed like other fats and lipid-soluble compounds [3,16]. Because of this, feeding astaxanthin along with dietary fats has been shown to increase bioavailability and biological activity. Studies have shown that in humans, feeding carotenoids along with a "fatty meal" increased absorption from 15% to 47% [54], and bioavailability was increased in rats fed astaxanthin solubilized in olive oil as compared to astaxanthin alone [3,16]. In the human model, a single 100 mg dose of *H. pluvialis* biomass was shown to be bioavailable and was found to be present in plasma, though incorporation into cell membranes was not measured [55].

3.2.2. Dosing of Astaxanthin

Little research has been published into an optimal dose of astaxanthin for horses. One unpublished preliminary study claimed that dosing astaxanthin at 750 mg/day had no significant improvement over 75 mg/day [56], indicating diminishing returns with an increased dose. Another study supplemented horses at 250 mg/day for up to four months with no adverse effects or changes in hematological parameters [57].

3.2.3. Toxicity of Astaxanthin

In humans and other animals, no toxic doses of astaxanthin have been reported [3,16,58]. Human studies have used doses as high as 40 mg per day (0.36-0.78 mg/kg BW) with no noted ill health effects [3],while rats have been fed astaxanthin at up to 17,076 mg/kg BW with negative impacts only attributed to deficiencies in other nutrients [59]. A rodent study found that the duration of supplementation had no effect on plasma concentrations, indicating toxic bioaccumulation was unlikely [59]. However, it has been reported that very high levels of astaxanthin supplementation can cause yellow/red skin pigmentation [16,59].

3.3. Antioxidative Properties and Metabolic Functions of Astaxanthin

Astaxanthin has several mechanisms of action for addressing oxidative stress and free radicals. It is able to quench a variety of radicals including singlet oxygen, which is known to radicalize biomolecules leading to a chain reaction of oxidation [16,60]. In addition, its position within cell membranes makes it better suited to inhibiting lipid peroxidation than many other antioxidant compounds [16]. Work done in rodent models has shown that astaxanthin may increase plasma SOD, GPx, and catalase, and reduce TBARS and the

production of singlet oxygen [52,61]. It is also thought to have a positive effect on mitochondrial function and energy metabolism by preventing the oxidation of CPT 1 transporters in mouse models [6]. In some canine studies, astaxanthin was associated with decreased nitric oxide production, markers of oxidative damage to proteins and DNA, mitochondrial oxidative damage, and improved mitochondrial function [20]. It also was shown to increase the animals' GSH/GSSG ratio, indicating decreased OS [20]. In humans, 8 weeks of supplementation prevented an increase in markers of oxidative damage to DNA [28]. Looking at the equine model, Arabian racehorses dosed with astaxanthin at 250 mg/day (0.52-0.58 mg/kg BW) displayed increased total antioxidant status and decreased TBARS and glutathione reductase at rest, and decreased GPx, glutathione reductase, and TBARS after exercise [29]. Additionally, in vitro research on adipose-derived stromal cells from horses with and without Equine Metabolic Syndrome (EMS) suggests that astaxanthin offsets signs of dysfunction typically found in EMS cells as compared to healthy cells, including excessive apoptosis, SOD efficiency and activity, total endogenous antioxidant capacity in EMS cells, and the expression of several genes related to cellular energy metabolism. The researchers observed improved overall mitochondrial function as measured by enhanced membrane potential and decreased percentage of cells with depolarized mitochondria, which were greater in EMS cells compared to healthy ones, suggesting one possible mechanism by which astaxanthin decreases OS and apoptosis [58].

Astaxanthin supplementation has been shown to have a wide range of other beneficial effects on health. Because immune cells have high concentrations of PUFAs in their cell membranes, the antioxidant action of astaxanthin can contribute to anti-inflammatory and immunomodulatory effects. Astaxanthin was shown to decrease several pro-inflammatory

cytokines in both in vitro lipopolysaccharide-treated microglia cells [57]. Additionally, astaxanthin may play a role in intestinal health and regulation of the microbiome. This has been shown repeatedly in the rodent model, including mice who were immunodeficient, obese, or with induced alcoholic fatty liver disease [62–65]. Additionally, astaxanthin was able to modulate β-diversity in polo horses, however the effect of deconditioning and reconditioning was greater [66].

3.3.1. Effects of Astaxanthin on Exercise

As an antioxidant, astaxanthin is also interesting in the context of exercise. The CPT I transporter, which regulates the entry of fatty acids into the mitochondria, is a major target for ROS generated in the mitochondria during exercise. This oxidative damage decreases the ability for the mitochondria to use lipids as an energy source, resulting in an increased reliance on carbohydrate stores and anaerobic metabolism [6]. However, rodent studies support the idea that astaxanthin supplementation can ameliorate this effect.

In mice undergoing a maximal running test, astaxanthin treatment decreased oxidation of CPT I transporters, which has not been seen with other antioxidant supplements, such as vitamin E or C [6]. Researchers also found that astaxanthin treated mice had increased running time to exhaustion and decreased fat tissue accumulation, while also displaying an increase in fat utilization and a decrease in carbohydrate utilization during exercise as indicated by the respiratory exchange ratio [6]. Similarly, in mice subject to various intensities of swimming exercise, astaxanthin treatment increased swimming time to exhaustion, decreased blood lactate, increased plasma non-esterified fatty acids and plasma glucose, and decreased adipose tissue accumulation, all suggesting an increase in lipid utilization as an energy source [67].

In human trials, astaxanthin supplementation may have a positive effect in athletic performance. Supplemented athletes have been shown to have improved performance in a cycling time trial, power output [68], mitigated rise in serum lactic acid [69], CK, and aspartate aminotransferase [70]. Similarly, in racing Arabian horses (for whom exercise is primarily aerobic), after a single training session horses fed astaxanthin at 250 mg/day showed increased total antioxidant status and decreased glutathione reductase and SOD as compared to controls [60]. However, no difference between treatments in number of races completed or number of wins was found between supplemented and unsupplemented horses. Another study demonstrated that thoroughbred racehorses supplemented with astaxanthin along with L-carnitine, had decreased CK and lactate dehydrogenase isoenzyme-5 after exercise, indicating the supplement decreased EIMD [56]. This was also demonstrated by a significant decrease in clinical signs of EIMD [56].

4. Carnitine

4.1. Overview

Carnitine is a naturally occurring chemical compound found in all mammals, along with some plants and microbes [71]. Among its many functions in the body, carnitine is required for the transport of fatty acids into and out of the mitochondrial matrix [71].

4.1.1. Sources of Carnitine

Carnitine is produced through biosynthesis almost exclusively in the liver, with the primary substrates are lysine and methionine. Additionally, vitamin B_6, nicotinic acids, vitamin C, and folate are required as co-enzymes [71]. Despite the ability of the mature

animal to produce carnitine, it has been shown that neonates are unable to synthesize enough carnitine to meet metabolic requirements. Instead, neonates rely on the carnitine present in milk [71]. Because of this, plasma total carnitine increases steadily during the first three years of life, after which it remains more stable [72]. Although some plants do contain carnitine for their own energy metabolism, research has shown that significant dietary contribution comes from animal derived products, which is typically irrelevant in equine nutrition, and studies have shown that vegetable oils contain very little if any carnitine [71].

In the body, skeletal muscle is the primary reservoir of carnitine, with middle gluteal muscle concentrations being 200x that of plasma [73]. This carnitine is present in multiple forms, the primary being free carnitine. However, carnitine can also bind to acetyl groups, forming acetylcarnitine, or to long-chain fatty acids (LCFAs), forming carnitine esters [71]. Within the muscle of the horse, 88% is present as free carnitine and 7% acetylcarnitine [74]. The remaining carnitine is primarily found in the blood, with typical concentrations for horses ranging from 15 to 55 μmol/l with large variations between individuals [72,75–77]. Of this, 70-80% is present as free carnitine, 7% as carnitine esters with medium to long-chain fatty acids, and the remainder as acetylcarnitine [72,73,75,78].

Both single bouts of exercise (acute exercise) and consistent training have an effect on carnitine in blood and tissues. Acute exercise has been shown to trigger a dramatic shift from free carnitine to acetylcarnitine in plasma and muscle, due to the increased transport of fatty acids into the mitochondria [74,77,78]. Researchers have found that with low to moderate intensity exercise, acetylcarnitine concentrations doubled with an equal reduction in free carnitine [78]. However, at high intensities, acetylcarnitine reached a peak concentration similar to that of moderate exercise, and instead a shift to a rapid increase in

lactate and glycerol-3-phosphate was observed [78]. This indicates the formation of acetylcarnitine may play a key role in regulating the mitochondrial acetyl CoA to CoA ratio. Alternatively, it could serve as a protective mechanism that limits the entry of excess fatty acids, transported as acylcarnitine, into the mitochondria during high-intensity exercise, when they are unable to significantly contribute to energy production. It was also observed that this effect was short-lived, with concentrations returning to normal within 30 minutes after intense exercise [78]. Although no changes in total carnitine were observed with acute exercise, consistent training over time has been shown to increase total carnitine. Trained 3- to 6-year old horses had 30% greater basal plasma free carnitine than age-matched, untrained horses, potentially due to an increase in biosynthetic ability, or adaptations to retain carnitine within the body [72]. This effect was not true for middle gluteal muscle carnitine, where basal concentrations did not significantly differ between trained and untrained horses [77].

4.1.2. Chemical Structure of Carnitine

L-carnitine is categorized as a quaternary amine [71,79]. Theoretically, the asymmetric structure at carbon 2 allows for the existence of two enantiomeric forms, however while D-carnitine does not exist in nature but can be synthesized [71]. Its structure also lends to carnitine's highly polar and primarily water-soluble nature [71]. More specifically, carnitine is zwitterionic and chemically similar to lecithin, allowing it to also cross lipid membranes [71].

