Equine High-Intensity Exercise, Articular Biochemistry, and Nutritional Antioxidant Supplementation

by

Jennifer L. MacNicol

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ABSTRACT

EQUINE HIGH-INTENSITY EXERCISE, ARTICULAR BIOCHEMISTRY, AND NUTRITIONAL ANTIOXIDANT SUPPLEMENTATION

Jennifer L. MacNicol
University of Guelph, 2017

Advisor: Professor W. Pearson

The objective of this thesis was to document the response in horses to high-intensity exercise and antioxidant supplementation. In a pilot study, horses underwent an exercise test. Plasma and synovial fluid (SF) samples were taken 24 hr prior to exercise (BL), 0.5, 1, 2, 4, 8, and 24 hr following exercise. High-intensity exercise in horses resulted in a transient systemic inflammatory state and articular inflammation and cartilage breakdown. In a subsequent study, horses were provided with a supplement comprised of *Cucumis melo* pulp extract to determine whether antioxidant supplementation impacted parameters of exercise-induced systemic or articular inflammation/oxidative stress. Based on the prior time course, samples were taken at BL, 1 and 24 hr following exercise. Supplementation resulted in post-exercise mitigation of articular oxidative stress and cartilage breakdown, increased antioxidant status, reduced blood lymphocyte levels, and 24 hr following high-intensity exercise supplemented horses displayed reduced inflammation and muscle breakdown.
DEDICATION

This thesis is dedicated to my Grandma Hood who always believed in me and was my kindred spirit, I wish I could share this with you now, but I know you’re here with me. I also want to dedicate this to my Mum, you’ve gone through so much this year and yet you still carried me through this. Your strength is always an inspiration.
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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Historically horses have been used for labor, transport, food, and sport. Whilst the modern horse in the developed world is no longer relied upon for work, sport persists as a significant component of the equine industry. Exercise is a physiological stress and has been associated with oxidative stress and inflammation (Bessa et al., 2016; Marlin et al., 2002). Exercise also places mechanical strain on articulating joints. Osteoarthritis (OA) is a degenerative joint disease which results in cartilage degradation and a loss of joint function (Bijlsma et al., 2011; Buckwalter et al., 2004). Oxidative stress and inflammation are implicated in the pathogenesis of OA, and stresses induced by high-intensity exercise increase the risk of OA development (Moller and Van Weeren, 2017; Riggs, 2006). OA is a common ailment in horses and can result in lameness, lost training time, and, depending on the severity, can terminate a horse’s athletic career (de Souza, 2016; Riggs, 2006; Kidd et al., 2001). Thus it is important to understand the specific oxidative and inflammatory responses of horses to intense exercise so that their negative impact on development of OA can be mitigated.

Exercise results in elevated markers of lipid peroxidation (Alessio and Goldfarb, 1998), inflammatory cytokines (Peake et al., 2015; Ostrowski et al., 1999) and has been likened to the physiological response observed following acute trauma or sepsis (Pedersen et al., 1998; Ostrowski et al., 1998). In horses elevations in whole blood inflammatory cytokines and synovial fluid (SF) nitrite have been observed following exercise (Lampe et al., 2008). Nitric oxide has been linked to the pathogenesis of OA and joint degeneration (Balaganur et al., 2014; Jang and Murrell, 1998; Pelletier et al., 1998). However, in horses, there has not been a detailed analysis of the time course of significant markers involved in inflammation and oxidative stress in the first 24 hr following high-intensity exercise in SF.

The use of antioxidant supplements to strengthen exercise performance, or assist in recovery, is common practice among athletes. This practice has also been adopted by the equine community and there are many commercial antioxidant supplements available for competition horses.
Similarly, antioxidants are often used to assist in the support and maintenance of optimum joint health in athletic horses. Some studies have demonstrated that the use of nutraceutical supplements composed of novel ingredients such as natural eggshell membrane, collagen hydrolysate, or rosmarinic acid as oral nutraceutical supplements have improved measures of cartilage synthesis, as well as reduced cartilage degradation and synovitis (van de Water et al., 2016; Wedekind et al., 2015; Pearson et al., 2012). Nevertheless, a systematic review of nutraceutical supplements for equines found limited evidence supporting their efficacy (Vandeweerd et al., 2016). Efficacy has not been tested in the majority of equine supplements on the market.

The primary goal of the research presented herein was to form a comprehensive timeline concerning specific markers of oxidative stress and inflammation both systemically and in the carpal joint of horses following a bout of high-intensity exercise. Subsequently, the systemic and articular antioxidant/anti-inflammatory potential of a dietary encapsulated antioxidant supplement, composed of melon pulp, in exercising horses was evaluated.

1.2 Objectives

The specific objectives of this research were to:

1) define the time course and magnitude of change in biomarkers of oxidative stress and inflammation, both systemically and within the local articular space of the intercarpal joint, during the first 24 hr following high-intensity exercise in horses, and

2) evaluate the effect of 3 weeks of daily supplementation with encapsulated Cucumis melo pulp extract on those systemic and articular changes in horses undergoing an acute bout of high-intensity exercise.
CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Exercise is a physiological phenomenon, involving several interacting biological events. Many of the typical responses to exercise have been connected to concurrent upregulation in oxidative stress and inflammation. Exercise strains multiple systems, and the strain it places on the joints is of particular interest due to the association between athletics and joint degeneration. An increase in many of the classical markers of joint inflammation and osteoarthritis (OA) is observed following exercise. A greater understanding of the connection between exercise and OA will improve the health and well-being of athletes. Antioxidant supplements are often used to combat oxidative stress in a variety of situations, and are popular ergogenic aids. However, the efficacy and safety varies between antioxidant compounds, and when combined antioxidants can interact and alter their biological effects. It is therefore of great interest to describe the bioactivity and mechanisms of action of supplements which display beneficial outcomes on health. *Cucumis melo* extract (CME) is an antioxidant extract produced from the pulp of *Cucumis melo*, with a growing body of evidence supporting potential beneficial health outcomes. This review will focus on: 1) interactions between exercise, oxidative stress, inflammation and muscle damage; 2) OA; 3) antioxidant supplements; and 4) *Cucumis melo* supplements. The purpose is to develop a comprehensive understanding of the complex relationships within and between these areas of interest.

2.2 Interactions Between Exercise, Oxidative Stress, Inflammation, and Muscle Damage

Exercise is a physiological stress which is associated with increased inflammation and production of reactive oxygen species (ROS) (Marlin et al., 2002; Pedersen et al., 1998). Exercise has many well-characterized benefits, such as improved cardiovascular health, reduced risk of chronic disease, and improvements in mitochondrial biogenesis (Drake et al., 2017; Wilson et al., 2015; Watson and Barr, 2014). Nevertheless, it does place increased strain on many biological systems. Some physiological adaptations occur immediately following the onset of exercise such as elevated heart rate and respiratory rate, the release of adrenaline, and the
redirection of blood flow to working muscles (Marlin and Nankervis, 2002). The upregulation of ROS, inflammation, and muscle damage associated with exercise are interconnected and occur to some degree following all exercise stress.

**Oxidative Stress and Exercise**

Consistent low levels of oxidants are produced during regular metabolism and, although their levels are controlled by antioxidants, they are never entirely eliminated. ROS have several important functions, including a role as cell signalling molecules which influence gene expression (Powers et al., 2011a). However, certain physiological stressors such as disease and exercise can result in heightened concentrations of ROS. Oxidative stress occurs when there is an imbalance between the production of oxidants relative to the antioxidant capacity of an organism, such that there is net oxidative activity (Powers et al., 2011a; Soffler, 2007). This stress can result from increased ROS production, insufficient endogenous antioxidant activity, or a combination of both. Oxidative stress can result in damage to lipid membranes, proteins, and/or DNA (Lykkesfeldt and Svendsen, 2007; Deaton and Marlin, 2003). Oxidants commonly produced in the body include radicals such as superoxide (O’), hydroxyl radical (H O’), nitric oxide (NO’), as well as non-radical oxidants such as hydrogen peroxide (H2O2) (Lykkesfeldt and Svendsen, 2007).

There is much debate concerning the sources and physiological role of ROS during exercise. Although superoxide radical is produced by mitochondria during resting conditions, its production is low (~0.15%) and this appears to decrease when mitochondria are in a more active state, as occurs during exercise (Powers et al., 2011a; Powers and Jackson, 2008). However, the generation of xanthine oxidase during ischemic conditions, the inflammatory cell response to exercise-induced tissue damage, auto-oxidation of catecholamines, oxyhemoglobin or methemoglobin have also been proposed as additional sources of ROS during exercise (Vollaard et al., 2005; Deaton and Marlin, 2003). It is now recognized that mitochondrial sources likely only play a small role in exercise-induced upregulation of ROS and that other cellular components such as xanthaine oxidases and NADPH oxidases are larger contributors (Gomez-Cabrera et al., 2015). ROS play a role in maximal force production during exercise, as well as
contribute to fatigue (Reid, 2008; Powers et al., 2011a). The ideal degree of oxidation required to enable optimal muscle function has yet to be described. Therefore, although low levels of ROS generated by exercise are necessary, excessive production of ROS during exercise is still considered undesirable.

Following exercise, measures of lipid peroxidation such as malondialdehyde (MDA; a non-specific measure of oxidative stress) are elevated in horses (Sara et al., 2012; Chiaradia et al., 1998). A more comprehensive understanding of exercise-induced oxidative stress can be generated by using both measures of oxidative damage and antioxidant enzyme activity. Decreased glutathione peroxidase (GPx) and vitamin C concentrations in exercising horses, despite a steady concentration of plasma α-tocopherol, is suggestive of exercise-induced oxidative stress (Hargreaves et al., 2002a). In horses completing a 140 km endurance race, Marlin et al. (2002) observed significantly elevated concentrations of plasma total barbituric reactive substances (TBARS; a measure of the lipid peroxidation) as well as significant reductions in total glutathione (an antioxidant marker). However, the glutathione redox ratio and plasma ascorbic acid concentrations of the horses were unchanged. Thus the authors concluded that the horses did not demonstrate classical oxidative stress following this endurance exercise. These results highlight the necessity of using multiple markers to demonstrate the oxidant/antioxidant balance within an organism at any given time.

The many factors influencing the oxidant/antioxidant system make integration of data across studies challenging. For example, an analysis of baseline pre-race GPx activity in a group of horses preparing for an endurance race in 4°C weather was significantly higher than the baseline GPx activity collected from horses in 28°C pre-race temperature (Hargreaves et al., 2002b). There is also a difference between tracks and treadmills; horses exercised on a treadmill have significantly higher concentrations of plasma lactate and protein thiol, a marker of oxidative stress, compared with horses exercising on a track (de Moffarts et al., 2006). Oxidative stress or antioxidant capacity will also be influenced by the particular organ or tissue which is sampled. Liu et al. (2000) observed that not only did exercise differentially influence antioxidant status of different organs, but antioxidant activity in the same organ responds differently to acute compared to chronic exercise. Although it might be expected that age would significantly affect
the extent of lipid peroxidation following an exercise test, no effect of age was found when comparing lipid hydroperoxide in mature horses (age 12±2 yr) compared with senior horses (age 22±2 yr) (Williams et al., 2008). This is inconsistent with the human literature, which demonstrates increased susceptibility to oxidative damage and reduced antioxidant activity following exercise in older adults compared to younger adults (Bouzid et al., 2014). This may suggest that the equine antioxidant system is more adaptable to exercise or the 10 year age gap between the mature and senior horses used in this study might be insufficient to detect age-dependent changes in antioxidant systems. Nonetheless, it is clear there are numerous factors capable of altering the oxidative response to exercise in a subject, organ, or tissue of interest. Future studies must therefore clearly and precisely quantify variables with the potential to influence outcomes.

There is a large base of evidence which indicates that training is a key feature which can influence basal antioxidant activity as well as the degree of oxidative stress induced by exercise. A post-exercise increase in lipid peroxidation is prevented when rats undergo daily exercise training for 18 weeks (Alessio and Goldfarb, 1988). This is similar in horses, as an 8-week training program also reduces post-exercise oxidative stress (Williams et al., 2008). Horses in consistent and intensive exercise training have significantly higher basal activity of major antioxidant enzymes (GPx, superoxide dismutase; SOD, catalase; CAT) as well as plasma total antioxidant status (TAS) (Krumrych, 2010). A study by Kinnunen et al. (2005) found that horses trained for endurance had higher measures of total antioxidant capacity, vitamin E concentration, GPx, glutathione-S-transferase, and glutathione reductase compared to horses trained as trotters. It is noteworthy that these comparisons were made across two separate studies. Thus, other variables such as breed, nutrition, and management may have contributed to the observed differences. Nevertheless, these findings underscore the possibility that all types of exercise training may not have the same influence on the antioxidant defense system. There is strong evidence that the training level and the type of training undertaken can impact an animal’s basal antioxidant system as well as its the ability to combat oxidative stress during an exercise challenge (Azizbeigi et al., 2014; Lima et al., 2013). The level of training of individuals should be considered when comparing data across studies.
Despite the negative ramifications of excessive ROS production, there is an increasing awareness of the important role ROS play during exercise, recovery, and adaptation (Powers et al., 2016). Under resting conditions, ROS are required for muscle force production (Powers et al., 2011a; Reid, 2001a). Within muscle, ROS exert a biphasic effect (Reid, 2001b). A small increase in ROS concentration amplifies force production, whereas large increases have the opposite effect and reduce muscular force. This model has been described by Reid et al. (1993) as an inverted U.

Contracting skeletal muscle generates ROS which act as cell signalling molecules and alter gene expression (Mason et al., 2016; Powers et al., 2011b). ROS produced during exercise activate the nuclear factor κβ (NF-κβ) pathway responsible for the upregulation of several antioxidant enzymes (Gomez-Cabrera et al., 2008). In rats treadmill running to exhaustion caused a rise in NF-κβ activation (Ji et al., 2004). NF-κβ and the resultant amplification in endogenous antioxidant enzyme expression following exercise is prevented by the use of the antioxidant allopurinol (Gomez-Cabrera et al., 2005). Exercise stimulates mitochondrial biogenesis, increasing the quantity and quality of skeletal muscle mitochondria (Drake et al., 2017). Peroxisome proliferator-activated receptor-γ cofactor-1α (PGC-1α) is a transcription factor which induces mitochondrial biogenesis (Wu et al., 1999) and is responsible for the regulation of several mitochondrial genes (Vega et al., 2000). Evidence indicates PGC-1α is responsible for skeletal muscle adaptations associated with exercise (Olesen et al., 2010). When ROS were increased in exercising rats by the provision of diethyl maleate, a glutathione depleter, PGC-1α mRNA was significantly enhanced (Strobel et al., 2014). Thus, ROS appear to be an important control mechanism for mitochondrial biogenesis associated with exercise (Powers et al., 2011b). Exercise-induced ROS appear to follow the principles of hormesis (Ji et al., 2016; Ji et al., 2006). A low/moderate level of exercise generates controlled amounts of ROS and promotes the health benefits associated with exercise. Although exercise-induced production of ROS was initially considered to be only an injurious process (Powers et al., 2016), it is now evident that ROS play a complex role during exercise and adaptation.

Nitric oxide (NO) has the ability to generate other free radicals and induce cellular damage. However, like ROS, NO is important during exercise and recovery. A significant linear relationship between post-exercise NO metabolites and rectal temperature is observed in horses.
following a standardized exercise test (Alberghina et al., 2015), indicating an involvement of NO in thermoregulation. In horses the inhibition of NO by L-NAME, a non-selective NO inhibitor, results in increased core body temperature, decreased sweating (Mills et al., 1997) and heightened plasma lactate (Mills et al., 1999). NO is involved in many important processes during exercise such as metabolic control, exercise capacity, and the cardiovascular benefits which accompany chronic exercise (Kingwell, 2000). In exercising rats infused with L-NAME, mean arterial pressure is significantly elevated while both vascular conductance and hind-limb blood flow become reduced (Ferguson et al., 2016). Eight weeks of exercise training in rats increases neuronal nitric oxide synthase (NOS1) expression in ventricular myocytes and induces cardiac hypertrophy (Roof et al., 2013). However, in NOS1 knockout mice exercise training does not induce the same cardiac adaptations (Roof et al., 2013) illustrating the involvement of NO in the beneficial cardiac response to exercise. Thus, it is evident NO is required for recovery from and adaptation to exercise.

The relationship between exercise and oxidative stress is complex. ROS are necessary to ensure normal muscle function and to provide many of the positive benefits associated with exercise. Yet, when produced beyond the body’s capacity to cope, the resultant oxidative stress leads to cellular and tissue damage. The threshold values below which ROS are beneficial and above which they are harmful are not clear, and may be a moving target, very much dependent upon the individual in question. However, development of ROS and NO target ranges, similar to those used for blood variables such as creatine kinase (CK) or aspartate amino transferase (AST), could be useful during exercise or training to help determine whether an individual is undergoing adaptive ROS exposure or harmful oxidative stress.

**Inflammation and Exercise**

Inflammation and oxidative stress are closely related. Immune cells are attracted to sites of injury where they undergo respiratory burst leading to the production of ROS. Inflammatory cytokines induce immune cells to generate ROS, which stimulate transcription factors leading to the production of more ROS and other inflammatory cytokines (Sallam and Laher, 2016). Oxidative stress and concurrent inflammation are implicated as interacting factors involved in many
diseases including diabetic nephropathy (Elmarakby et al., 2012) and cancer (Reuter et al., 2010). During exercise ROS and inflammation are associated through muscle metabolism and injury (Peake et al., 2007).

Cytokines are small proteins which are primary mediators of inflammation as they communicate inflammatory/anti-inflammatory signals between cells (Floege, 2012). Cytokines are pleiotrophic, can act in a paracrine or autocrine fashion, can act synergistically or antagonistically, and often regulate their own expression (Peake et al., 2015; Feghalie and Wright, 1997). Cytokines are involved in regulating the inflammatory response to the exercise-induced muscle damage. It has been proposed that the mechanical damage to muscle cells which occurs during exercise is a stimulus for cytokine production (Pedersen et al., 1998). However, along with their role in regulating the inflammatory and anti-inflammatory responses to exercise, cytokines are also involved in muscle repair and regeneration following muscle damage (Tidball et al., 2014). Interleukin-1β (IL-1β), which becomes elevated following muscle injury, increases myoblast proliferation, thus stimulating muscle repair (Otis et al., 2014). The characterization of cytokine profiles during and following exercise provides insight into an organism’s biochemical response to exercise.

The cytokine profile changes throughout the exercise period. Interleukin-6 (IL-6) becomes elevated within 30 minutes of the onset of exercise, peaks at exercise cessation (Ostrowski et al., 1998) and is significantly correlated with muscle damage following eccentric exercise (Bruunsgaard et al., 1997). Interleukin-1α (IL-1α) increases during low exercise stress, whereas tumor necrosis factor α (TNFα) only becomes elevated during high-intensity exercise, implicating damaged skeletal muscle as a source (Kimura et al., 2001). While sampling throughout the exercise period is a logistically challenging task, cytokine flux prior to, during, and following exercise is significant. Many cytokines have opposing actions. Thus, to generate a comprehensive picture of the response to exercise, cytokines with antagonist activities must be considered. The inflammatory cytokines IL-1β, IL-6, and TNFα become elevated following a marathon along with the anti-inflammatory cytokine interleukin 10 (IL-10) (Ostrowski et al., 1999). It is likely that IL-10 becomes elevated along with inflammatory cytokines following exercise as a safeguard against uncontrolled inflammation. In order to understand how and why
the body responds to exercise in a particular manner it is essential to comprehensively describe cytokine fluctuations as well as the activity of antagonistic cytokines.

Prostaglandins, a subclass of eicosanoids, are involved in inflammation and the pain response. In man, prostaglandin E₂ (PGE₂) is raised in marathon runners immediately following exercise (Demers et al., 1981). Following eccentric weight training in man, PGE₂ peaks at 24 hr and remains elevated until 48 hr after exercise cessation (Smith et al., 1993). However, horses undergoing a treadmill test do not show a change in plasma PGE₂ immediately following exercise, nor 2, 6, 24, 48, or 72 hr thereafter (Donovan et al., 2007), suggesting there may be a species specific component in the magnitude of response of PGE₂ to exercise. More work needs to be done to clarify the timing of the PGE₂ response following exercise in different species as well as to better define its role during the post-exercise period. Following exercise, PGE₂ production has been proposed to be, at least in part, generated by macrophages, which become activated following exercise-induced muscle damage and by circulating inflammatory cytokines (Pedersen et al., 1998). Cytokines, PGE₂, and muscle-cell damage have an intricate relationship during and following exercise.

Muscle Damage and Exercise

Muscle damage following exercise is often associated with plasma CK. CK is an enzyme predominantly found within muscle cells (Thornton and Lohni, 1979). Thus when muscle enzymes, such as CK, are present within extracellular fluids, this is indicative of specific muscle cell damage. Elevated levels of CK have also been observed during muscular dystrophy, which is associated with muscle degeneration (Aston et al., 1984).

There is a strong connection between the muscle damage caused by exercise and the inflammatory response to exercise (Lewis et al., 2012; Cheung et al., 2003). It has been suggested that skeletal muscle damage resulting from exercise activates IL-6 production which stimulates the production of the inflammatory prostaglandin PGE₂ (Pedersen et al., 1998). IL-6 is also involved in leukocyte stimulation and recruitment of mononuclear cells (Hunter and Jones, 2015). Neutrophils and other immune modulating cells involved in cellular clean up become
activated by exercise-induced muscle damage and release inflammatory cytokines compounding the inflammatory response to exercise (Lewis et al., 2012; Chung et al., 2003; Pedersen et al., 1998). Neutrophil count, IL-6, and CK are elevated immediately following 45 minutes of downhill running (Peak et al. 2005). These markers were further increased at 1 hr post-exercise. However, by 24 hr IL-6 and neutrophils had returned to baseline whereas CK was still significantly elevated. Although this demonstrates that inflammatory cells, inflammatory cytokines, and muscle damage are all elevated following exercise, there does not appear to be a clear, direct relationship between the magnitude of increase in individual cytokines and exercise intensity.

There are many factors which can influence the degree of exercise-induced oxidative stress and muscle damage, including ambient temperature, gender and age. Exercising in high ambient temperature elicits increases in markers of oxidative stress which are not evident when the same exercise protocol is performed in moderate or cool temperatures (Quindry et al., 2013). In horses exercise-induced oxidative stress is dependent upon exercise duration and ambient conditions (Mills et al., 1996). However, exercise performed in hot conditions does not appear to be associated with increased markers of muscle damage (Nybo et al., 2013; Starkie et al., 1999). The lack of correlation between oxidative stress and muscle damage during the heat illustrates that although ROS might be involved in muscle damage (Kanter et al., 1988; Maughan et al, 1989) and participate in weakening muscle cell membrane integrity (Bloomer and Goldfarb, 2004), they do not appear to be the primary mediator of exercise-induced muscle damage.

Unlike season (Harris et al., 1990), both gender and age influence the relative concentrations of plasma CK and AST in racing thoroughbreds (Harries et al., 1998; Harris et al., 1990). CK levels, as a marker of muscle damage, are also influenced by training. Male runners have significantly higher resting CK activity, accompanied by a significantly lower CK response one day following a 45-minute bout of high-intensity exercise compared with untrained men (Evans et al., 1986). Both concentric and eccentric training reduce muscle damage (Nosaka and Newton, 2002). In rats, 4 weeks of either high or moderate intensity training reduces CK following prolonged treadmill running (Hyun et al., 2016). It is apparent that gender, age, and training can all influence the extent of muscle damage following exercise. However, the mechanisms behind
how these factors alter muscle damage is unclear. Future investigations should focus on the cellular mechanisms mitigating exercise-induced muscle damage.