4.2. Absorption, Bioavailability, and Dosing of Carnitine

4.2.1. Absorption of Carnitine

When supplemented orally, L-carnitine is absorbed primarily in the proximal jejunum of the small intestine. While intestinal absorption is thought to be higher in younger animals as to absorb the carnitine present in milk [71], absorption of supplemented carnitine has not yet been measured. Exact measurement of oral availability of oral carnitine in adult horses has not been measured but based on several supplement studies it is assumed to be low [73,75]. In one study, horses administered 10 g of carnitine orally were compared to horses given the same dose intravenously, which indicated an estimated oral absorption of 5.3% based on urinary excretion [73]. Another study fed up to 60 g of carnitine per day and found only 3.5% to 7.5% of the dose was excreted in urine within 24 h [75].

4.2.2. Bioavailability of Carnitine

Despite low urinary excretion estimates, it has been shown that the small proportion of L-carnitine that is absorbed orally does notably increase plasma concentrations in the horse [76]. Horses that received 10 g of L-carnitine orally showed doubled blood carnitine concentrations within 4 hours. Values gradually returned to normal but had not fully by 12 hours [73]. While this response was considered small as compared to intravenous administration, which raised levels from 25 to 1000 μmol/l [73], it still displays physiologic changes as a result of supplementation.

However, research has shown that it is unlikely that oral supplementation has a substantial impact on muscle carnitine concentrations in the horse, potentially due to changes being more difficult to detect due to greater carnitine concentrations in muscle than plasma

[71]. Supplementation of carnitine to trained horses for 8 weeks, starting at 10g and increasing to 60g, showed a trend towards an increase in middle gluteal muscle carnitine concentrations [75], whereas untrained horses supplemented with carnitine showed no increase in muscle concentrations [73]. Even when carnitine was administered intravenously for 26 days, horses in light work did not show an increase in muscle concentrations [73].

In horses, some research indicated that effects from oral supplementation may not be dose dependent. It was found that the increase in plasma carnitine concentrations was greater in horses receiving 1g of carnitine than 6g, and even the control treatment of soya oil was able to increase carnitine concentrations [71]. One theory is that this is due to the timing of administration [75], as evidence indicates that intestinal transport capacity is a limiting factor in the ability for the ability for oral carnitine concentration to cause changes in plasma carnitine concentration [71]. In one study single doses of 10-30g showed a peak in plasma concentrations between hours 2 and 4 after consumption, versus when the dose was split between morning and evening feed, the increase in plasma carnitine was only slightly greater but levels remained more stable during the day [75].

4.2.3. Dosing of Carnitine

In the horse, a wide range of doses of carnitine have been tested, but no ideal dose has been identified. Doses as low as 1g have been shown to have an effect on plasma concentrations [37]. However, most studies on the physiological impacts of L-carnitine have used 10-60g per day for chronic supplementation [73,75].

4.2.4. Toxicity of Carnitine

No toxic effects of L-carnitine supplementation have been reported in horses, even at doses as high as 60g/day [75]. However, in human research oral administration has been reported to cause dyspepsia, and an increase in pro-atherogenic factors linked to read meat consumption [80]. Generally, 2g of carnitine daily is considered safe for chronic supplementation in humans [80]

4.3. Antioxidative Properties and Metabolic Functions of Carnitine

The primary function of L-carnitine in the body is in aerobic fat metabolism [71,81]. Because long-chain acyl-CoA derivatives cannot cross the inner mitochondrial membrane alone, it requires a carrier in order to be usable for β-oxidation. In the cytosol, acyl-CoA and carnitine are converted into acylcarnitine. The reaction is catalyzed by CPT-1, which is located on the surface of the inner mitochondrial membrane. Then, acylcarnitine is transported into the mitochondrial matrix in a 1:1 exchange with free carnitine, mediated by the inner mitochondrial membrane protein carnitine-acylcarnitine translocase. Meaning, for each carnitine ester that crosses the inner mitochondrial membrane, a free carnitine has to be transported back to the opposite direction. Typically, acylcarnitine is entering the mitochondrial matrix and free carnitine is exiting, but this can operate in the opposite direction based on thermodynamic equilibrium [71]. Inside the mitochondrial matrix, Palmitoyl-transferase-2 converts acylcarnitine back into free carnitine and acyl-CoA, which is then available for β-oxidation.

Carnitine also plays a role in fat metabolism under stress conditions such as ketosis, diabetes, starvation, and hypoxic exercise [71,82]. These states cause an accumulation of

acetyl-CoA in the mitochondria [71,82], which triggers the transfer of the acetyl moiety of acetyl-CoA to carnitine, forming acylcarnitine which can then be transported back to the cytosol [71,83]. This raises the acetyl-CoA to CoA ratio in the mitochondria, which is required to maintain equilibrium for the TCA cycle [83] and formation of ATP [84]. This decrease in free carnitine limits the transport of LCFAs into the mitochondria, which under hypoxic conditions such as intense exercise are unable to contribute to energy metabolism [71], further supporting carnitine's function in maintaining the acetyl-CoA/CoA ratio [71,74].

In addition, carnitine has been observed to have antioxidant properties. Research shows that in vitro, carnitine can inhibit the oxidation of blood plasma proteins and is particularly effective in scavenging hydrogen peroxide and superoxide [85]. This is mirrored in vivo; rats with ischemia-reperfusion induced damage to gastric mucosa and treated with carnitine had reduced lesion scores and TBARS concentrations, and lessened decreases in catalase and prostaglandin E_2 [86]. Similarly in rats treated with ethanol to induce OS, carnitine supplementation was able to improve levels of antioxidant enzymes and markers of oxidative damage in both the brain and serum, some of which back to a level equivalent to control animals not treated with ethanol [87]. Additionally, in humans with type II diabetes, daily carnitine supplementation decreased TBARS and conjugated dienes while also improving oxidized LDL, LDL cholesterol, triglycerides, apolipoprotein A1 and apolipoprotein B-100 [88].

4.3.1. Effects of Carnitine on Growth and Maintenance

Despite low oral bioavailability, carnitine has proven effective as a supplement in both the horse and other mammals. In humans supplemented with 2-3g of carnitine a day for two weeks, improved plasma glucose and ammonia, and lowered heart rate were observed,

although it did not show any effect on fat or carbohydrate metabolism [79,89,90]. Pregnant mares supplemented with carnitine showed no changes in foal birth weight, weight gain, or other growth parameters, and researchers were unable to establish a direct relationship between carnitine concentrations in milk and concentrations in foal plasma until 28 days of age [91]. Further research showed yearlings supplemented with 10g of L-carnitine a day had greater daily gain, efficiency of utilization for DE and digestible protein [71]. Other findings included increased plasma glucose and decreased triglycerides, possibly indicating increased fat utilization and decreased carbohydrate utilization for energy. In healthy adult horses, carnitine may increase leptin, but had no effect on insulin sensitivity, indicating it likely is not effective in relieving metabolic conditions [92]. However, many have concluded that it is unlikely that carnitine supplementation will benefit athletic performance in animals not in regular training [93].

4.3.2. Effects of Carnitine on Exercise

Carnitine is primarily of interest in the context of exercise as a way of improving the efficiency of energy metabolism. In humans, carnitine supplementation has been shown to increase exercise performance due to its function in maintaining the acetyl CoA/CoA ratio in the mitochondria, decreasing lactate production [94]. Studies have shown both acute and chronic supplementation improves exercise capacity and endurance [95]. Additionally, football players given a single dose of 3-4g of carnitine had faster running speed and lower heart rates at corresponding lactate levels to untreated individuals [96], while another study demonstrated increased muscle mass [97,98]

In exercising horses at rest, carnitine supplementation was shown to increase plasma glucose and creatine and decrease plasma lactate and CK [71]. One study demonstrated that

horses who were supplemented with carnitine at 10g/d for 5 weeks and regularly conditioned, and then deconditioned for 5 weeks while supplementation continued, demonstrated an increased muscular response to training [93]. Those horses displayed a 46% increase in gluteal muscle total carnitine content [93]. Additionally supplemented horses had an increased percentage of type IIa fibers, reduced cross-sectional area of type I fibers, a 40% increase in capillary-to-fiber ratio, and a 35% increase in capillary density, but these difference partially or fully disappeared after deconditioning [93].

During exercise and recovery, carnitine supplementation at 5 g/day has been shown to mitigate increases in lactate, CK, and lactate dehydrogenase activity in the horse after exercise [71]. Additionally, 6 g of L-carnitine a day led to a quicker recovery of blood glucose levels after submaximal exercise than horses getting 1 g or a control treatment [104]. However, the effect of carnitine supplementation on lipid metabolism in the horse appears to be dependent upon the specific exercise tests studied. Plasma non-esterified fatty acids were shown to have a greater increase in horses supplemented with carnitine who were trotted over a 2600 m distance, [104], whereas a lesser increase was seen in supplemented horses subject to 1280 m of submaximal galloping exercise [105]. Supplemented trotting horses also had a reduced increase in triglycerides as compared to unsupplemented horses, whereas a greater increase was seen among galloping horses. Nevertheless, L-carnitine supplementation at 10 g/d for 5 weeks increased muscular response to training [93].

Additionally, carnitine may be helpful in exercise recovery. One study showed a protective effect against muscle damage, indicated by increased insulin-like growth factor-binding protein-3 concentrations prior to and after exercise [99]. Additionally, carnitine was shown to alleviate pain and tenderness and decrease CK after exercise, indicating reduced

muscle tissue damage resulting in cytosolic protein leakage [100]. It is suggested that carnitine can improve delivery of oxygenated blood to muscle tissues during exercise and thereby reducing subsequent muscle tissue damage and delayed onset muscle soreness [99,101–103]. It is believed that this is due to its activity as an antioxidant and in promoting lipid metabolism as opposed to anaerobic metabolism, which can lead to further generation of ROS. This ability to combat oxidative damage relieves stress on endothelial tissue, promoting blood flow and oxygen delivery [103]. This is echoed by MRI imaging that identified decreased muscle tissue disruption after exercise in humans administered intravenous carnitine [99,101].

5. Vitamin E

5.1. Overview

Vitamin E is a class of several compounds that are known to be an essential nutrient for horses and other animals [5]. The primary purpose of vitamin E is as a non-enzymatic antioxidant [5]. As a lipophilic compound, it incorporates into cellular membranes [5], making it effective in preventing phospholipid peroxidation and membrane damage [106,107]. Vitamin E also plays a role in maintaining immune function, as immune cells are at high risk of OS due to high concentrations of PUFAs in their cell membrane [106]. Vitamin E also impacts the transcription rate of certain genes [106], such as CD36 [108], α-tropomyosin [109], and collagenase [110]. Other functions of vitamin E independent of antioxidant ability include inhibition of cellular proliferation [111], activation of nuclear factor NF-κB [112], platelet aggregation [113], monocyte adhesion [114], and stabilization of plasma membranes [115].

Vitamin E deficiency has a range of deleterious effects. Because of the role of vitamin E in maintaining immune cells, in many species a dietary deficiency can impair T- and B-cell function [116]. Vitamin E deficiency is also implicated in the development of several motor neuron diseases, such as white muscle disease, equine degenerative myeloencephalopathy, neuroaxonal dystrophy, equine motor neuron disease, and vitamin E-deficient myopathy [5,106].

Vitamin E is also used in the treatment of some neurological diseases in the horse. Recommended therapeutic doses range from 1,500-2,000 IU/500kg/d based on belief that vitamin E may be neuroprotective, even in disorders not related to vitamin E deficiency [106].

5.1.1. Sources of Vitamin E

The levels of vitamin E in feedstuffs vary [5], but fresh forages or forages harvested at immature stage generally have the greatest concentrations at 30-100 IU/kg DM, while grains have lower concentrations at 20-30 IU/kg DM [5]. Additionally, natural vitamin E declines in feeds over time with storage; as much as 53-73% in alfalfa stored for 12 weeks [5]. Vitamin E is usually included in most fortified feeds, most often in its synthetic form [5]. It can also be supplemented individually and has been shown to be the most commonly supplemented antioxidant in horses [4]. However, evidence shows that labeling in supplements may be inconsistent with the true concentration [117].

5.1.2. Chemical Structure of Vitamin E

There are 8 distinct forms of vitamin E, all of which include a chromanol ring and a 16-carbon side chain [5]. Each of these 8 forms have differences in biological activity

[5,106]. They can first be separated by the saturation of the side chain; tocopherols are saturated while tocotrienols are unsaturated. Within those two groups, each have 4 isoforms- α, β, γ, and δ, all differing in number and placement of methyl groups on chromanol ring [106]. The most biologically relevant form, α-tocopherol, has 8 stereoisomers [106]. RRR-α-tocopherol, often referred to as "natural vitamin E" has the greatest biological and antioxidant activity, with 1.49 IU/mg [5,106]. Alternatively, a racemic mix of all 8 α-tocopherol stereoisomers, often referred to as "all-rac-α-tocopherol" or "synthetic vitamin E" [5,106] is more common and used to set the international standard of 1 mg = 1 IU [106].

The mechanism of action of α-tocopherol as an antioxidant comes from the phenolic group, which donates a hydrogen atom to lipid peroxyl radicals and converts them to a staple peroxyl, which can then be degraded by GPx. In this process, the α-tocopherol is converted to a tocopherol radical, which is much more stable than the lipid peroxyl radical and can be converted back to a reduced state by vitamin C [5].

5.2. Absorption, Bioavailability, and Dosing of Vitamin E

5.2.1. Absorption of Vitamin E

The intestinal absorption of vitamin E functions similarly to other lipids [106]. It has been shown that the absorption and transport of vitamin E requires normal fat absorption and metabolism [5]. Following dietary fat intake, pancreatic esterases and bile acids are secreted, resulting in the formation of micelles including vitamin E. These micelles are incorporated into intestinal enterocytes through passive uptake, before being passed along to chylomicrons in the lymphatic system for hepatic uptake and distribution throughout the body [106]. In

humans, it has been shown that supplementing vitamin E along with a high-fat meal increased concentrations in both plasma and chylomicrons [118].

In other species vitamin E status can be limited by high dietary concentrations of PUFAs [5], although this has not yet been shown in the horse [119]. This may be offset by the fact that many dietary PUFA sources naturally contain relatively high amounts of vitamin E [5].

5.2.2. Bioavailability of Vitamin E

The bioavailability of vitamin E is dependent on the chemical composition of the vitamin, the formulation of the product, and the process of micellization. Studies have shown that the alcohol form of the vitamin is more biologically active and is preferentially incorporated into rat tissues as compared to the acetate form [120]. It is thought that hydrolysis of the acetate form limits absorption in the small intestine as compared to the alcohol form [120]. Commercially available forms of vitamin E supplements include powdered, lipid, emulsified, and micellized [120]. One study demonstrated that over seven days the powdered supplements, specifically the powdered acetate and alcohol forms of the vitamin, had the lowest bioavailability [120]. However, another study comparing powdered versus liquid supplements did not find a difference between the formulations [121]. Evidence also suggests that bioavailability is increased by the processing of micellization, a technique that mimics the body's natural process of emulsifying fats. The vitamin E is surrounded by surfactants or emulsifiers similar to bile acids, forming small, spherical structures known as micelles that can combine with water [120]. Comparatively, micellized alcohol powders may have had slightly greater bioavailability than non-micellized powders, but the greatest bioavailability was found with the liquid micellized formulation after 7 days of

supplementation [120]. The authors of that study theorized this was because the micelles transported the vitamin E more efficiently through the intestinal tract than non-micellized forms [120].

As mentioned, RRR-α-tocopherol has the greatest biological activity of the different forms of vitamin E [5,106]. It is also more readily absorbed than synthetic forms resulting in higher serum α-tocopherol concentrations, due to selective uptake by the liver and release into the bloodstream [42,122]. Studies in the horse have shown 1 IU of RRR-α-tocopherol is equivalent to 2 IU of all-rac-α-tocopherol in increasing plasma concentrations [106].

There is consistent evidence that vitamin E supplemented to horses can be absorbed and incorporated into tissues. For instance, feeding α-tocopherol at 300 IU/kg DM significantly increased both serum and middle gluteal muscle α-tocopherol concentrations compared to lower levels of 44 or 80 IU/kg DM [123]. Similarly, racehorses given 1,400 IU/day of D-α-tocopherol showed higher plasma tocopherol levels 8 hours after training, with even greater concentrations observed before, immediately after, and 8 hours post-training by day eight of supplementation [124]. Supplementation with 4,000 IU/day of various forms of vitamin E led to significant increases in serum tocopherol after just one and two weeks, regardless of the form of the supplement used [122]. Plasma α-tocopherol levels also rose significantly after 10 days of supplementation at both 1,000 and 10,000 IU/day, with the higher dose group showing a more pronounced effect [125]. Notably, supplementation with 10,000 IU/day of RRR-α-tocopherol for 24 days resulted in a marked increase in cerebrospinal fluid (CSF) α-tocopherol concentrations [126]. These findings collectively demonstrate that vitamin E, particularly in the form of RRR-α-tocopherol, is

effectively absorbed and distributed throughout the body in response to dietary supplementation.

5.2.3. Dosing of Vitamin E

Currently, dietary requirements for vitamin E are set by the National Research Council. For horses at maintenance, the requirement is set at 1 IU/kg BW based in maintenance of erythrocyte stability in vitamin E deficient foals, enhancement of humoral immune function in mature horses, and maximization of tissue stores of vitamin E [5]. For growing horses under 2 years of age, there is a lack of research, but current evidence suggests that the recommended 2 IU/kg BW should be more than sufficient (NRC, 2007, p. 116). For horses in work, the requirement is also increased due to evidence that exercise induces lipid oxidation [5] and regular training depletes serum vitamin E when fed at deficient to moderate levels (0.3-0.16 IU/kg BW) [5,123]. While a single submaximal exercise bout did not alter plasma tocopherol concentrations [4,123], 150-250 IU/kg DM of vitamin E was necessary to prevent a drop in serum α-tocopherol for horses in regular work [5]. Other research shows that horses supplemented with 5000 IU/d had a decrease in white blood cell apoptosis and plasma CK activity, during and after a simulated 55km endurance race [5,127].

5.2.4. Toxicity of Vitamin E

Vitamin E toxicity in horses is very rare, as unlike other fat-soluble vitamins, it does not accumulate in the body [128]. Excess vitamin E is regulated through the cytochrome P450 system, and can be sequestered in the liver, rapidly metabolized, or excreted, and at a certain point tissue and plasma concentrations will plateau and additional supplementation

will have no effect [128]. Currently, according to the NRC the upper limit for vitamin E is set to 20 IU/kg BW, or 10,000 IU for a 500kg horse based on reports of coagulopathy and impaired bone mineralization in other species [5]. Additionally, there is some evidence that in exercising horses, doses of 10 times the NRC requirement may have had a negative impact on beta-carotene absorption [107].

5.3. Antioxidative Properties and Metabolic Functions of Vitamin E

Under normal conditions, 90% of vitamin E in the body is stored in adipose tissue [129]. Because of this, normal plasma levels vary widely between healthy horses [130]. In general, plasma concentrations <1.5 μg/mL are considered deficient, 1.5-2 is thought to be marginal, and >2 is generally adequate [5,106]. Despite this variation, plasma concentrations are thought to accurately reflect adipose tissue, hepatic, and muscular concentrations based on current research [131,132]. Variation may also be dependent on age, as foals, weanlings, and yearlings have statistically lower plasma concentrations than mature horses. Even with no apparent neuromuscular abnormalities and regardless of season or diet, 80% of weanlings were vitamin E deficient while only 20% of adults were when using a cutoff of 1.2 μg/mL [133]. This effect is thought to be due to low levels of α-tocopherol in the mare's milk and colostrum [134,135].

Currently, there is no strong evidence to suggest a benefit in supplementing vitamin E to healthy, inactive horses who are already receiving adequate dietary concentrations [106]. However, there may be some benefits in various body systems such as immune function. For example, horses fed supplemental vitamin E and selenium had increased IgG after receiving a vaccine [136], and horses supplemented with all-rac α-tocopherol for 16 weeks had

increased IgG_a, IgG_T, and increased bacterial killing capacity of monocytes and neutrophils [137].