Conclusion

Initiation, propagation, and recovery from exercise induces physiological adaptation responses as measured by temporal changes in biomarkers of oxidative stress, inflammation, and muscle damage, and these outcomes interact in a complex fashion. Increased oxygen uptake during exercise triggers oxidative stress resulting in inflammation which in turn produces more ROS. ROS cause lipid peroxidation and weaken membrane integrity causing muscle enzyme leakage and cell damage, which leads to more inflammation and the cycle continues. These processes do not occur in isolation and cannot entirely be separated. The elaborate interrelationships make it challenging to understand the effects of exercise on an organism and likely contribute to the occasionally conflicting results found within the literature. More work must be done in order to better understand the interrelationships which occur during exercise. In this way comparisons between studies can be made with more confidence and future research can be designed with these elements in mind. This will also provide athletes and those involved in sport with constructive and applicable information which they can use during their training, competitions, and recovery.

2.3 OA

Degenerative joint disease, otherwise known as OA, is one of the primary causes of lameness, lost training days, and early retirement for horses (de Souza, 2016; McIlwraith et al., 2012; Kidd et al., 2001). This disease is not unique to the horse and is also common in humans (Litwic et al., 2013; Buckwalter et al., 2004) and canines (Henrotin et al., 2005). The aetiology of OA has not yet been conclusively determined. Trauma is commonly considered a causal factor of OA, and it is believed that inflammation along with ROS are involved with its progression (Kidd et al., 2001). A diagnosis of OA covers a wide array of clinical signs. Thus, OA is considered a multifactorial disease of which the cumulative result is cartilage degradation and a loss of joint function.
Markers of OA

Direct imaging techniques such as radiography, scintigraphy, magnetic resonance imaging, and arthroscopy (Kidd et al., 2001) are often used to visualize damage to the joint structure. A major limitation of all these techniques is that they are unable to detect the very early stages of pathological change which occur prior to visible cartilage degeneration (Oei et al., 2014; Chiaradia et al., 2012). Early diagnosis and treatment of OA enables a reduction in structural damage and can prolong the time to clinical disease. An emerging area of interest is the use of articular biomarkers to assist in early detection of OA, as well as provide insight into dynamic changes occurring within a joint at a particular point in time (Henrotin et al., 2016, Lotz et al., 2013; Bijlsma et al., 2011). Biomarkers indicate local articular catabolic or anabolic processes. They can be classified either as direct, including products of cartilaginous and boney tissues or molecules specific to those tissues; or indirect, including compounds not derived from joint tissues but which may have an effect on the physiological processes within the joint (McIlwraith, 2005). Currently, as there is no one single biomarker which can effectively identify the precise state of OA progression, articular biomarkers of both cartilage breakdown and synthesis can help to develop a more inclusive image of joint health (de Grauw, 2011).

Inflammation is closely associated with OA and, regardless of the initial insult, inflammation is involved in the propagation of articular damage (de Grauw, 2011; Kidd et al., 2001). PGE$_2$ is an important biological signal during articular inflammation (Lee et al., 2013) and is involved in several pathological processes within the joint such as chondrocyte apoptosis and structural damage (Matel-Pelletier et al., 2003). Therefore, it is often measured as a biomarker of OA-affected joints. PGE$_2$ is significantly elevated in the SF of diseased equine carpal joints (Gibson et al., 1996) and the fetlock joints of fetlock-lame horses (de Grauw et al., 2006). The concentration of PGE$_2$ is significantly elevated in the SF from osteoarthritic equine joints (Kirker-Head et al., 2000). An experimental model of OA in horses also increased SF PGE$_2$ (Frisbie et al., 2008). The presence of PGE$_2$ in SF has been identified as having excellent predictive ability as a single value to distinguish the presence of OA in adult horses (Bertone et al., 2001). The recurrent presence of PGE$_2$ in diseased joints provides strong evidence that inflammation is involved in the pathogenesis of equine OA. Future research should focus on
further defining the role of inflammation in the development of clinical signs. Furthermore, it is important to consider what impact the reduced inflammation will have. Blunting inflammation may have some beneficial effects, including reduced damage, and delayed disease progression, but it may also just provide symptomatic relief without improving, or possibly impairing, articular cartilage structure and function.

Oxidative stress and inflammation are interrelated and hence it is not surprising that signs of oxidative stress have also been identified in diseased joints. The mechanical stresses placed on joints induce the production of ROS which act as catabolic factors and cause chondrocyte DNA damage and apoptosis (Yui et al., 2016). Dimock et al. (2000) demonstrated an association between oxidative stress and equine OA by identifying elevated levels of SF protein carbonyl content (a measure of ROS damage) in diseased equine joints. Intra-articular NO, a free radical often associated with inflammation, may also be indicative of arthritis (Jang and Murrell, 1998). When arthritis was induced in Sprague Dawley rats, the provision of a NO synthase inhibitor decreased synovial inflammation and tissue destruction (McCartney-Francis et al., 1993). Thus demonstrating the involvement of NO in degenerative processes within the arthritic joint. However, NO also displays chondroprotective actions (Jang and Murrell, 1998) including prostaglandin inhibition, increased collagen synthesis, and inhibition of apoptosis (Abramson, 2008). Thus its complete inhibition in the joint may not be desirable. It is likely, however, that similar to many other ROS (Ji et al., 2016), NO within the joint has hormetic qualities and when the concentration of SF NO exceeds the necessary threshold for health it contributes to joint damage.

The defining feature of OA is a decline in cartilage structure and function. Thus, the impact of a particular stimulus can be assessed using biomarkers of cartilage breakdown and synthesis. Proteoglycan fragments, such as glycosaminoglycans (GAG), are released into the SF during cartilage breakdown (Garvican et al., 2010; Lohmander et al., 1992). However, the quantification of SF GAG content in diseased equine joints has led to controversial results with some studies showing elevated concentrations (Palmer et al., 1995; Alwan et al., 1991) and others concluding that there is no difference between the SF GAG content in healthy and unhealthy equine joints (van den Boom et al., 2004a; Fuller et al., 2001). The use of criteria with
differing sensitivity to identify osteoarthritic and healthy horses, as well as different assay
standard and sample preparation may have contributed to the conflicting results. Research into
GAG assay protocols and various methods of SF digestion have found significant differences in
the final concentrations of GAGs recovered from samples (Oke et al., 2003) and thus its use as a
practical biomarker of proteoglycan catabolism is debatable. However, the use of metabolomics
in plasma and SF is a novel, accurate, and sensitive way to assess joint biochemistry as well as
identify new markers of OA (Hertonin et al., 2016; Zhang et al., 2016; Mickiewick et al., 2015;
Garvican et al., 2010).

Intra-Articular Injections

A transient model of arthritis can be induced by injecting an irritant into the intra-articular space.
This model allows for a controlled examination of the joint biochemistry during inflammation, as
well as the time course and magnitude of relevant biomarkers. Lipopolysaccharide (LPS) is
commonly used as an irritant in equine models of induced arthritis. The inflammatory marker
PGE\(_2\) peaked 12 hr after intra-articular injections of LPS into the radiocarpal joints of Quarter
horses and remained elevated for 336 hr (Lucia et al., 2013). Similarly, Pearson et al. (2012)
observed a significant increase in SF PGE\(_2\) and GAG 12 hr following the injection of LPS into
equine intercarpal joints. Other studies have detected significant increases in SF PGE\(_2\) (Wang et
al., 2015; de Grauw et al., 2009) and GAG (de Grauw et al., 2009) 8 hr following LPS
stimulation of equine joints. However, SF GAG continued to rise until it peaked at 24 hr post-
injection, and returned to baseline by 168 hr post-injection (de Grauw et al., 2009). An intra-
articular injection of autologous processed plasma also induced a transient rise in SF PGE\(_2\) which
was followed by an increase in GAG chondroitin sulfate at 24 hr post injection (Moreira et al.,
2015). These responses suggest that PGE\(_2\) is inherent to joint inflammation regardless of
stimulus and that inflammation and cartilage breakdown both occur in irritated joints.
Additionally, it appears that joint irritation may exert effects on cartilage metabolism for
prolonged periods of time, potentially as long as a week following the initial stimulus. The
extensive period of time during which joint integrity may be compromised following a stimulus
has implications for rehabilitation and recovery following traumatic injury and warrants further
investigation.
Exercise and Joint Biochemistry

Athletic horses are at a high risk of osteochondral injury and OA, which can severely limit or even end their careers (Riggs, 2006). A greater understanding of how exercise influences equine joint health will enable development of appropriate training methods for young horses to support developing joints, as well as prolong athletic careers and promote equine welfare.

Roos et al. (1993) assessed the change in levels of SF proteoglycan fragments after different forms of exercise in human athletes (treadmill running, road running, and soccer). Paired analysis comparing the concentration of proteoglycan fragments in SF before vs after exercise in the same athlete demonstrated significant exercise-induced increases, perhaps as a consequence of increased mechanical loading of the joints. Another study assessing the response of knee cartilage in human athletes following exercise (treadmill running, road running, soccer) found an increasing trend following exercise in all markers of cartilage breakdown investigated including aggregcan, stromelysin, and tissue inhibitor of metalloproteinase (Roos et al., 1995). Procollagen II C-peptide, a measure of matrix synthesis, was unchanged by the exercise. These results suggest that exercise induces cartilage degradation but not concurrent synthesis. However, post-exercise samples were taken between 30-60 minutes following exercise cessation and this may not have provided sufficient time for synthesis to become evident. Studies in horses have reported a transient rise in PGE₂ post-exercise (van den Boom et al., 2005) indicative of synovial inflammation. However, matrix metalloproteinases (MMPs), a group of enzymes involved in cartilage degradation and OA, were not altered by exercise (van den Boom et al., 2004b). The response of cartilage matrix metabolism to an acute bout of exercise depends on the type, intensity, and duration of the exercise performed. Thus different forms of exercise should be investigated individually. Also, increased SF GAG may indicate amplified cartilage catabolism and/or increased matrix turnover. Hence, measurement of both proteoglycan breakdown and synthesis should be undertaken to resolve the net effect of exercise on cartilage structure.

The influence of a singular bout of exercise on cartilage metabolism does not describe how chronic exercise impacts joint health. A moderate exercise regime improves cartilage GAG content (Roos and Dahlberg, 2005) and helps maintain cartilage integrity (Sun, 2010). Joint
movement and loading are necessary for joint health. Joint immobilization and unloading result in articular cartilage thinning (Nomura et al., 2017). Immobilized rabbit knee joints display signs of degenerative changes and reduced extractable proteoglycan content when compared to the joints from regularly exercised rabbits (Tammi et al., 1983). Hence a moderate exercise routine appears to preserve joint health when compared to joint immobilization (Tammi et al., 1983). However, the effects of intense exercise on joint health are not equivalent to those of moderate exercise. A systematic literature review of observational studies from 2000-2014 found that former elite human athletes from either team or individual sports are at higher risk for OA, particularly of the lower extremities, than the general population (Gouttebarge et al., 2015). In a rat model both strenuous running and immobilization result in osteoarthritic changes including macroscopic cartilage degradation and reduced cartilage proteoglycan content (Ni et al., 2016). A study which exercised 2 year old quarter horses/quarter horse crosses for 6 months 5 day/wk on high speed treadmills resulted in lameness of all exercised horses, significantly greater effusion of the intercarpal and metacarpophalangeal joints, as well as gross articular cartilage erosion of the metacarpophalangeal joints when compared to the controls (Kawcak et al., 2000). Joint immobilization and unloading, extreme exercise stress, long term, heavy mechanical loading of joints, or exercise strain placed on unhealthy joints are generally considered detrimental to cartilage integrity and health.