5.3.1. Effects of Vitamin E on Exercise

Where vitamin E truly shows its function as a supplement is under stress conditions such as exercise and training. In humans, vitamin E reduced EIMD, as measured by CK and lactate dehydrogenase levels [11]. In the horse, the evidence of an effect on EIMD is more unclear. One study found that after a simulated endurance race, horses supplemented with 5,000 IU/day had a significant reduction in lactate and CK [127]. However, other studies have shown that doses of 5,000 and 10,000 IU/day did not change lactate or CK levels in unfit horses after a treadmill interval test [107], and doses up to 300 IU/kg DM did not have an impact on serum AST or CK after a repeated submaximal exercise test [123].

Vitamin E shows promise as a supplement targeting antioxidant status in the exercising horse. Supplementation at 1.8 IU/kg BW/day significantly altered GPx levels immediately and 24 hours after moderate exercise in unfit horses [138]. Additionally, racehorses fed 1,400 IU/d of α-tocopherol for one day had greater Trolox equivalent antioxidant capacity (a general measure of a sample's resistance to oxidation) than controls 8 hours after exercise, and after 8 days supplemented horses had decreased MDA 8 hours after exercise and had greater Trolox equivalent antioxidant capacity than controls before, after, and 8 hours after exercise [124]. When it comes to markers of oxidative damage, horses receiving RRR-α-tocopherol had reduced levels of plasma protein oxidation after exercise compared to horses receiving synthetic α-tocopherol [122]. However, other research has found that supplementation up to 300 IU/kg DM did not have an impact on middle gluteal muscle TBARS or conjugated dienes after a repeated submaximal exercise test [123].

There is also evidence of a relationship between vitamin E and markers of lipid peroxidation such as MDA/TBARS in the exercising horse. In fit horses, plasma vitamin E and MDA have been shown to be inversely correlated [106,139], and is middle gluteal muscle vitamin E and TBARS [5].

Across species, results on the effect of vitamin E supplementation on athletic performance is inconsistent. Studies have measured changes in aerobic capacity, EIMD, muscle recovery, exercise-induced insulin sensitivity, muscle damage markers, muscle soreness, sports performance metrics, muscle function and strength, muscle mass gains, acute stress response, and systemic markers of inflammation without consistent results [140]. In the horse, a study comparing supplementation with natural versus synthetic vitamin E at various doses, combined with a 6-week training protocol, found that none of the treatments affected TBARS, total glutathione, protein carboxylation, CK, IL-10, IL-1b, or IL-6 [42]. However, supplementation at 4000 IU/d of natural vitamin E did mitigate decreases in stride duration and lead to lower TNFα and AST levels as compared to horses given synthetic vitamin E at either 4000 or 1000 IU/day [42].

6. Conclusion

ROS are natural byproducts of cellular metabolism and serve important roles in normal physiological functions. However, when ROS production exceeds the body's ability to neutralize and eliminate them, OS occurs. This imbalance can result in cellular and tissue damage, ultimately damaging overall health and function.

In performance horses, several routine management practices can contribute to both acute and chronic OS. Exercise, while critical for conditioning and musculoskeletal development, is one of the most significant contributors. It increases mitochondrial oxygen consumption, leading

to elevated ROS production. When exercise is repeated without sufficient recovery or antioxidant support, the resulting oxidative burden may intensify tissue damage and has been linked to the development of various equine conditions detrimental to health and performance.

To counteract the effects of OS, nutritional interventions and commercial supplements have become an increasingly common strategy. Supplementation with antioxidant compounds is one such approach, designed to either enhance the body's endogenous antioxidant defenses as does vitamin E, or to neutralize free radicals directly like astaxanthin.

In addition to direct antioxidant support, improving energy metabolism is another avenue for reducing OS. Carnitine has been shown to decrease ROS formation by supporting more complete oxidation of lipids, especially during periods of high energy demand. In doing so, carnitine may indirectly mitigate OS and improve recovery in exercising horses.

The goal of these interventions is to protect cellular integrity, promote efficient recovery, and enhance athletic performance. By proactively managing oxidative stress, equine caretakers can better support the performance, welfare, and long-term health of their horses.

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CHAPTER II

EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON OXIDATIVE STRESS IN YOUNG EXERCISING HORSES

1. Introduction

Oxidative stress (OS) is defined as a condition under which the concentration of free radicals exceeds the capacity of endogenous antioxidant mechanisms [1,2]. Free radicals, and specifically reactive oxygen species (ROS), are chemical compounds containing an unpaired electron, making them highly reactive and prone to oxidizing biomolecules. Conversely, antioxidants are a class of molecules that are able to neutralize the damaging effects of free radicals while remaining stable.

It is well-established that ROS contribute to many metabolic processes [1,3,4], and a moderate increase in ROS during exercise has a positive effect as well [5]. ROS are necessary for force production in skeletal muscle [6], training-induced adaptations in endurance [7], adaptation of endogenous antioxidant defense systems to exercise [8], and initiating the repair of damaged muscle fibers [9] in humans.

However, exercise-induced increases in ROS production have the potential to reach a level that can no longer be sufficiently regulated by antioxidant systems. This leads to oxidative damage, a hallmark of oxidative stress, which has detrimental impacts on health and athletic performance. Effects include increased muscle fatigue [6,10], muscle soreness [11], and systemic inflammation [12], as well as reduced exercise tolerance [13], and decreased overall athletic performance [11,13]. An increase in oxygen intake seen with exercise increases production of ROS, through increased mitochondrial respiration, as well

as an association with prostanoid metabolism and catecholamine activity [14,15]. Additionally, trauma to muscle fibers can lead to the disruption of iron-containing proteins, inflammation, and accumulation of leukocytes and macrophages, which will also increase the production of ROS [14]. Historically, OS has typically been evaluated by the measurement of antioxidative compounds or byproducts of oxidative damage [1]. However, little research has been done evaluating gait kinematic parameters such as stride duration and range of motion as a measure of OS in the horse. Stride duration has been studied in relation to serum vitamin E concentrations after supplementation in the horse [16], but the relationship between movement and oxidative stress biomarkers was not analyzed. Gait kinematics has however been used in the horse as an indirect measure of damage to joint structures and joint discomfort [17–19], both known sequelae of chronic OS [15,20]. Others have shown that strenuous exercise has a direct impact on gait kinematics in the horse [18,21]

The objective of this study was to evaluate the use of a commercial antioxidant supplement on OS and gait kinematic parameters in young horses beginning a typical training program. We hypothesize that administration of the antioxidant supplement will result in increased oxidative capacity and decreased oxidative damage.

2. Materials and Methods

2.1. Ethical Approval

The study protocol was approved by Colorado State University Animal Care and Use Committee (Approval number, 5151; Approval date, 13 December 2023).

2.2. Animals

This study included 18 stock-type horses (*Table 1*) consigned to a university colt-training program and housed at the Colorado State University Equine Teaching and Research Center. Seventeen horses were registered American Quarter Horses, and one horse was a registered American Paint Horse. Horses were stratified by sex and age and randomly assigned into a treatment group (SUPP) and a placebo group (CON) of nine horses each. Throughout the 60-day study period, body weight (BW) was recorded weekly using a livestock scale, and Henneke body condition score (BCS) [22] was recorded every other week by two independent trained evaluators and averaged.

Table 1
Horse demographics and initial body measurements

Treatment	N	Age (months)[a]		Weight (kg)[b]		BCS[c]		Height (cm)[d]	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
SUPP	9	28.2	6.0	457.4	49.5	5.5	0.7	144.8	5.1
CON	9	29.7	7.2	457.8	43.9	5.4	0.5	146.3	2.9
Overall	18	28.9	6.5	457.6	45.4	5.5	0.6	145.5	4.1

[a] Determined using AQHA/APHA registered date of birth
[b] Measured on day 0 using calibrated livestock scale
[c] Measured on day 0, scores were recorded by two independent evaluators and averaged
[d] Measured using a standard horse height measuring stick on day 30

2.3. Supplementation and Feeding

Beginning on day 1, horses were fed 15g of either the supplement (SUPP; Medical Muscle, Full Bucket) or a placebo product (CON) once daily. The supplement contained 6000 mg of L-carnitine, 75 mg of astaxanthin, and 1200 IU d-alpha-tocopherol. The CON product was visually identical to SUPP but contained no active ingredients. Horses were fed 100 g of a minimally fortified sweet feed (Sweet Mix Plus Feed, Country Acres) with a small amount of water to facilitate intake of the supplement. Researchers were blinded to the

treatment for the duration of the study and during data analysis. In addition to supplementation, horses were fed a grass-alfalfa hay mix ad libitum and 0.91 kg of a ration balancer (Enrich Plus, Purina Mills) to meet all dietary requirements. Horses had ad libitum access to water and salt.

2.4. Exercise and Housing

Horses were in light work [15] prior to the start of the study. Period 1 (days 1 through 30) was used as a deconditioning period where horses were kept at maintenance with no forced exercise. Horses were group-housed in dry lot paddocks (520 m^2) of 4-6 horses each. Starting in period 2 (days 31 through 60), horses were kept at moderate work and housed individually in box stalls (13.4 m^2) with daily outdoor turnout. Workload was typical of an introductory training program and included both groundwork and ridden work at all three gaits, for a minimum of 90 minutes 3 times per week.

On day 31 all horses received routine veterinary care, which included vaccinations, dental care under sedation, blood draws, and a lameness exam. Additionally, all but two horses (one from each treatment group) were subjected to arthrocentesis as part of another research study.

2.5. Data Collection

2.5.1. Gait Kinematic Analysis

On days 30 (end of period 1) and 60 (end of period 2), and prior to any exercise that day, horses were video recorded using an iPad Pro (Apple, CA, 11”) at the walk and trot to evaluate stride duration and range of motion.

Stride duration (DUR) was measured in milliseconds (ms) and was defined as the time it took for the horse to complete one stride, beginning and ending when the right front hoof made contact with the ground. Knee range of motion (KRM) and hock range of motion (HRM) were considered to be the difference between the minimum and maximum angle of that joint within a single stride, which was measured in degrees (°).

To allow for accurate visualization and consistent measurements, duct tape markers were placed on relevant anatomical landmarks by the same trained individual for each horse on both days. Forelimb markers were placed on the cubital, midcarpal, metacarpophalangeal, and proximal interphalangeal joints. Hindlimb markers were placed on the femorotibial joint, calcaneus, and metacarpophalangeal joint.

A familiar handler led the horses from the left side over an approximately 9m distance, while the horses' right side was recorded. This was repeated until three videos with at least three normal strides with no interruptions were captured for each gait. In total, six videos were captured per horse on each of the two days.

Videos were uploaded to Kinovea motion analysis software (*v 2023.1.2*) for analysis. Within each video, the three most clearly visualizable strides were measured for DUR, KRM, and HRM. Within Kinovea, DUR was measured by subtracting the timestamp of the first frame of the stride from the timestamp of the last frame of the stride. Both KRM and HRM were measured using the angle tool, with the points on either the knee or hock serving as the center of rotation, and the joints directly proximal and distal to it were used to calculate angle. For each video, the angular kinematics function was used to export data into excel, where minimum and maximum measurements for each stride were extracted. All parameters were measured separately for each stride and then averaged across each video for statistical

analysis. Additionally, parameters for walk videos (W-DUR, W-KRM, W-HRM), were analyzed separately from parameters for trot videos (T-DUR, T-KRM, T-HRM).

2.5.2. Standardized Exercise Test

On days 30 and 60, a standardized exercise test (SET) was conducted. Horses were worked on the ground without restraint in a 9.14 m round pen by a familiar handler. The test consisted of 5 minutes at the walk, 10 minutes at the trot, 6 minutes at the canter, and finally 5 minutes at the walk, with the direction being reversed at the halfway point for each gait. In total, the SET was 26 minutes in duration, intended to simulate a typical workout for a young horse in training.

2.5.3. Heart Rate

Horses were fitted with a heart rate monitor (*Polar, Equine V800 Science*) during the SET to record average (HR_{avg}) and maximum (HR_{max}) heart rate to evaluate exercise intensity and evaluate changes in exercise intensity. Heart rate data was uploaded to the Polar FlowSync application and subsequently exported to Microsoft Excel (v 16.98) to extract mean and maximum values for each SET session.

2.5.4. Blood Sample Collection

Blood samples were collected from each horse on days 0, 30, 31, 60, and 61. Samples from day 0 (d0) were used to establish baseline values prior to supplementation. On days 30 (d30) and 60 (d60), samples were collected immediately prior to the gait kinematic analysis (PRE), immediately upon completion of SET (POST), and then one hour (1h), and 24 hours

(24h) after completion of the SET. This made for 9 total collection timepoints (denoted as d0, d30-PRE, d30-POST, d30-1h, d30-24h, d60-PRE, d60-POST, d60-1h, d60-24h).

Samples were collected via jugular venipuncture into 10ml tubes containing 158 USP units of EDTA (*BD Vacutainer, Franklin Lakes, NJ*). The tubes were then centrifuged at 7000×g for 7 minutes, and transfer pipettes were used to aliquot the plasma layer into 2ml microcentrifuge tubes which were stored at -20°C until analysis.

2.5.5. Analysis of Oxidative Stress Biomarkers

Commercially available colorimetric assay kits were used for evaluation of Glutathione Peroxidase Activity (GPx), Superoxide Dismutase Activity (SOD), and Thiobarbituric Reactive Acid Substances Concentration (TBARS) (*Caymen Chemical, Ann Arbor, MI*).

For analysis of GPx, samples were diluted 1:3 with the provided sample buffer in order to reach a concentration within the kit's dynamic range of 50-344 nmol/min/ml. Samples were run in triplicate, and reacted with the provided nicotinamide adenine diphosphate, GPx co-substrate, and cumene hydroperoxide in an optically clear plate. Absorbance was measured at 340 nm and GPx activity in nmol/min/ml was calculated according to kit directions. The intra-assay coefficient of variation was 3.33%, and the inter-assay coefficient of variation was 8.26%.

SOD analysis was conducted in duplicate, after being diluted with the provided sample buffer at a ratio between 1:5 up to 1:20, in order to reach a concentration within the kit's dynamic range of 0.005-0.050 U/ml. The diluted samples were pipetted into an optically clear plate and combined with the provided tetrazolium salt solution and xanthine oxidase and incubated at room temperature for 30 minutes. Absorbance was measured at 450 nm

using a plate reader, and then compared against a standard curve of known SOD concentrations to calculate enzyme activity in U/ml. The intra-assay coefficient of variation was 3.17%.

For the measurement of TBARS concentration, plasma was aliquoted into 1.5 ml locking microcentrifuge tubes and reacted with sodium dodecyl sulfate, thiobarbituric acid, and an acid solution. Microcentrifuge tubes were incubated in a dry bath at 95°C for one hour before being quickly removed and cooled on ice and then centrifuged at 1600×g for 10 minutes. The supernatant was transferred to an optically clear plate in duplicate, and the absorbance was measured at 535 nm using a plate reader. Absorbance values were compared to a standard curve and calculated according to kit directions in order to calculate concentration in μM. The intra-assay coefficient of variation was 0.93%.

2.6. Statistical Analysis

Mixed linear regression models were constructed for each response variable based on conformity to assumptions of linearity, homogeneity, and normality. All models included fixed effects for treatment (SUPP or CON), sex, and age (in months). Additionally, a variable for time was included in each model. This was an indicator for week for BW, BCS, and HR models, an indicator for each of the nine sampling timepoints (d0, d30-PRE, etc.) for OS biomarkers, and an indicator for day (30 or 60) for gait kinematic measurements. Random effects were included for individual horse. In addition, models for OS biomarkers and gait kinematic measurements included an interaction between treatment and time, and models for DUR included height (in cm) as a fixed effect.

Assumptions of linearity, homogeneity of variance, and normality were evaluated using diagnostic plots. Assumptions for all models were sufficiently met with the exception of the model for GPx, which was remedied using a log-transformation of the GPx values.

Fixed effects were evaluated using F-tests with the Kenward-Roger method for degrees of freedom. Estimated marginal means were calculated for factors with significant effects. When evaluating estimated marginal means for treatment, only timepoints from d30 and d60 were included because d0 was prior to the initiation of treatment. Statistical significance was considered $p < 0.05$, and trends as $p < 0.10$.

The relationship between OS biomarkers and gait kinematic measurements were quantified using Pearson correlation with Holm p-value adjustments. OS biomarker values from each timepoint were considered separately and the correlation coefficient was calculated for gait kinematic measurements from the same day. Additionally, the change in OS biomarker values from timepoints found to be statistically different from one another were calculated and compared to gait kinematic measurements from the same day. Correlations with an adjusted *p*-value < 0.05 were considered significant.

Data were analyzed with R (version 4.4.1), including packages tidyverse, dplyr, performance, car, emmeans, lme4, lmerTest, and correlation.

3. Results and Discussion

3.1. Body Measurements

Initial body measurements are recorded in *Table 1*. In the model for the body measurements over time, there was no effect of treatment on BW ($p = 0.68$) or BCS ($p = 0.33$). There was however an effect of week for BW ($p < 0.001$) and BCS ($p = 0.01$). Average

BW decreased from week 1 at 457kg (CI: 441-473kg) to week 5 at 434kg (CI: 417-450kg) before steadily increasing through week 9 to 451 kg (CI: 435-467kg). Average BCS however steadily decreased throughout the study, from week 1 at 5.5 (CI: 5.3-5.7) to week 9 at 5.3 (CI: 5.1-5.5). Age was found to have an effect on BW (p = 0.001) and BCS (p = 0.003), with older horses being 5.02kg (CI: 2.73-7.31) heavier on average and having a BCS 0.1 (CI: 0.0-0.1) points greater. Sex was found to be significant for BCS (p = 0.048), with geldings having a BCS 0.39 (CI: 0.06-0.72) points greater than mares on average.

Though this could be interpreted to indicate an increase in horses' muscle mass and a decrease in fat tissue, the effect by week on BCS and BW is likely of minimal practical significance. The difference between the maximum and minimum average BW by week was 27 kg, which is unlikely to have a substantial impact on horse health or performance. Similarly, the range of weekly mean BCS was only 0.17, which again is of minimal, if any, practical importance. One contributing factor likely was cold weather, which may have contributed to the decrease in BW and BCS seen over period 1, which became less influential when horses were stalled and blanketed during period 2 leading to a recovery in BW. Additionally, increased workload during period 2 likely accounted for the continuous loss in BCS even while BW recovered, as energy stored in subcutaneous fat was utilized to increase skeletal muscle mass.

Due to technological error, HR was only recorded for six of the 18 horses on day 30. Of the horses for whom HR was recorded on day 30, two belonged to SUPP and four belonged to CON. Therefore, the six recorded horses on day 30 were compared against all 18 horses on day 60. Despite this, there was no evidence to suggest a difference between day 30 and day 60 for either HR_{max} (p = 0.54), or HR_{avg} (p = 0.17). Mean HR_{max} was 202 (CI: 176-

228) bpm on day 30 and 193 (CI: 181-206) bpm on day 60. Mean HR_{avg} was 125 (CI: 106-143) bpm on day 30 and 111 (CI: 102-120) bpm on day 60, corresponding with the definition of "heavy" to "very heavy" work [15], though this may have been influenced by stress. The goal of the SET was to induce more intense exercise than that to which horses were acclimated, and these results suggest that the intensity of the SET was sufficient.

There was no evidence of an effect by treatment on HR_{max} ($p = 0.87$), or HR_{avg} ($p = 0.34$), indicating that treatment did not have an effect on cardiovascular fitness or function. Prior research has shown that moderate levels of ROS contribute to exercise adaptation [5], and some have suggested that antioxidant supplementation may disrupt this process [8]. With the current data we saw no evidence that this level of supplementation inhibited or promoted aerobic fitness. However, due to the fact that HR was only recorded for 6 of the 18 horses on day 30, it may be misleading to draw conclusions about any effects on day 30.

3.2. Oxidative Stress Biomarkers

No significant treatment-by-time interaction was observed for GPx ($p = 0.442$), SOD ($p = 0.688$), or TBARS ($p = 0.907$). Therefore, results for differences over time were averaged over treatment and results for treatment comparisons were averaged over time points.