Current diagnostic techniques, such as radiography, lack sensitivity for subtle joint lesions (Kawcak et al., 2000) and early OA (Oie et al., 2014). Thus biomarkers of joint inflammation are becoming more practical as diagnostic aids. However, since both exercise and OA initiate changes within joint biochemistry, investigators must differentiate between changes resulting from exercise and those indicating disease. Some biomarkers show increased responsiveness to OA as compared with exercise, including GAG, epitopes CS846, CPII, Col CEQ, type 1 and 2 collagen degradation fragments, osteocalcin, type 1 collagen and PGE$_2$ (Frisbie et al. 2008). Serum markers CS846, CPII, GAG, and type 1 and 2 collagen fragments are also useful indicators for detecting early OA, and for differentiating the effects of exercise alone (Frisbie et al., 2008). While there is potential for biomarkers to differentiate between OA and normal articular adaptation to exercise, reliable reference ranges which can be applicable to clinicians have not been established.
Repeated Arthrocentesis

Arthrocentesis is the only method available to collect SF for analysis in the live, standing animal. Thus, the influence of arthrocentesis on the joint must be clarified to avoid confounding effects of the procedure on the outcome of interest. Repeated arthrocentesis results in a gradual increase in the concentration of SF TNFα and MMP. At least 14 days between arthrocentesis procedures are recommended for accurate analysis of MMP (van den Boom et al., 2004b). Similarly, repeated arthrocentesis in horses has been identified as a potentially confounding factor when analysing SF for NO, PGE₂, and GAG, and a week or more is recommended between samples (van den Boom et al., 2005). Conversely, others have not found an effect of arthrocentesis 4 times in 48 hours on SF PGE₂ (Lamprecht and Williams 2012). The cause of the dissimilar response of PGE₂ to repeated arthrocentesis is not readily apparent. Although the arthrocentesis procedure carries a higher risk than blood sampling due to the reduced immune surveillance within the micro-environment of the joint, it is a fairly unobtrusive procedure and can be done quickly with little trauma to the joint itself. The aforementioned studies appeared to perform a similar aseptic arthrocentesis technique. However, the needle sizes were not reported in the above mentioned studies and it is plausible this may have contributed to differences in inflammation following the procedures. Arthrocentesis is the only in vivo method currently available to access SF in the live, standing horse. Thus, more exploration into its influence on SF biomarkers will help refine the procedure and limit its impact on biomarkers of interest.

Conclusion

OA is a substantial problem in athletic horses. Horses suffering from OA can become lame and the condition can be career-ending. Changes in the chemical markers within the SF of diseased joints are often present before radiographic changes become evident and are a promising avenue to assist veterinarians in diagnosing OA at an early stage. Early diagnosis may enable maintenance and other supportive measures to be put in place, slowing the progression of the disease and prolonging the animal’s career. Although moderate exercise promotes joint and cartilage health, excessive exercise or exercising compromised joints is detrimental. Exercise results in metabolic changes in the joint, reflected by the biomarkers appearing in SF. Future research should define changes in biomarkers occurring due to disease as distinct from those...
resulting from exercise, given that many of the horses at risk for developing OA are also undergoing exercise training. Arthrocentesis is currently the only technique available to obtain SF samples for biomarker analysis from live, standing horses. Thus, improved characterization of the effects of repeated sampling will limit confounding effects of the procedure on outcomes of interest.

2.4 Antioxidant and Joint Supplements

The production of ROS is intrinsic within an organism, and as such, there are several endogenous antioxidant systems in place to prevent oxidative stress. The oxidative stress during exercise can be influenced by multiple factors including exercise type, duration, and intensity (Pingitore et al., 2015). As such, a general recommendation concerning the use of antioxidant supplements during exercise is not practical. Antioxidant supplementation is popular among athletes. However, in some instances it may be detrimental to performance and prevent some of the health benefits associated with exercise (Merry and Ristow, 2016; Braakhuis and Hopkins, 2015; Pingitore et al., 2015; Gomex-Cabrera et al., 2008). Dietary antioxidants are a necessary part of an organism’s antioxidant defense system and supplementation may improve antioxidant capacity (Powers and Jackson, 2008; Soffler, 2007). Therefore, there is an interest in the use of antioxidant supplements during times of heightened oxidative stress including disease, ageing, and exercise. As horses are often used for sport, equine antioxidant supplements represent an expanding market.

**Vitamin E**

The term “vitamin E” refers to a group of 8 compounds known as tocopherols and tocotrienols (Davies, 2009). Alpha-tocopherol is the only form found in significant quantities in equine tissues (Frape, 2010). Vitamin E is an essential antioxidant and plays a role in protecting lipid membranes from oxidative damage. In the equine diet, vitamin E is found primarily in fresh forage and pasture.

Vitamin E is an important antioxidant for exercising muscle as it is the principle antioxidant involved in lipid membrane protection (Gerster, 1991). Vitamin E is one of the most popular
antioxidant supplements provided to horses (Williams, 2010), and contributes to maintainance of plasma $\alpha$-tocopherol during training (Siciliano et al., 1997). Daily vitamin E supplementation (approximately 15 mg/kg) in the diets of racing greyhounds improved plasma levels of $\alpha$-tocopherol and prevented its decrease following a sprint race (Scott et al., 2001). In exercising polo ponies, a vitamin E intake of 42 IU kgDM$^{-1}$ was sufficient to reach near saturation levels in plasma (McMeniman and Hintz, 1992). However, daily vitamin E supplementation (approximately 300 IU kgDM$^{-1}$) significantly increases the concentration of reduced glutathione following intense exercise in horses (Duberstein et al., 2009). It seems that additional vitamin E supplementation above recommendations assists in supporting endogenous redox systems and maintaining plasma vitamin E levels in animals which undergo intense exercise.

Although the provision of vitamin E may assist in the maintenance of plasma $\alpha$-tocopherol during exercise it is often provided to optimize performance. Williams et al. (2005) investigated the impact of vitamin E levels on the performance of Arabian horses following an 80 km endurance race, and the provision of vitamin E beyond the dietary recommended level was not found to improve performance. Alaskan sled dogs also undergo intense endurance races, and when pre-race blood samples were taken from dogs competing in the 1998 Iditarod race it was found that dogs with higher pre-race vitamin E status displayed enhanced endurance and improved performance (Piercy et al., 2001). However, both studies were field trials and as such there were multiple factors beyond the vitamin E status of the animals which could have impacted performance. The use of self-reporting surveys of animal dietary intake, a singular blood sample to determine vitamin E status, different housing conditions and ownership could all have influenced study results. A meta-analysis examining the use of vitamin E supplements on exercise performance found no significant impact of vitamin E supplementation on exercise-induced lipid peroxidation or muscle damage (Stepanyan et al., 2014). However, the authors noted that the high variability between studies in this field make comparisons difficult and recommended stricter more unified procedures for future research. In the skeletal muscle of rats undergoing 10 weeks of swimming training increases in muscle oxidative capacity, expression of factors associated with mitochondrial biogenesis, and increases in antioxidant capacity were prevented by vitamin E supplementation (Venditti et al., 2014a). Additionally, the livers of trained rats supplemented with vitamin E demonstrated reduced antioxidant activity and
increased susceptibility to oxidation (Venditti et al., 2014b). Based on the current evidence it appears that supplementation of vitamin E beyond requirements is not recommended for healthy individuals in exercise training.

Although it appears that additional vitamin E assists in maintaining plasma levels of \( \alpha \)-tocopherol the influence of this on performance needs to be clearly defined. Field trials have come to conflicting conclusions and therefore more controlled trials evaluating the influence of vitamin E on specific parameters of performance need to be undertaken. Additionally, the long term benefits of vitamin E supplementation in relation to the adaptations of skeletal muscle and other organs to exercise training, regardless of immediate performance enhancement, should also be considered.

\textit{Vitamin C}

Vitamin C is a water soluble vitamin that assists in the regeneration of vitamin E. It is not considered essential in the horse because they can synthesize it in their liver from glucose (Stillions, et al., 1971). Vitamin C is involved in collagen synthesis, hormone synthesis, and acts as an antioxidant (Brown, 2000). The provision of supplemental vitamin C to horses may be beneficial during times of increased stress or in geriatric animals (Worth, 2010). Both an 80 km and 160 km endurance race reduces plasma ascorbic acid in horses (Hargreaves et al., 2002a). Due to the increase in oxidative stress associated with exercise (Powers et al., 2016; Deaton and Marlin, 2003; Kanter et al., 1994) the addition of supplemental vitamin C to the diets of horses which undergo intensive exercise or exercise training may be useful.

Vitamin C is not as commonly supplemented to exercising horses as vitamin E. However, its use has been investigated. In Thoroughbred race horses, intravenous administration of 5 g of ascorbate prevented an exercise-induced increase in TBARS following a simulated 1000 m race (White et al., 2001). Additionally plasma ascorbate, antioxidant capacity, and total antioxidant reactivity were increased when compared to basal levels. However, post-race plasma CK was unaltered. This supports the hypothesis that intravenous ascorbate mitigates subsequent oxidative stress but not muscle damage.
High doses of vitamin C can result in pro- as opposed to anti-, oxidant activity (Paolini et al., 1999). Thus over supplementation of vitamin C can be a concern as it may have the potential to enhance oxidative stress and impair performance. The use of vitamin C supplementation in both humans and rats undergoing endurance training significantly attenuates improvements in endurance capacity, and increases in the expression of transcription factors associated with mitochondrial biogenesis and/or antioxidant mRNA (Gomez-Cabrera et al., 2008). Racing greyhounds supplemented orally with 1 g of vitamin C daily for 4 weeks ran a 500 m sprint race, on average, 0.2 s slower than un-supplemented dogs (Marshall et al., 2002). Although vitamin C supplementation did appear to slow the dogs, it was noted that the 500 m sprint did not increase the parameters of oxidative stress which were measured. Hence, it is possible that during situations which induced a greater degree of oxidative stress supplemental vitamin C may provide an advantage. Nevertheless, the use of high doses of vitamin C in exercising individuals or those in training should be undertaken with caution as it may hamper performance or training efficiency.

It has long been established that vitamin C demonstrates a sparing effect on vitamin E (Sharma and Buettner, 1993) and as such they are often supplemented together. Williams et al. (2004) provided horses with a daily dose of either 5000 IU vitamin E alone or with an additional 7 g vitamin C for 3 weeks prior to an 80 km endurance competition. Plasma ascorbic acid was significantly increased in the group with added vitamin C, however, all other parameters of antioxidant activity and muscle leakage were unaffected by the additional vitamin C. A study conducted in humans found that a supplement providing 1000 mg/day vitamin C and 400 IU/day vitamin E blocked the insulin sensitising effects of exercise as well as the up-regulation of endogenous antioxidant enzymes induced by exercise (Ristow et al., 2009). This suggests that the transient increase in exercise-induced ROS is adaptive and necessary for some of the short and long term beneficial effects of exercise. In a double blind, randomised control study on the effect of vitamin E and C supplementation during endurance training, a vitamin E+C supplement (235 mg vitamin E and 1000 mg vitamin C) blunted the training induced increase in expression of factors associated with mitochondrial biogenesis in skeletal muscle (Paulsen et al., 2014). However, supplementation did not affect the improvements in VO$_{2\text{max}}$ and running performance
observed after training. A similar study, which examined the effects of a vitamin E+C mix (1000 mg/day vitamin C and 400 IU/day vitamin E) also found supplementation hampered skeletal muscle increases in SOD activity and mitochondrial transcription factor A following 4 weeks of endurance training (Morrison et al., 2015). More work investigating interactions between various antioxidant vitamins is necessary to clarify if, when, and how much supplementation is advantageous and at what point it becomes detrimental. Based on the current information available, supplementing both vitamin E and C appears to be unwarranted and may, in fact, prevent some of the benefits associated with exercise and training.