Average GPx varied by time point ($p < 0.001$; *Table 2*). On day 30, GPx activity at d30-1h was greater compared to d30-PRE ($p = 0.013$) and d30-POST ($p = 0.005$). Conversely, no significant changes were found throughout day 60. Pre-exercise values increased across the reconditioning period, from d30-PRE to d60-PRE ($p = 0.005$). Sex was not significant ($p = 0.183$), nor was age ($p = 0.461$).

SOD was also found to have a significant difference by time point (p = 0.042; *Table 2*). No changes were seen throughout day 30 when horses were deconditioned. After the reconditioning period, d60-PRE values were lower relative to day 0 (p = 0.016) and a trend towards a decrease was found compared to d30-PRE (p = 0.088). Compared to d60-PRE, there was a trend towards an increase at d60-POST (p = 0.056) and a significant increase at d60-1h (p = 0.037), suggesting the SET triggered an increase in SOD activity. In addition, there was evidence for a trend for an effect of sex (p = 0.042), with geldings on average having mean SOD activity 0.09 (CI: -0.01-0.19) U/ml greater than that of mares (p = 0.092). Age was not significant (p = 0.191).

Table 2

Oxidative Stress Biomarkers by Timepoint

	Day 0	Day 30				Day 60			
	d0	PRE	POST	1h	24h	PRE	POST	1h	24h
Glutathione Peroxidase (GPx) Activity (nmol/ml/min)									
Mean	97.40[a]	121.70[a]	96.80[a]	160.60[b]	n/a	174.10[b]	179.10[b]	153.40[b]	n/a
SE	6.10	7.62	6.25	10.40	n/a	10.90	11.20	10.20	n/a
Superoxide Dismutase (SOD) Activity (U/ml)									
Mean	0.799[a]	0.664[ab]	0.809[ab]	0.768[a]	n/a	0.538[b]	0.677[ab]	0.726[a]	n/a
SE	0.062	0.062	0.066	0.064	n/a	0.062	0.062	0.066	n/a
Thiobarbituric Acid Reactive Substances Concentration (TBARS) (µM)									
Mean	6.23[a]	5.90[ac]	3.79[bc]	4.87[c]	5.96[a]	5.66[a]	3.70[b]	5.61[ac]	5.81[a]
SE	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.23	0.22

[a,b,c] Means with differing superscripts denote significantly different results at p≤ 0.05. Estimated marginal means are averaged over treatment and reported along with standard error (SE).

Increases in GPx activity following a single exercise bout have been described previously [11,23], as have increases in SOD activity [20,23,24]. However, other studies have also found a decrease [11,25] or no change [10,11,13,20,24–26] in GPx and/or SOD activity after a single exercise bout (i.e. acute exercise). The variation between these results

is widely thought to be a reflection of differences in horses' fitness, acclimation to OS, and the intensity and duration of exercise.

A similar relationship between GPx and SOD has previously been noted by Ott et al. [13], which followed horses through an 8-week reconditioning protocol similar to that of the present study. They found that when GPx was elevated, SOD remained at baseline, and vice versa. The authors of that study suggest GPx and SOD may have a "synergistic" relationship, which the present results appear to support.

TBARS also differed by time point (p <0.001; Table 2). Concentrations decreased from PRE to POST on both day 30 (p <0.001) and day 60 (p <0.001) with no difference in PRE (p = 1.000) or POST (p = 1.000) values. Compared to d30-PRE, TBARS was still decreased at d30-1h (p = 0.028) and did not fully recover to pre-exercise values until 24h (p = 0.363). Meanwhile, on d60 TBARS recovered to being similar to d60-PRE levels by d60-1h (p = 0.698).

Our observed changes in TBARS due to the SET on both days 30 and 60 suggest the SET did successfully influence oxidative damage in the form of lipid peroxidation. However, exercise would typically be expected to increase TBARS [14], while in the present study we observed a decrease in TBARS from PRE to POST on both days. Others have observed a similar effect in horses [11,24] and humans [27], though typically only in conditioned individuals. Some have proposed that this result is due to training-induced adaptations in antioxidant capacity [24,27]. We theorize that the SET used in this study was sufficient to trigger an antioxidant response without producing enough ROS to significantly increase lipid peroxidation, resulting in a decrease in TBARS. This would concur with the results for both GPx and SOD, which found that the SET did create an antioxidant response on both days.

However, this still does not explain why a decrease in TBARS was seen on day 30 when horses were deconditioned.

Another explanation that warrants discussion is that the TBARS assay has previously been criticized for lack of specificity in measuring lipid peroxidation end products [28]. The assay was developed to primarily measure malondialdehyde, a main end product of lipid peroxidation, however other species react with TBA and produce compounds with similar absorbances, including aldehydes and products of protein and carbohydrate breakdown [28]. Therefore, the changes seen in the present results could be reflective of other physiological changes resulting from exercise. Despite this, TBARS is still a common and long-standing measure of lipid peroxidation and is considered a reliable indicator of OS especially when used alongside other assays [28].

TBARS also tended to be affected by treatment ($p = 0.061$), unlike GPx ($p = 0.443$) or SOD ($p = 0.840$), when averaged over all time points. Averaged across all time points after supplementation began, mean TBARS was 0.33 (CI: -0.69-0.04) μM lower for SUPP than CON ($p = 0.076$), indicating the supplement likely did have an effect in decreasing OS. Due to the lack of a treatment-by-time interaction, it can be inferred that this effect was independent of acute exercise or the reconditioning protocol.

This trend for a treatment effect on TBARS is supported by the current understanding of the mechanism of action of each of these ingredients. Astaxanthin is a lipid-soluble antioxidant and in vitro, it has an antioxidant capacity 100 times greater than that of alpha-tocopherol [29]. Astaxanthin's chemical structure allows it to act both within cell membranes and at the cell surface, making it highly effective in combating lipid peroxidation as well as other types of oxidative damage [30]. In the horse, a preliminary study showed that long-

term, high dose astaxanthin supplementation resulted in increased total antioxidant capacity and decreased TBARS [31], which was not seen with the dose and duration used in the present study. Similarly, vitamin E primarily functions to prevent the peroxidation of lipids; by imbedding itself in the cell membrane it can protect polyunsaturated fatty acids from oxidative damage [32]. Dietary supplementation of vitamin E can decrease TBARS but has not been shown to effect GPx or SOD in the exercising horse [16,33–36], which is echoed by our results. [33][34][35]. L-carnitine functions differently than vitamin E or astaxanthin, as it mainly facilitates the transport of fatty acids into the inner mitochondrial matrix [37]. There is evidence it does exhibit some antioxidative behavior [38,39] and in humans reduces lipid peroxidation while also mitigating hypoxic stress and muscle damage [28]. These results support the hypothesis that the supplement did have a physiological effect in the horse and effectively mitigated aspects of OS.

Age has also been shown to have measurable impacts on OS and antioxidant capacity in the horse, which was observed in our results. Previous studies report that following exercise, untrained yearlings had lower OS and greater antioxidant status than mature untrained horses [40–42]. However, in one study both groups were then subjected to two weeks of training, after which yearlings had significantly greater OS than mature horses indicating they were less able to adapt to exercise training [42].

3.3. Gait Kinematics

W-DUR demonstrated a trend for a treatment-by-day effect ($p = 0.099$; figure 1A), but not an effect of treatment ($p = 0.946$) or day ($p = 0.149$). No significant differences were found between treatments on either day 30 ($p = 0.535$) or day 60 ($p = 0.622$). However, CON

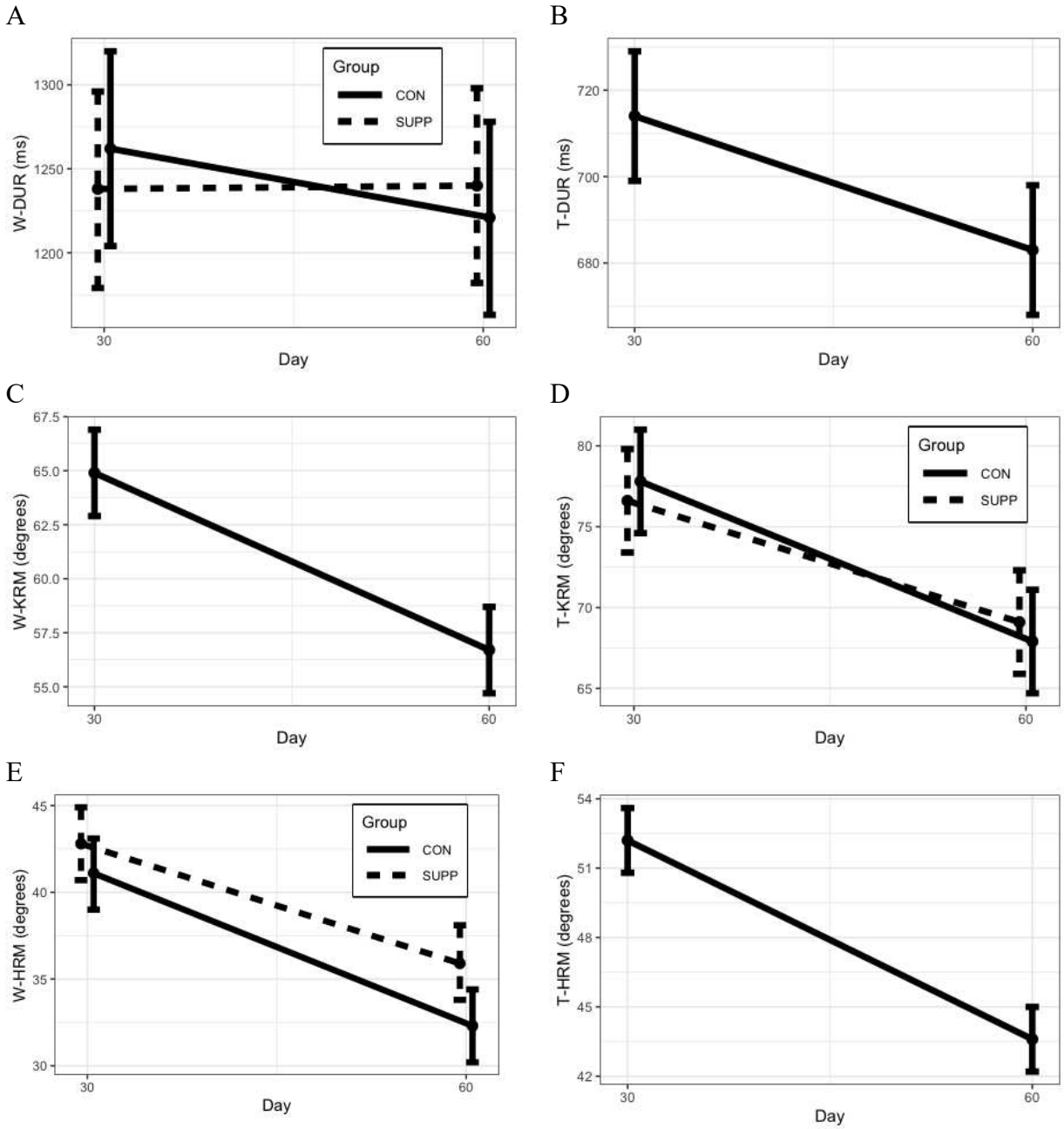

Fig. 1

Changes in gait kinematic parameters from day 30 to 60, as a result of transitioning from maintenance (no enforced exercise) to moderate work. Horses were walked (W) and trotted (T) over a 9 m distance, and video was analyzed using motion analysis software to determine stride duration (DUR), knee range of motion (KRM) and hock range of motion (HRM). For results where treatment x day was not significant, results were averaged over treatment. (A) W-DUR by treatment and day and (B) T-DUR by day were both measured in milliseconds (ms). (C) W-KRM by day, (D) T-KRM by treatment and day, (E) W-HRM by treatment and day, and (F) T-HRM by day were all measured in degrees.

experienced a 41.5ms (CI: 4.13-78.80) decrease in W-DUR between days 30 and 60 (p = 0.030) while SUPP did not experience a significant change (p = 0.882).

T-DUR had no evidence of an effect of treatment-by-day (p = 0.438) or treatment (p = 0.120), but a significant effect by day (p <0.001; figure 1b), and a trend for an effect by height (p = 0.057). Averaged over both treatment groups, T-DUR decreased 28.2ms (CI: 23.50-38.40) between days 30 and 60.

W-KRM had no effect by treatment-by-day (p = 0.94) or treatment (p = 0.987), but did have an effect by day (p <0.001; figure 1c). Averaged over both groups, there was an 8.2° (CI: 7.12-9.28) decrease between days 30 and 60.

T-KRM was found to have a significant effect by treatment-by-day (p = 0.014; figure 1d), in addition to an effect by day (p < 0.001) but no effect by treatment (p = 0.996). Both groups experienced a decrease in joint mobility from day 30 to day 60; SUPP decreased 7.44° (CI: 6.04-8.84) and CON decreased 9.95° (CI: 8.55-11.35).

W-HRM demonstrated a trend for an effect by treatment-by-day (p = 0.061; figure 1e), in addition to an effect by day (p < 0.001) and a trend for treatment (p = 0.065). There was no difference between treatment groups on day 30 (p = 0.241), however on day 60, SUPP on average had a W-HRM 3.61° (CI: 0.63-6.59) greater than CON. From day 30 to 60, CON decreased 8.73° (CI: 7.33-10.13) while SUPP decreased 6.83° (CI: 5.42-8.25).

T-HRM displayed no treatment-by-day effect (p = 0.13) or treatment effect (p = 0.85), but there was an effect of day (p <0.001; Fig 1f). Averaged over both treatment groups, average T-HRM decreased 8.61° (CI: 7.70-9.51) between days 30 and 60.

The results for the 6 gait kinematic measures largely lead to the same conclusion: while the reconditioning protocol led to decreased stride duration and joint mobility, SUPP

was able to offset some of those effects. Stride duration has previously been used in the horse as an indicator of joint discomfort [17] and delayed onset muscle soreness [16]. A related parameter, stride length, has been associated with increased risk of musculoskeletal injury in racehorses [43], and increased asymmetry of movement [44]. However, articular ROM has been described as a more direct indicator of joint comfort [17]. Decreased joint mobility is a common finding in joint diseases such as synovitis and osteoarthritis[45,46], and has been associated with increases in the pro-inflammatory cytokine IL-1β [17]. Beyond the health implications of gait kinematics, there is also applications for equine performance and industry. Increased stride duration and hock ROM have been significantly correlated with a judge's subjective score of gait quality in young warmbloods at the trot [47], and increased knee ROM has been interpreted as a more supple and animated forelimb [48]. Additionally, various measures of gait kinematics have been used to accurately predict jumping faults in eventing horses [49].

OS has been implicated in joint soreness and the pathogenesis of joint diseases [15,20]. It has been proposed that ROS target hyaluronan, thereby decreasing the viscosity of synovial fluid and further increasing friction and inflammation within the joint [50]. This is supported by the fact that both acute and chronic lameness have been associated with increased concentrations of ROS [51], and inflamed joints contain greater concentrations of superoxide-producing phagocytes [48].

Similarly, OS is a known cause and consequence of muscle damage. Exercise-induced trauma to muscle tissue is perpetuated by ROS produced both during the exercise bout and during recovery [11,15]. This damage can have significant impacts, namely delayed-onset muscle soreness, systemic inflammation, and reduced athletic performance [11,20].

Vitamin E supplementation in horses has mitigated exercise induced muscle damage, and lessened the decrease in trot stride duration after acute exercise [16,52]. Fagan et al. [16] also found serum alpha-tocopherol was correlated with a lesser decrease in stride duration, suggesting that the anti-lipid peroxidative action of vitamin E may have prevented muscle damage and delayed onset muscle soreness.

Past evidence suggests that L-carnitine supplementation may have also significantly impacted gait kinematics. Johnson et al. [26] found that horses that received a supplement containing carnitine, as well as other antioxidants including d-alpha-tocopherol, were able to retain greater fetlock mobility after an exercise test to exhaustion. The authors of that study suggested L-carnitine may have supported faster exercise recovery and reduced soft tissue strain. While the present study did not evaluate changes in gait kinematics after the SET, our observation of a decrease in knee and hock mobility after 30 days of reconditioning may reflect a similar physiological process.

To the authors' knowledge, the effect of astaxanthin supplementation on gait kinematics in horses has not previously been studied in horses. However, one study found that Arabian racehorses supplemented with astaxanthin daily showed no difference in several inflammatory cytokines compared to controls [53]. Other studies have shown that astaxanthin effectively decreased lactate and creatine kinase during exercise in both mice [54] and humans [55]. In racing thoroughbreds supplemented with both astaxanthin and L-carnitine, the supplement decreased markers of exercise-induced muscle damage [56]. Additionally, they found a significant reduction in the incidence of clinical signs of lameness, including muscle pain and stiffness. This supports the theory that these two ingredients may

work best in tandem, as astaxanthin is particularly well suited to preventing the oxidation of the CPT-1 transporter, while L-carnitine improves the efficiency of it [56].

With the current body of scientific knowledge, it is unknown why some parameters displayed only a day effect and not a treatment-by-day effect, and vice versa. Gait may be a notable factor; out of the three parameters displaying a treatment-by-day effect, two were at the walk. Prior research utilizing gait kinematics in relation to various supplements have also found a greater treatment effect at the walk, but so far have not been able to confirm an underlying mechanism. Some have suggested that because the walk does not have a suspension phase, movement at the walk is less dependent on athletic ability or training and therefore the effects of supplementation become more evident [17]. Alternatively, many study designs evaluating both gaits will record horses at the walk prior to the trot. It is possible that walking first allowed the horses to warm up, causing them to move more fluidly at the trot [17,48].

3.4. Correlation of Oxidative Stress Biomarkers and Gait Kinematics

No statistically significant correlations between any of the gait kinematic measures and GPx, SOD, or TBARS from any time point on either day were found (*see Appendix I*). There are several reasons why we may not have found a significant correlation between OS biomarkers and gait kinematic parameters, even though changes were seen in gait kinematics in relation to reconditioning, which is known to be associated with OS. One consideration is the fact that OS can influence musculoskeletal function [15,20,48–51], but its effects on gait are likely mediated by multiple processes such as inflammation and muscular force exertion [15,20], weakening direct correlations. It also may be the case that these specific biomarkers are reflective of systemic OS, not necessarily localized oxidative processes in joints or muscles

directly affecting stride mechanics. Another consideration is the limitations associated with the very acute nature of gait kinematic measurements. OS may produce gradual tissue effects [15,20] not captured in a single kinematic measurement session, perhaps manifesting hours or days after an exercise session. Future studies should consider conducting gait kinematic measurements post-exercise or repeating measurements several times during the recovery period.

4. Conclusion

In summary, 30 days of reconditioning in young horses led to changes in antioxidant enzyme activity while also causing restricted movement, demonstrated by a decreased stride duration and joint range of motion. A single bout of moderate intensity exercise resulted in increased GPx activity when horses were deconditioned, increased SOD when horses were conditioned, and decreased TBARS regardless of fitness. The supplement treatment tended to reduce TBARS as compared to placebo and attenuated some changes in mobility resulting from reconditioning. Overall, this commercial antioxidant supplement was effective in combating some aspects of oxidative stress in young horses beginning a typical training program.