*Antioxidant Blends, Extracts, and Whole Food Additives*

**Antioxidant Blends**

Antioxidants are sometimes provided as isolated compounds, as described above, but more frequently are offered as part of a heterogeneous mixture of feed additives. Antioxidant blends offer an intriguing avenue for supplementation due to the often interrelated and synergistic activities of many antioxidant compounds (Wang and Zhu, 2017). An antioxidant supplement regime (vitamin E, A, and C for 60 days, the free radical scavenger allopurinol for 15 days, and N-acetylcysteine for 3 days) in healthy male non-athletes blunted exercise-induced increases in plasma IL-1β, TNFα and IL-6 following 45 minutes of bicycle riding at 70% VO₂max (Vassilakopoulos et al., 2003). Similarly, Alaskan sled dogs fed an antioxidant blend of 400 units vitamin E, 3 mg β-carotene, and 20 mg lutein daily for a month prior to 3 days of running exercise showed significantly decreased markers of DNA oxidative damage, as well as increased resistance of lipoproteins to *in vitro* oxidation compared with both sedentary controls and exercised but unsupplemented dogs (Baskin et al., 2000). American Foxhounds provided with a novel antioxidant blend prior to a two day hunt had reduced plasma AST, CK, and improved tracking scores compared to dogs receiving only commercial kibble (Huntingford et al. 2014). However, a major drawback of this study was a lack of randomisation of the groups; thus the data should be interpreted with caution. However, the data do support the hypothesis that combinations and novel blends of antioxidant supplements can impact oxidative stress and muscle damage following exercise, with some blends of antioxidant compounds showing more effect than any of the components alone. Future studies should explore why certain combinations
may affect change whereas others may not. This may help unravel the complex interactions between various antioxidant systems.

**Extracts and whole foods**

When added to the diet, an extract (a preparation concentrating the active ingredients of a particular substance) or whole food provides the advantage of having the interactions of multiple ingredients in the optimal ratios present in nature (Pingitore et al., 2015). The documented efficacy of different extracts is varied and comparison across studies is difficult as often circumstances are unique to a particular study. In army dogs which underwent exercise training, increases in MDA and reactive oxygen metabolites were observed following exercise (Kukovska et al., 2015). However, no significant changes were noted when dogs were supplemented with either rosehip or grapeseed extract. In this study it is difficult to ascertain whether these extracts were ineffective antioxidant supplements, or whether another factor, such as the preparation, diminished the extract potency. Another study in dogs, found that whole blueberries added to the diet of unfit Alaskan huskies significantly improved their plasma antioxidant potential immediately post-exercise when compared to un-exercised controls (Dunlap et al., 2006). A challenge when testing antioxidant activity of whole foods is the difficulty in definitively identifying distinct bioactive compounds or metabolites. It is possible that a particular compound in blueberries is a very strong antioxidant or that a particular combination of compounds present have synergistic activity. Nonetheless, there are many health benefits associated with the consumption of fruits and vegetables (Liu, 2013; Slavin and Lloyd, 2012). Hence, whole foods are an excellent resource to base further investigations.

Extracts have been provided to exercising horses, often in the form of liquid doses which can be supplied via nasogastric tube. Delivering the supplements in this form during research ensures that each horse receives a full and equal dosage. In horses administered either orange peel or black tea extracts via nasogastric tube 1 hr prior to a graded exercise test, both extracts modulated the cytokine response to exercise and orange peel extract reduced VO$_2$ recovery time (Streltsova et al., 2006). A similar study which dosed horses with either cranberry or ginger extract via nasogastric tube prior to a graded exercise test found horses dosed with the cranberry extract displayed significantly lower mRNA expression of TNFα following exercise (Liburt et
The horses provided with the ginger extract had a significantly reduced post-exercise VO$_2$ recovery time. However, a study by Smarsh et al. (2010) found that the provision of orange peel, black tea, cranberry, or ginger extract via nasogastric tube 1 hr prior to a graded exercise test in horses did not reduce plasma lipid hydroperoxides, total glutathione, or GPx. The authors concluded that a single dose of nutraceutical extract 1 hr prior to exercise does not reduce oxidative stress or improve antioxidant capacity. All studies used healthy, untrained Standardbred mares, the same graded exercise test, applied the extracts in 2 l aqueous doses via nasogastric tube 1 hr prior to exercise, and used the same time course for sampling. The chief differences between these papers lies in the outcome measures which were examined. Despite having a limited impact on measures of plasma oxidative stress or antioxidant activity, these extracts seemed to exert effects on mRNA expression of inflammatory cytokines and improve parameters of recovery in the horses. These results are not necessarily mutually exclusive and when assessed together it is possible that these supplements are impacting performance through a route other than reduced oxidative stress. On the other hand, it is possible the extracts did reduce oxidative stress but the markers chosen were inappropriate to demonstrate these results. Further mechanistic examinations will provide insight into how the antioxidant system can be altered or influenced, enabling more targeted development of new products and allowing for evaluation of potential side effects.

**Joint Supplements**

Equine joint supplements are commonly available on the equine market due, at least in part, to the prevalence of OA in athletic horses and the absence of a drug ‘cure’ for the condition. There are pharmaceuticals on the market such as phenylbutazone (PBZ) which can be used to treat the symptoms of OA. However, there are multiple documented side effects to the use of PBZ including gastric irritation and ulceration, renal dysfunction, right dorsal colitis, hypoproteinemia and neutropenia (McConnico et al., 2008; Collins and Tyler, 1984; Snow et al., 1979). The toxicity of PBZ in horses has a strong dose-response relationship (Lees and Toutain, 2013; Lees and Higgins, 1985). Nevertheless, these significant side effects highlight the need for long term efficacious alternatives. Nutraceuticals and dietary supplements represent a potential means of fulfilling this need. However, the general lack of research concerning the safety and efficacy of
the majority of equine supplements, joint supplements included, creates a potentially hazardous environment for the consumer and their horse.

PBZ is a frequently prescribed nonsteroidal anti-inflammatory drug, and is administered to horses to treat pain and discomfort associated with OA. Oral provision of PBZ following stimulation of equine joints with LPS did not reduce the SF markers of cartilage breakdown GAG or C2C, but it did reduce the marker of collagen synthesis CPII (de Grauw et al., 2014). This suggests that, while PBZ may be effective in treating acute pain, it may be at the cost of cartilage resynthesis. Therefore, further research exploring various compounds which may reduce cartilage inflammation following an insult, as well as protect cartilage against potential damage is of interest.

*In vitro* research using equine chondrocytes provides a non-invasive method of screening potential drugs and nutraceuticals for activity. This method of testing can also offer insight into potential modes of action. Several cartilage explant studies have investigated the effects of nutraceuticals and other dietary compounds on the release of biomarkers such as PGE2, GAG, and NO into culture medium following explant stimulation with LPS or IL-1β (Pearson et al., 2010; Pearson et al., 2007ab; Tung et al., 2002). The difference between supernatant concentrations in biomarkers can provide insight into the mechanism of action in suspected anti-arthritic compounds. Exposing chondrocytes to compounds which have undergone a simulated digestion and hepatic metabolism (Pearson et al., 2010) provides results which are more valid and applicable with regards to the live animal as this procedure imitates nutrient metabolism. The GAG release from equine cartilage explants co-cultured with IL-1β and 100 μmol/L curcumin was not significantly different than in unstimulated controls (Clutterbuck et al., 2009). The limited GAG release may be a result of a reduction in inflammatory induced breakdown. However, curcumin is toxic to chondrocytes at high levels and since no measures of chondrocyte viability were used in this study it is unknown whether curcumin may have resulted in chondrocyte death leading to diminished GAG release. It is necessary to examine various gauges of activity in order to develop a comprehensive image of the joint biochemistry under stimulation. *In vitro* research allows for a detailed and precise investigation of joint biochemistry. By utilizing simulated digestion and hepatic metabolism procedures, as well as
investigating multiple markers of chondrocyte activity, a reasonable understanding of the influence of a nutraceutical or drug on cartilage can be gained. However, the isolated nature of *in vitro* research unfortunately cannot account for the entire complexity within the whole organism. Thus *in vivo* research is unavoidable when exploring the efficacy of potential joint supplements.

A model of induced arthritis can be used in horses to establish the efficacy of a product intended for use as an anti-arthritic supplement. When irritants such as IL-1β or LPS are injected into the intra-articular space they induce an inflammatory response similar to OA, however, the reaction is transient. A model of intra-articular inflammation employing LPS in horses was used to examine the impact of an oral supplement composed of spearmint with an enhanced concentration of rosmarinic acid (Pearson et al., 2012). Inclusion of this nutraceutical supplement into the diet of horses for 24 days resulted in significantly lower SF concentrations of GAG and PGE₂. A similar study investigating a different dietary nutraceutical, composed of a proprietary blend of mussel, shark cartilage, abalone and *Biota orientalis* lipid extract, found this supplement prevented IL-1β induced increases in SF PGE₂ and GAG (Pearson et al., 2009). It is clear that certain dietary nutraceuticals can reduce inflammation and cartilage breakdown in joints stimulated with an irritant. However, there is still a missing link describing the applied use of these products in clinically healthy and unhealthy horses. Before any product can be employed with full confidence its effects in a field setting must be described.

Multiple nutraceuticals have been investigated for their use in reducing OA symptoms, progression, or development. However, there is a lack of basic characterization of the mechanisms involved in nutrient uptake into the synovial space as well as by articular cartilage (Goggs et al., 2005). The articular environment is isolated and responses of synoviocytes and chondrocytes to various chemical signals are complex. Thus, speculating upon the response of the articular environment to a particular dietary supplement is a difficult task. Therefore, research into nutraceuticals for joint health typically use *in vitro* models to characterize the biochemical response, use *in vivo* models of joint inflammation to demonstrate the articular response, and use field trials to determine the clinical and applied benefit of the product. However, field trials present practical difficulties such as identifying homogenous control and
clinically affected groups. There are also ethical considerations if it is necessary to withhold pharmaceutical treatment in lame horses or those in pain. Unfortunately, due to these considerable challenges, the expense of research and the lack of returns it generates, specifically in the equine industry, a majority of companies cannot supply the necessary information concerning the safety and proficiency of their products. This lack of research prevents the use of supplements to their full potential and allows unsafe or inefficacious products to persist in the marketplace.

**Conclusion**

The equine market is inundated with a multitude of different supplement products. Currently antioxidant supplements and joint supplements are some of the most popular and commonly used. Vitamin E, a well-known free radical scavenger, is often used to optimize performance of endurance horses. It does improve plasma α-tocopherol concentrations (Siciliano et al., 1997; Scott et al., 2001), however as of yet there is limited evidence indicating improved parameters of performance. Likewise, supplemental vitamin C improves plasma ascorbate (Williams et al., 2004; White et al., 2001), yet it may negatively impact performance (Marshall et al., 2002) or prevent beneficial adaptations to exercise (Morrison et al., 2015; Paulsen et al., 2014).

Nevertheless, there are countless antioxidant extracts and whole food supplements which, perhaps due to the natural blend of compounds, may hold promise as valuable aids during exercise and recovery. There is a need for safe and efficacious nutraceutical supplements on the market. Supplements for joint health should be tested *in vitro* and *in vivo* to evaluate their influence on joint inflammation and turnover. However, it is difficult to identify and enroll homogenous control and treatment groups for clinical and field trials which makes applied testing of these products a significant challenge. Nonetheless, product testing for efficacy and safety are of the utmost importance in order to ensure equine safety and improve the quality of life in athletic horses.

**2.5 Cucumis Melo**

As described earlier, antioxidant systems play key roles in the control and maintenance of a homeostatic environment. SOD is an endogenous antioxidant enzyme that is present in most
living organisms (Fridovich, 1995; Marklund, 1984). SOD catalyses the conversion of the ROS superoxide to hydrogen peroxide, which gets further metabolized to water and oxygen by the enzymes CAT or GPx (Romao, 2015). Superoxide is produced several ways including within the mitochondria by NADPH oxidases and xanthine oxidase (Powers et al., 2011a; Fridovich, 1995). The conversion of superoxide is of particular importance as it is a highly reactive molecule which can perpetuate further oxidation.

*Cucumis Melo*

The provision of SOD as an antioxidant supplement is an intriguing concept given that SOD is an enzyme and therefore not subject to consumption during a reaction. However, SOD from different sources is not equally efficacious with respect to radical scavenging abilities. An evaluation of injected bovine, human, and rat SOD (500 mg/kg) in a rat model of paw edema found that human and bovine SOD were superior anti-inflammatory agents (Baret et al., 1984). A particular type of non-genetically modified cantaloupe melon extract (*Cucumis melo*. extract; CME) has particularly high levels of SOD activity (Carillon et al., 2013a; Vouldoukis et al., 2004a). Although CME typically also contains other antioxidants in addition to SOD, SOD-depleted CME loses virtually all measurable antioxidant activity (Vouldoukis et al., 2004a). Methanolic extracts from different parts of *C. Melo* demonstrated that the flesh of the melon provided the highest methanolic yield; while the the leaf extract demonstrated the greatest total phenolic content, as well as significantly higher antioxidant activity as assessed by DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl radical scavenging activity, β-carotene bleaching activity, and total antioxidant activity (Ismail et al., 2010). *C. melo* represents an easily accessible source of vegetal SOD that has the potential to be harnessed and utilized as an antioxidant supplement.

Oral administration of free SOD has poor bioavailability. The improved bioavailability of SOD following liposomal encapsulation, which provides protection of the molecules during the gastric phase of digestion (Regnault et al., 1996), indicates that degradation by gastric enzymes is the most probable explanation for the reduced activity of ingested free SOD (Giri and Misra, 1983). No modifications of endogenous antioxidants are observed when oral CME is provided to mice.
without a gastroprotective delivery mechanism (Vouldoukis et al., 2004b). However, when CME is encapsulated with wheat gliadin, its ingestion promotes endogenous antioxidant defenses in the mice. When the activity and toxicity of CME using three different encapsulation coatings (palm oil, shellac, and gum arabic) at 3 doses (10, 40, and 160 IU SOD/day) was examined no irregularities with respect to blood hematology were noted (Carillon et al., 2013b). Furthermore, at the highest dose of CME hepatic (Cu, Zn, and Mn) SOD expression was significantly elevated and IL-6 was significantly decreased, regardless of the coating used. It is clear that encapsulation of CME is a viable delivery option which protects its activity from gastric degradation.

The anti-inflammatory potential of CME is evident from the elevated levels of endogenous SODs and reduced inflammatory markers identified following its consumption for 28 days by rats (Carillon et al., 2013b). The addition of CME to an in vitro model using mouse peritoneal macrophages prevents the production of superoxide anion and peroxynitrite following the addition of IgG1/anti-IgG1 immune complexes (Vouldoukis et al., 2004a). In vivo supplementation of mice with encapsulated CME for 28 days counters the production of free radicals, TNFα, and significantly enhances the production of IL-10 when mouse macrophages are harvested and assessed ex vivo following an injection of the pro-inflammatory cytokine interferon gamma (Vouldoukis et al., 2004a). Similarly, feeding CME to mice for 28 days enhances circulating and hepatic SOD, CAT, and GPx activities, as well as improves RBC resistance to oxidative induced hemolysis (Vouldoukis et al., 2004b). Encapsulated CME supplementation augments endogenous antioxidant status and possibly improves RBC membrane integrity, assisting in the prevention of oxidation induced cell destruction.

**Health Consequences of Oxidative Stress and the Potential Role of CME Supplementation**

Many important diseases, including diabetes (Piconi et al., 2003), HIV (Pace and Leaf, 1995), and renal disease (Galle, 2001) are associated with oxidative stress, though it is often unclear whether oxidative stress is a causal factor or an effect of the disease.

Due to the improvement in baseline antioxidant status following the ingestion of encapsulated CME (Romao, 2015), it has been investigated as a supplement in disease models known to be
associated with oxidative damage. Renal oxidative damage in a rodent model of type 2 diabetes was reduced following the addition of encapsulated CME into the diet (8 IU/g of diet) for 12 weeks (Naito et al., 2005). CME supplementation in hamsters fed an atherogenic diet significantly heightened blood and liver SOD activity, reduced aortic fatty streak area and the production of superoxide anion (Decorde et al., 2010). When encapsulated CME was added into the diet of weanling piglets at either 12.5 or 50 IU/kg of the diet, GIT stress protein levels were reduced (Lalles et al., 2011). Additionally, a pig model of ischemia/reperfusion injury demonstrated that 14 days of oral supplementation (10,000 IU/day) with encapsulated CME prior to surgery significantly reduced whole blood DNA damage, NO release, and spinal cord apoptosis (Kick et al., 2007). These results suggest an associated reduction of oxidative cell injury following the consumption of CME. Cats fed encapsulated CME supplement daily for 30 days (100 mg of a proprietary oral SOD complex) displayed significantly elevated levels of erythrocyte SOD and a heightened CD4+:CD8+ ratio after acute infection with feline immunodeficiency virus (Webb et al., 2008). These data suggest that encapsulated CME supplementation yields improvements in endogenous antioxidant activity, as well as reductions in parameters of oxidative stress and tissue damage in multiple species during various disease states. Due to the chronic nature of many diseases in which ROS likely play a role (ie diabetes, heart disease) it seems likely that CME may be most beneficial as a preventative measure. CME may provide a valuable tool which can be utilized to reduce the chance of disease development in high risk populations.

In humans the influence of oral CME supplementation was investigated with respect to hyperbaric oxygen (HBO)-induced oxidative stress (Muth et al., 2004). An oral encapsulated CME supplement (1000 UI-NBT) was provided for 2 weeks prior to HBO exposure, which reduced HBO-induced DNA damage and blood isoprostane levels (a recognized marker of oxidative stress). Consistent with several of the animal studies (Decorde et al., 2010; Naito et al., 2005; Vouldoukis et al., 2004ab), encapsulated CME supplementation protected against induced oxidative damage. The data are in contrast, however, to those describing no improvements in antioxidant levels, markers of oxidative stress, or perceived fatigue in 50-65 yr old women with unexplained fatigue consuming encapsulated CME (approximately 16 IU/day) for 12 weeks (Houghton et al., 2011). There are several possible explanations for these negative results. No
oxidative parameters were involved in the study inclusion criteria, thus it is possible the fatigue experienced by the subjects may not have been related to oxidative stress, fatigue may not be responsive to antioxidant supplementation, the dosage may have been too low or the supplement provided for insufficient time to exert any physiological effects, or the supplement may have been damaged. No adverse effects of ingestion were noted in either study, supporting the hypothesis that CME does not produce measurable adverse effects on health. Additionally, conditions known to induce significant oxidative damage, such as HBO exposure or ischemia/reperfusion injuries, may achieve a degree of protection from CME supplementation.

Encapsulated CME has been used across a range of species (Lalles et al., 2011; Decorde et al., 2010; Webb et al., 2008; Muth et al., 2004; Vouldoukis et al., 2004b) and in a variety of oxidative stress related conditions (Kick et al., 2007; Naito et al., 2005; Muth et al., 2004). Although there appears to be evidence supporting the antioxidant activity of encapsulated CME (Decorde et al., 2010; Webb et al., 2008; Vouldoukis et al., 2004ab) further characterization is required. There is a need to precisely define if there are tissue-specific actions of CME, and whether the measured inhibition of oxidative damage results in physiologically relevant improvements in health. A better understanding of why encapsulated CME does not consistently augment endogenous antioxidant activity will be important for its widespread application in the future.

**SOD Supplementation and Exercise**

Exercise is a physiological stressor which can result in excessive production of ROS (Soffler, 2007). Antioxidant supplements are often used prior to exercise with the goal of reducing oxidative stress in order to optimize performance, improve recovery, or reduce muscle damage. Encapsulated CME as a source of SOD supplementation is fairly recent. However, interest in the potential use of exogenous SOD to act as an ergogenic aid has been considered prior to the development of CME. In rats, an injected SOD derivative bound to albumin significantly reduced plasma and muscle TBARS as well as plasma xanthine oxidase immediately following a bout of exhaustive exercise (Radak et al., 1995). This early evidence indicates the ability of exogenous SOD to mitigate oxidative stress induced by intense exercise.
In a blinded study, either encapsulated CME or a placebo was provided as an oral daily supplement (500 mg of the encapsulated CME product GliSODin®) to members of the Polish rowing team participating in a 6-week training camp (Skarpanska-Stejnborn et al., 2011). Prior to and following supplementation, subjects underwent a 2000 m time trial on a rowing ergometer. Following supplementation with CME, erythrocyte SOD activity was significantly elevated and plasma C reactive protein (an inflammatory marker) was significantly reduced. Although, supplementation appeared to reduce inflammation and improve SOD status, no alterations in measures of oxidative damage following the exercise test were noted in the CME group. However, although the results of this human trial appear positive, further characterization in more controlled settings and following different intensities of exercise are necessary to substantiate the use of CME in athletes.

In addition to human athletes, CME has been explored in equine athletes. No differences were found in erythrocyte SOD, total glutathione, GPx, inflammatory cytokine gene transcripts, or SF PGE2 and chondroitin sulfate between mares supplemented with either placebo or CME for 6 weeks prior to a repeated sprint exercise test (Lamprecht and Williams, 2012). Conversely, 60 days of CME supplementation (520 IU/day) improved RBC resistance to hemolysis and reduced serum CK in racehorses compared to unsupplemented controls (Notin et al., 2010). Compared with the former study (Lamprecht and Williams, 2012), the latter study (Notin et al., 2010) employed a larger number of horses which increased the power of the study, and potentially improved its ability to discern the effects of supplementation. Additionally, the horses were in active race training for a minimum of 4 months prior to the exercise test and remained in training throughout the study period. Training is known to influence resting (Krumrych, 2010) and post-exercise (Miyazaki et al., 2001) concentrations of antioxidants and oxidative stress. It is possible that training participated in a synergistic relationship with CME supplementation to strengthen/stimulate the antioxidant system. Further work is required to ascertain whether encapsulated CME supplementation is capable of influencing parameters of exercise related oxidative stress. If CME does improve antioxidant potential, the groups of athletes in which supplementation will afford the greatest advantage need to be identified.
Oral CME Supplementation: A Potential Mechanism of Action

The mechanism of action of encapsulated CME supplementation has not yet been established. Since SOD is a protein it is unlikely to directly cross the gut barrier, but oral SOD supplementation has initiated health promoting outcomes despite its inability to be absorbed as an intact enzyme (Carillon et al., 2013a). Several studies have demonstrated an upregulation in endogenous SOD activity, and occasionally other antioxidant enzymes such as CAT and GPx following the ingestion of encapsulated CME (Lalles et al., 2011; Decorde et al., 2010; Webb et al., 2008; Vouldoukis et al., 2004b). Thus, it has been proposed that the consumption of encapsulated CME may lead to an upregulation of endogenous antioxidant systems, perhaps via the upregulation of associated gene transcripts, a cascade of events beginning in the intestine where the SOD would be released (Romao, 2015; Carillon et al., 2013ab). Nevertheless, a number of studies have found no changes in endogenous antioxidant activity associated with encapsulated CME supplementation (Lamprecht and Williams, 2012; Houghton et al., 2011; Notin et al., 2010; Muth et al., 2004). Thus, although this theory potentially explains some of the response to CME supplementation it may not capture the entire picture. More research is essential to clarify how CME interacts with the gut environment in order to characterize the potential cascade of events which may lead to an upregulation of the endogenous antioxidant systems. A better understanding of how CME stimulates the gut will lead to a more comprehensive image of which factors are necessary to initiate systemic change. This will in turn help to explain alterations in the antioxidant outcome measures which have been observed.