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APPENDIX I

CORRELATIONS OF OXIDATIVE STRESS BIOMARKERS AND GAIT KINEMATIC MEASUREMENTS

Time Point	Oxidative Stress Biomarker	Gait Kinematic Measure	Correlation Coefficient (r)	CI Minimum	CI Maximum	p-Value
d30-PRE	GPX	W-DUR	0.05	-0.43	0.50	0.99
d30-PRE	GPX	T-DUR	-0.02	-0.49	0.45	0.99
d30-PRE	GPX	W-KRM	0.62	0.21	0.84	0.74
d30-PRE	GPX	T-KRM	0.27	-0.22	0.66	0.99
d30-PRE	GPX	W-HRM	0.16	-0.33	0.58	0.99
d30-PRE	GPX	T-HRM	-0.01	-0.48	0.46	0.99
d30-PRE	SOD	W-DUR	-0.35	-0.70	0.14	0.99
d30-PRE	SOD	T-DUR	-0.04	-0.50	0.43	0.99
d30-PRE	SOD	W-KRM	-0.08	-0.52	0.40	0.99
d30-PRE	SOD	T-KRM	-0.08	-0.53	0.40	0.99
d30-PRE	SOD	W-HRM	0.25	-0.24	0.64	0.99
d30-PRE	SOD	T-HRM	-0.12	-0.56	0.37	0.99
d30-PRE	TBARS	W-DUR	0.02	-0.45	0.48	0.99
d30-PRE	TBARS	T-DUR	-0.19	-0.60	0.30	0.99
d30-PRE	TBARS	W-KRM	0.23	-0.27	0.63	0.99
d30-PRE	TBARS	T-KRM	0.05	-0.42	0.51	0.99
d30-PRE	TBARS	W-HRM	-0.01	-0.48	0.46	0.99
d30-PRE	TBARS	T-HRM	-0.35	-0.70	0.13	0.99
d30-POST	GPX	W-DUR	-0.06	-0.52	0.44	0.99
d30-POST	GPX	T-DUR	0.16	-0.35	0.60	0.99
d30-POST	GPX	W-KRM	-0.23	-0.64	0.28	0.99
d30-POST	GPX	T-KRM	-0.29	-0.68	0.22	0.99
d30-POST	GPX	W-HRM	0.02	-0.47	0.49	0.99
d30-POST	GPX	T-HRM	0.13	-0.38	0.57	0.99
d30-POST	SOD	W-DUR	0.01	-0.47	0.49	0.99
d30-POST	SOD	T-DUR	0.10	-0.40	0.56	0.99
d30-POST	SOD	W-KRM	-0.03	-0.51	0.46	0.99

Time Point	Oxidative Stress Biomarker	Gait Kinematic Measure	Correlation Coefficient (r)	CI Minimum	CI Maximum	p-Value
d30-POST	SOD	T-KRM	0.34	-0.17	0.71	0.99
d30-POST	SOD	W-HRM	0.08	-0.42	0.54	0.99
d30-POST	SOD	T-HRM	0.19	-0.32	0.61	0.99
d30-POST	TBARS	W-DUR	-0.60	-0.84	-0.16	0.99
d30-POST	TBARS	T-DUR	-0.33	-0.70	0.18	0.99
d30-POST	TBARS	W-KRM	-0.08	-0.54	0.42	0.99
d30-POST	TBARS	T-KRM	-0.23	-0.64	0.28	0.99
d30-POST	TBARS	W-HRM	0.12	-0.38	0.57	0.99
d30-POST	TBARS	T-HRM	0.04	-0.45	0.51	0.99
d30-1h	GPX	W-DUR	0.15	-0.35	0.59	0.99
d30-1h	GPX	T-DUR	0.36	-0.15	0.72	0.99
d30-1h	GPX	W-KRM	0.29	-0.22	0.68	0.99
d30-1h	GPX	T-KRM	0.39	-0.12	0.73	0.99
d30-1h	GPX	W-HRM	-0.10	-0.55	0.40	0.99
d30-1h	GPX	T-HRM	-0.06	-0.53	0.43	0.99
d30-1h	SOD	W-DUR	-0.13	-0.58	0.37	0.99
d30-1h	SOD	T-DUR	0.11	-0.39	0.56	0.99
d30-1h	SOD	W-KRM	0.32	-0.19	0.70	0.99
d30-1h	SOD	T-KRM	-0.10	-0.55	0.40	0.99
d30-1h	SOD	W-HRM	0.49	0.02	0.79	0.99
d30-1h	SOD	T-HRM	-0.08	-0.54	0.41	0.99
d30-1h	TBARS	W-DUR	-0.30	-0.68	0.21	0.99
d30-1h	TBARS	T-DUR	-0.37	-0.72	0.14	0.99
d30-1h	TBARS	W-KRM	0.05	-0.44	0.52	0.99
d30-1h	TBARS	T-KRM	0.10	-0.40	0.55	0.99
d30-1h	TBARS	W-HRM	-0.32	-0.70	0.19	0.99
d30-1h	TBARS	T-HRM	0.25	-0.26	0.65	0.99
d30-24h	TBARS	W-DUR	0.28	-0.22	0.66	0.99
d30-24h	TBARS	T-DUR	0.14	-0.35	0.57	0.99
d30-24h	TBARS	W-KRM	0.58	0.15	0.82	0.99
d30-24h	TBARS	T-KRM	0.15	-0.34	0.58	0.99
d30-24h	TBARS	W-HRM	0.02	-0.45	0.49	0.99
d30-24h	TBARS	T-HRM	-0.06	-0.51	0.42	0.99
d60-PRE	GPX	W-DUR	0.23	-0.27	0.63	0.99

Time Point	Oxidative Stress Biomarker	Gait Kinematic Measure	Correlation Coefficient (r)	CI Minimum	CI Maximum	p-Value
d60-PRE	GPX	T-DUR	0.24	-0.26	0.64	0.99
d60-PRE	GPX	W-KRM	0.08	-0.40	0.53	0.99
d60-PRE	GPX	T-KRM	0.17	-0.32	0.59	0.99
d60-PRE	GPX	W-HRM	-0.59	-0.83	-0.17	0.99
d60-PRE	GPX	T-HRM	-0.26	-0.65	0.23	0.99
d60-PRE	SOD	W-DUR	0.07	-0.41	0.52	0.99
d60-PRE	SOD	T-DUR	-0.12	-0.56	0.37	0.99
d60-PRE	SOD	W-KRM	0.07	-0.41	0.52	0.99
d60-PRE	SOD	T-KRM	0.22	-0.27	0.62	0.99
d60-PRE	SOD	W-HRM	-0.57	-0.82	-0.14	0.99
d60-PRE	SOD	T-HRM	-0.21	-0.62	0.28	0.99
d60-PRE	TBARS	W-DUR	-0.24	-0.64	0.25	0.99
d60-PRE	TBARS	T-DUR	-0.63	-0.85	-0.23	0.62
d60-PRE	TBARS	W-KRM	0.22	-0.27	0.62	0.99
d60-PRE	TBARS	T-KRM	0.08	-0.40	0.53	0.99
d60-PRE	TBARS	W-HRM	0.18	-0.31	0.60	0.99
d60-PRE	TBARS	T-HRM	0.28	-0.21	0.66	0.99
d60-POST	GPX	W-DUR	0.03	-0.44	0.49	0.99
d60-POST	GPX	T-DUR	0.09	-0.39	0.53	0.99
d60-POST	GPX	W-KRM	-0.12	-0.56	0.37	0.99
d60-POST	GPX	T-KRM	-0.02	-0.49	0.45	0.99
d60-POST	GPX	W-HRM	-0.42	-0.74	0.06	0.99
d60-POST	GPX	T-HRM	-0.15	-0.58	0.34	0.99
d60-POST	SOD	W-DUR	-0.20	-0.61	0.30	0.99
d60-POST	SOD	T-DUR	0.07	-0.41	0.52	0.99
d60-POST	SOD	W-KRM	0.07	-0.41	0.52	0.99
d60-POST	SOD	T-KRM	-0.11	-0.55	0.38	0.99
d60-POST	SOD	W-HRM	0.00	-0.47	0.47	0.99
d60-POST	SOD	T-HRM	-0.44	-0.75	0.03	0.99
d60-POST	TBARS	W-DUR	0.26	-0.24	0.65	0.99
d60-POST	TBARS	T-DUR	-0.35	-0.70	0.14	0.99
d60-POST	TBARS	W-KRM	0.31	-0.19	0.68	0.99
d60-POST	TBARS	T-KRM	0.28	-0.21	0.66	0.99
d60-POST	TBARS	W-HRM	0.00	-0.47	0.46	0.99

Time Point	Oxidative Stress Biomarker	Gait Kinematic Measure	Correlation Coefficient (r)	CI Minimum	CI Maximum	p-Value
d60-POST	TBARS	T-HRM	0.31	-0.18	0.68	0.99
d60-1h	GPX	W-DUR	0.31	-0.22	0.70	0.99
d60-1h	GPX	T-DUR	0.26	-0.27	0.67	0.99
d60-1h	GPX	W-KRM	-0.04	-0.52	0.47	0.99
d60-1h	GPX	T-KRM	0.19	-0.34	0.62	0.99
d60-1h	GPX	W-HRM	-0.63	-0.86	-0.19	0.99
d60-1h	GPX	T-HRM	-0.27	-0.68	0.26	0.99
d60-1h	SOD	W-DUR	-0.39	-0.74	0.13	0.99
d60-1h	SOD	T-DUR	-0.26	-0.67	0.27	0.99
d60-1h	SOD	W-KRM	0.30	-0.23	0.69	0.99
d60-1h	SOD	T-KRM	-0.01	-0.50	0.49	0.99
d60-1h	SOD	W-HRM	0.24	-0.29	0.66	0.99
d60-1h	SOD	T-HRM	-0.11	-0.58	0.41	0.99
d60-1h	TBARS	W-DUR	0.54	0.06	0.82	0.99
d60-1h	TBARS	T-DUR	-0.04	-0.52	0.47	0.99
d60-1h	TBARS	W-KRM	-0.24	-0.66	0.29	0.99
d60-1h	TBARS	T-KRM	-0.12	-0.58	0.40	0.99
d60-1h	TBARS	W-HRM	-0.30	-0.69	0.23	0.99
d60-1h	TBARS	T-HRM	0.26	-0.27	0.67	0.99
d60-24h	TBARS	W-DUR	-0.24	-0.64	0.25	0.99
d60-24h	TBARS	T-DUR	-0.34	-0.69	0.15	0.99
d60-24h	TBARS	W-KRM	0.17	-0.32	0.59	0.99
d60-24h	TBARS	T-KRM	-0.03	-0.49	0.44	0.99
d60-24h	TBARS	W-HRM	-0.03	-0.49	0.45	0.99
d60-24h	TBARS	T-HRM	-0.07	-0.52	0.41	0.99