Conclusion

The variety of studies performed indicate that consumption of encapsulated CME is safe across a wide array of species. In several physiologically stressful situations it also enhances antioxidant defenses and reduces oxidative cellular damage. However, this is not always the case and a more detailed and specific characterization of when supplementation with encapsulated CME is valuable, including optimal conditions and target subjects, will provide direction for therapeutic applications. Furthermore, the putative mechanism of action for encapsulated CME must be verified, and the specific cascade of events following its ingestion defined. This will assist in focusing novel research in the most advantageous direction.
2.6 Conclusion

Exercise, oxidative stress, inflammation, and muscle damage are inextricably related. Exercise results in muscle damage, an upregulation of ROS, and an inflammatory response which varies depending upon multiple cofactors. Increasingly, the involvement of oxidative stress and inflammation are being recognized as contributors to OA. Thus the impact of exercise on joint health is relevant. Antioxidant supplements are used to reduce the strain of exercise as well as support joint health. There is likely common ground between these two functions which can be exploited for the benefit of athletes. However, many antioxidant compounds do not exhibit any influence on the antioxidant system or have be found to attenuate the long term benefits associated with exercise. Therefore, more work must be done to refine these products and promote only those with proven safety and efficacy. CME has reduced oxidative damage and promoted antioxidant defenses during multiple physiological challenges. It is a candidate for further exploration and may have additional health promoting benefits not yet discovered. The use CME as an ergogenic aid to promote antioxidant defenses in athletes, reduce oxidative damage, and improve recovery is a specific area that requires further attention.
CHAPTER 3: A TIME COURSE EVALUATION OF INFLAMMATORY AND OXIDATIVE MARKERS FOLLOWING HIGH-INTENSITY EXERCISE IN HORSES: A PILOT STUDY

Abstract

Exercise is a physiological stress resulting in reactive oxygen species and inflammatory mediators, the accumulation of which are thought to contribute to degenerative articular diseases. The horse is of particular interest in this regard as equine athletes are frequently exposed to repetitive bouts of high-intensity exercise. The purpose of this study was to provide a detailed description of the response of articular and systemic oxidative and inflammatory biomarkers following high-intensity, exhaustive exercise in horses. A group of horses (Ex; n=4) underwent repeated bouts of high-intensity exercise, at a target heart rate of 180 bpm, until voluntary exhaustion. Baseline plasma and synovial fluid (SF) samples were taken 24 hr prior to exercise, then at 0.5, 1, 2, 4, 8, and 24 hr following exercise cessation. This time course was repeated in a group of non-exercised control horses (Co; n=3). Plasma and SF samples were analysed for prostaglandin E2 (PGE2), nitrite, total antioxidant status (TAS), and glycosaminoglycans (GAG). The Ex group had significantly higher plasma nitrite at 0.5, 1, and 2 hr; and higher plasma PGE2 at 0.5 and 1 hr compared to Co. SF PGE2 and GAG were also higher in Ex horses at 8 hr compared with Co. It is concluded that high-intensity exercise in horses results in a rapid increase in systemic oxidative and inflammatory markers from 0.5-2 hr after exercise, which is followed by local articular inflammation and cartilage turnover by 8 hr post-exercise.

Key words: horse, oxidative stress, inflammation, exercise, physiology
Introduction

Exercise stress is associated with alterations in circulating inflammatory cytokines as well as increased markers of oxidative stress (Marlin et al, 2002; Pedersen et al, 1998). Elevations of plasma creatine kinase (CK), lactate dehydrogenase, and malondialdehyde (MDA) were observed in human athletes following an 80 km running race, indicating not only significant tissue damage, but also lipid peroxidation (Kanter et al, 1988). Oxidative stress and inflammation are closely linked and several reactive oxygen species (ROS), such as nitric oxide (NO), are also involved in inflammatory pathways. Both oxidative stress and inflammation can lead to damage at the level of the tissue, cell, protein, or DNA (Deaton and Marlin, 2003).

Although several physiological processes produce free radicals as by-products and this results in routine levels of oxidative stress, relatively little is known concerning when, where, and how much oxidative stress the body undergoes during physiologically taxing conditions, such as disease or exercise (Vollaard et al, 2005). Nevertheless, there is a degree of overlap between the cytokine response following exercise and the response to trauma (Ostrowski et al, 1998). These similarities establish exercise as a physiologically traumatic event and underscore the need for further investigation into the body’s adaptive response to it.

The response of equine athletes to acute, high-intensity exercise is still poorly understood. While it is known that mRNA expression of inflammatory cytokines including interferon-gamma, tumor necrosis factor alpha, interleukin 6, and interleukin 1 increase in plasma following exercise (Liburt et al, 2010), the impact of these responses on functional changes in protein expression, and ultimately their contribution to articular changes in the highly mobile joints, remains unclear. This is an important question because articular changes leading to osteoarthritis (OA) can often result in the termination of a horse’s athletic career. Articular inflammation in horses is associated with elevated local biomarkers of inflammation and oxidative stress (Gibson et al, 1996; Kidd et al, 2001; Kirker-Head et al, 2000). In particular prostaglandin E₂ (PGE₂; de Grauw et al, 2009) and NO (Jang & Murrell, 1998) are recognized as key participants in the disease pathway. Several biomarkers of cartilage metabolism and turnover are also influenced by exercise (Billinghurst et al, 2003; Frisbie et al, 2008). Glycosaminoglycans (GAG) have been
used as direct biomarkers of cartilage metabolism (McIlwraith, 2005), and thus can be useful indicators of the response of cartilage to a particular stimulus such as exercise. It is clear that exercise can result in an upregulation of various biochemical compounds involved in joint disease. However, to the authors’ knowledge, a comprehensive examination of the response of oxidative and inflammatory biomarkers to exercise in horses has yet to be undertaken.

The objective of this study was to characterize a time course of inflammatory and oxidative markers in both plasma and synovial fluid (SF) following an acute bout of high-intensity exercise in horses.

**Materials and Methods**

**Experimental Animals**

All experimental procedures and protocols were approved by the Nutraceutical Alliance Animal Care Committee (Campbellville, ON, Canada) prior to the beginning of this study in accordance with the Ontario Animals for Research Act and the Canadian Council on Animal Care guidelines. Eight horses of mixed breed, gender, and age were used (Table 3.1). Horses were maintained outside in group housing, with access to fresh water and hay ad libitum. All horses were clinically normal and underwent a health check, which consisted of checking temperature, pulse, respiration, hydration, menace reflex (a blink reflex in response to rapid movement) and gut sounds, prior to sampling. The horses had no known history of chronic joint inflammation or lameness, and were used for light pleasure riding. Owners provided written informed consent prior to the inclusion of their horse in the study. Four horses were randomly assigned to an exercise group (Ex) and 4 to a control group (Co). Exercise horse 1 only provided SF samples to the 1 hr time point. Control horse 8 was removed from the study due to intractability with the procedures and was not replaced.
Exercise

Horses on the Ex treatment were galloped in pairs under saddle around a half-mile dirt track. They were each fitted with a heart rate monitor (Polar Electro Canada, Lachine, QC). They were galloped for one lap around the track, with rider encouragement, at near maximal effort (at a target heart rate of 180 bpm) and then walked until their heart rates recovered to approximately 100 bpm (approximately 4 minutes of walking). This process was repeated until their time around the track increased by 10% over their fastest lap (Table 3.1).

Sample Collection

Baseline blood and SF samples were taken approximately 24 hr prior to exercise (BL), then 0.5 hr, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr after cessation of exercise. The sampling time course was also conducted in the unexercised Co group at a later date within the same month. Refer to Fig 3.1 for a schematic of the sampling time course.

Blood samples:

An area approximately 4 X 5 cm around the sampling area was clipped to reduce hair length to less than 2 mm. Emla cream (2.5% lidocaine, 2.5% prilocaine; AstraZeneca, Mississauga, ON) was applied to the left jugular groove and the left intercarpal joint approximately 30 minutes prior to sampling. Blood samples were collected directly into sodium heparin vacutainers (Becton-Dickenson, Mississauga, ON) from the jugular vein using a 21G 1.5” multiple sample needle. Blood samples were kept chilled at 4°C until processing, which occurred within 1-4 hr after sampling.

Synovial fluid samples:

The left carpal joint underwent aseptic arthrocentesis. The sample area was sterilized using iodine scrub followed by 99% isopropyl alcohol. 1-3 mL of SF was aspirated using a 22G 1”
needle into a sterile 3 cc syringe and transferred into a sodium heparin vacutainer. SF samples were kept chilled at 4°C until processing, which occurred within 1-4 hr after sampling.

Sample processing:

Blood and SF samples were centrifuged at 6000 x g for 15 minutes. Effluent was transferred into Eppendorf tubes and stored at -20°C until analysis.

Analysis of biological samples

Plasma samples were analyzed directly, whereas SF samples were pre-treated with hyaluronidase to improve assay precision (Jayadev et al, 2012). Hyaluronidase solution (4 mg/ml) was prepared in PBS. Synovial fluid samples were thawed to room temperature and centrifuged for 10 minutes at 10,000 rpm. 100 µl of supernatant was then mixed with 100 µl of hyaluronidase solution.

Samples were analysed using colorimetric spectrophotometric assays for nitrite (Griess Reaction; Molecular Probes, Eugene, OR), total antioxidant status (TAS; Cayman Chemical, Ann Arbor, MI), and glycosaminoglycan (GAG; dimethyl methylene blue; DMMB)(Chandrasekhar et al, 1987; Goldberg & Kolibas, 1990). Samples were analysed using a spectrophotometric immunoassay for prostaglandin E₂ (PGE₂; DetectX® Prostaglandin E₂ Enzyme Immunoassay, Arbor Assays, Ann Arbor, MI).

Statistics

Data are presented as means ± SEM unless otherwise stated. Marker concentrations (Yijk) were subject to repeated measures ANOVA using PROC MIXED (University Edition; SAS Institute Inc.) according to the following model:

\[ Y_{ijk} = \mu + \beta \times \text{initial} + \text{trt}_i + \text{time}_j + \text{trt} \times \text{time}_{ij} + \epsilon_{ijk} \]
where $\mu$ = overall mean, $\beta$ = covariate slope, initial = marker concentration at BL, trt = fixed effect of treatment ($i = 1$ or $2$), time = repeated measure of time ($j = 1$ to $7$) and $\epsilon_{ijk}$ = experimental error. Comparisons were made between treatments at each sampling time using estimate statements. Significance was set at $P \leq 0.05$.

Results

TAS

There was no effect of treatment on either plasma ($p = 0.7$) or SF ($p = 0.2$) [TAS]. Neither plasma nor SF TAS differed significantly between groups at any time point measured (Table 3.2).

NO

Plasma [nitrite] in the Ex group was higher than in Co at 0.5 hr ($p = 0.03$), 1 hr ($p = 0.04$), and 2 hr ($p = 0.04$) (Table 3.2; Fig. 3.2). There was also an effect of treatment ($p = 0.05$) and time ($p = 0.05$).

There was no effect of treatment on SF [nitrite] ($p = 0.9$) and SF [nitrite] did not differ significantly between groups at any time point measured (Table 3.2).

PGE$_2$

Plasma [PGE$_2$] in the Ex group was higher than in the Co group at 0.5 hr ($p < 0.0001$), and 1 hr ($p = 0.002$) (Table 3.2; Fig. 3.3). A treatment ($p = 0.02$), time ($p = 0.009$), and treatment by time interaction ($p = 0.003$) was observed.

There was no effect of treatment on SF [PGE$_2$] ($p = 0.1$). In the Ex group SF [PGE$_2$] was higher than Co at 8 hr ($p = 0.02$) (Table 3.2; Fig. 3.4).
There was no effect of treatment on plasma [GAG] (p = 0.4) and plasma [GAG] did not differ significantly between groups at any time point measured (Table 3.2).

There was no effect of treatment (p = 0.8) on SF [GAG] and no differences were observed between Ex and Co at any time. However, a time effect was observed (p = 0.04). This was likely due, at least in part to an aberrantly high SF [GAG] in horse 5 at 8 hr (766 µg/ml) which was beyond 4 SD of the mean. When this point was removed, there was no effect of treatment (p = 0.5). However, the Ex group SF [GAG] at 8 hr was higher than that of Co (p = 0.002) (Table 3.2; Fig. 3.5) and a treatment by time interaction was observed (p = 0.04).

Discussion

A growing body of evidence supports the hypothesis that high-intensity exercise contributes to a post-exercise inflammatory state, mediated at least in part by a cytokine profile akin to that seen during sepsis or trauma (Ostrowski et al., 1998; Pedersen et al., 1998). Our data further support this hypothesis, and indicate a time course of transient systemic inflammation as assessed by serum biomarkers which was evident 30 minutes following exercise and that lasted for approximately 2 hrs. This was followed by a localized articular inflammation (as evidenced by elevated PGE\textsubscript{2}) and increased accumulation of GAG in SF 8 hrs after cessation of exercise.

Biomarkers significantly influenced by high-intensity exercise in the current study included NO, PGE\textsubscript{2}, and GAG. Nitric oxide synthase (NOS) metabolises L-arginine into citrulline and NO (Kingwell, 2000), and can itself result in oxidative stress (Thérond et al., 2000). Three isoforms of NOS exist, the constitutively expressed endothelial (eNOS) and neuronal (nNOS) forms, as well as the inducible (iNOS) form which is stimulated by various inflammatory cytokines (Kleinert et al., 2003; Kingwell et al., 2000). NO is rapidly broken down into the stable end products nitrate and nitrite (Moshage et al., 1995). The half-life of these breakdown products is approximately 1.5 hr (Thérond et al., 2000) and thus the plasma concentrations of nitrate and
nitrite reflect recent changes in NO production. The Greiss reaction (used in the current study) is a common method of detecting a sample’s nitrite concentration (Bryan and Grisham, 2008; Moorcroft et al., 2001; Guevara et al., 1998; Green et al., 1982). In the current study, plasma NO was elevated in exercised horses between 0.5 and 2 hr following exercise. This is consistent with previous reports of increased NO in horses at 5 and 30 minutes following walk-trot exercise (Alberghina et al, 2015) and 2 hrs following repeated sprint exercise (Lamprecht et al, 2009). Skeletal muscle produces small amounts of NO during normal metabolism, but during exercise this production is markedly amplified (Reid, 2008).

The role of elevated NO during and following high-intensity exercise requires further study. NO is known to have several important physiological functions during exercise and is involved in vasodilation, skeletal muscle contraction and metabolism (Kingwell, 2000). Exogenous provision of nitrate, a stable precursor to nitric oxide, to human athletes results in marked improvement in maximal sprint and high-intensity intermittent running performance (Thompson et al, 2016), and significantly improved maximal work rate in untrained humans undergoing 3 weeks of sprint interval training (Muggeridge et al, 2017). Furthermore, inhibition of NOS in mice prevents the normalization of ion concentrations, plasma CK, TNF-α, and IL-6 following periodic whole-body acceleration following unaccustomed eccentric exercise (López et al, 2016). In equines a significant linear relationship between post-exercise body temperature and NO metabolites can be observed (Alberghina et al, 2015) indicating a role of NO in thermal adaptation to exercise. These data suggest that NO production may play an important beneficial role in adaptation to and/or recovery from high-intensity exercise, which may be dampened by provision of exogenous antioxidant substances.

PGE2 is a pro-inflammatory eicosanoid, which is a central chemical in the propagation and perception of inflammation and pain (Grösch et al, 2017; Kuehl & Egan, 1980). Alterations in both plasma and SF concentrations of PGE2 were detected in our study. In Ex horses, plasma PGE2 was significantly higher at 0.5 hr and 1 hr compared to Co. PGE2 plays a complex role in adaptation to and recovery from high-intensity exercise. In untrained humans there is an increase in post-exercise plasma PGE2 (Markworth et al, 2013). Inhibition of this increase through treatment with ibuprofen, a non-steroidal anti-inflammatory drug and non-specific inhibitor of
cyclooxygenase, resulted in concurrent blunting of lipoxins and protectins, lipid mediators associated with the resolution of inflammation and exercise recovery (Markworth et al, 2013). The associated between blunted PGE$_2$ and dampened inflammatory resolution indicates a mechanistic connection between elevated plasma PGE$_2$ post-exercise and the induction of physiological recovery from exercise.

PGE$_2$ in SF is an established biomarker of articular inflammation and joint disease (Moreira et al, 2015; Frisbie et al, 2008; Kirker-Head et al, 2000; Bertone et al., 2001), and is elevated in the SF of equine joints that have undergone a traumatic injury (Gibson et al, 1996). SF PGE$_2$ was not elevated until the 8 hr sampling time point post-exercise. The physiological significance of this increase is not completely understood and requires further study. We propose that the initial elevations in plasma levels of PGE$_2$ may have triggered inflammatory processes within the joint leading to PGE$_2$ production by synoviocytes and/or chondrocytes resulting in the lag time before the increase in SF PGE$_2$ became apparent. Future research investigating the impact of systemic PGE$_2$ production on the ensuing increase in SF PGE$_2$ will help to clarify whether the stimulus for joint inflammation following high-intensity exercise is chemical or mechanical in nature.

GAGs are structural breakdown products of the large aggregating proteoglycan molecules which are present in most connective tissue. They play an essential role in maintaining hydrostatic tension in cartilage, and are released during catabolism of proteoglycan (McIlwraith, 2005). In SF, GAGs have been identified as potential biomarkers of proteoglycan breakdown (de Grauw et al, 2014). Proteoglycan fragments have been documented to increase in humans following various types of exercise (Roos et al, 1993) and in patients following traumatic injury to the knee, being elevated in some patients for up to 4 years following an injury (Lohmander et al, 1989). However, reports of alterations in GAGs following exercise or injury are inconsistent throughout the literature. While no significant difference between SF GAG concentrations in equine OA-affected joints compared to unaffected contralateral joints are reported by some (Fuller et al, 2001), others (Frisbie et al, 2008) report SF GAG to be significantly greater during disease and exercise in horses. There are several methodological differences between these studies which may account for the discrepancies concerning their results. The latter (Frisbie et al, 2008) used horses in which OA was induced by the surgical creation of an osteochondral
fragment, perhaps controlling disease development and providing a more standardized environment than what would be possible when using clinical cases, as was the case in the former (Fuller et al, 2001). It is also possible that the use of the contralateral joint in OA-affected horses (Fuller et al, 2001) cannot be regarded as representative of, or equivalent to, a healthy equine joint. In humans, there are no differences in SF GAG concentration before and 30 to 60 minutes after bouts of various forms of exercise (soccer, treadmill running, and road running) (Roos et al, 1995). However, based on our results, the timing of these samples would not have been adequate to observe any potential SF GAG alterations. Our results indicate an approximate 8 hr delay between the termination of exercise and a significant increase in SF GAG concentration. The physiological implications of the post-exercise rise in SF GAG observed in our study is not known, and should be explored in further research. Others have suggested that increased post-exercise GAG in horses indicates accelerated breakdown of the cartilage matrix due to the chemical and/or mechanical stress imposed by exercise (Frisbie et al, 2008). However, it is not known to what extent proteoglycan synthesis is also occurring, a physiological process which is known to be increased with exercise (van den Hoogen et al, 1998). Further characterization of other enzymatic indicators of cartilage turnover such as matrix metalloproteases and cartilage oligomeric matrix protein (de Grauw, 2011; McIlwraith, 2005) will be necessary to accurately characterize the local articular response to exercise. Future research should explore the net effect of exercise on proteoglycan synthesis and breakdown, in order to better understand the overall effects of exercise on cartilage health.

Although exercise is commonly considered a physiological stressor that induces systemic as well as localized oxidative stress (De Moffarts et al, 2006; Vollaard et al, 2005), we found no changes in plasma or SF TAS concentrations. It is known that exercise of differing intensities results in varied measures of oxidative stress (Liu et al, 2000). Therefore, the lack of change in plasma TAS could indicate that, although our exercise test was of a high-intensity, it was not sufficiently rigorous, or was too short in duration, to stress the antioxidant equilibrium within the body to such a degree that a deficit was apparent.
Conclusion

An acute bout of high-intensity exercise in horses results in a time course of transient systemic inflammation beginning approximately 0.5 hr following exercise and lasting approximately 2 hr. This systemic inflammatory state is followed by increased GAG and PGE\(_2\) within the joint by 8 hr. These results provide insight into the systemic and articular biochemical responses of horses to an acute bout of high-intensity exercise.

Acknowledgements

Gratitude is extended to the owners of the horses participating in this study.

Grants

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<thead>
<tr>
<th>Horse</th>
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<th>Gender</th>
<th>Age</th>
<th>Group</th>
<th>Laps</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Morgan</td>
<td>Gelding</td>
<td>17</td>
<td>Ex</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Oldenberg</td>
<td>Mare</td>
<td>7</td>
<td>Ex</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Thoroughbred</td>
<td>Gelding</td>
<td>8</td>
<td>Ex</td>
<td>4</td>
</tr>
<tr>
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<td>7</td>
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<td>4</td>
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<tr>
<td>5</td>
<td>Halflinger X</td>
<td>Mare</td>
<td>5</td>
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<tr>
<td>6</td>
<td>Quarter horse</td>
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</tr>
<tr>
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<td>Morgan</td>
<td>Gelding</td>
<td>16</td>
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<tr>
<td>8</td>
<td>Andalusian</td>
<td>Gelding</td>
<td>7</td>
<td>Co</td>
<td>n/a</td>
</tr>
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</table>

Table 3.1. Description of age, gender, and breed of horses used in the study, and number of laps performed during the exercise test for exercised (Ex; n=4) and control (Co; n=3) horses included in the study.
Table 3.2. Plasma and synovial fluid (SF) markers of inflammation, oxidative stress, and cartilage breakdown in control (Co; n=3) and exercised (Ex; n=4 for plasma variables and n=4 until 1 hr following which n=3 for SF variables) horses. Co horses underwent the same sampling time course but were not exercised. Marker concentrations are means ± SEM. BL = baseline sample take approximately 24 hr prior to the exercise test.

* Significantly different from Co (p≤0.05).

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>BL</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Co</td>
<td>2.1 ± 0.31</td>
<td>1.8 ± 0.31</td>
<td>1.7 ± 0.31</td>
<td>1.4 ± 0.31</td>
<td>1.7 ± 0.31</td>
<td>1.8 ± 0.31</td>
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</tr>
<tr>
<td>Ex</td>
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<td>1.8 ± 0.21</td>
<td>1.7 ± 0.21</td>
<td>1.5 ± 0.21</td>
<td>1.8 ± 0.21</td>
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</tr>
<tr>
<td><strong>SF TAS (mM)</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Co</td>
<td>1.1 ± 0.51</td>
<td>1.8 ± 0.51</td>
<td>2.0 ± 0.51</td>
<td>2.5 ± 0.51</td>
<td>2.0 ± 0.51</td>
<td>2.4 ± 0.51</td>
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<td><strong>PNitrite (µM)</strong></td>
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<tr>
<td>Co</td>
<td>1.0 ± 0.46</td>
<td>1.4 ± 0.46</td>
<td>1.7 ± 0.46</td>
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<td>1.3 ± 0.46</td>
<td>0.8 ± 0.46</td>
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</tr>
<tr>
<td>Ex</td>
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<td>2.2 ± 0.32*</td>
<td>1.8 ± 0.32</td>
<td>1.9 ± 0.32</td>
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<td><strong>SF Nitrite (µM)</strong></td>
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</tr>
<tr>
<td>Co</td>
<td>1.7 ± 4.1</td>
<td>7.4 ± 4.1</td>
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<td>5.3 ± 4.1</td>
<td>13.2 ± 4.1</td>
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</tr>
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<td><strong>PGAG (µg/ml)</strong></td>
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<td></td>
</tr>
<tr>
<td>Co</td>
<td>133 ± 64.1</td>
<td>137 ± 64.1</td>
<td>194 ± 64.1</td>
<td>144 ± 64.1</td>
<td>132 ± 64.1</td>
<td>90 ± 64.1</td>
<td>148 ± 64.1</td>
</tr>
<tr>
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<td>153 ± 55.1</td>
<td>108 ± 55.1</td>
<td>102 ± 55.1</td>
<td>201 ± 55.1</td>
<td>265 ± 55.1</td>
<td>302 ± 55.1</td>
</tr>
<tr>
<td><strong>SF GAG (µg/ml)</strong></td>
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</tr>
<tr>
<td>Co</td>
<td>94 ± 47.6</td>
<td>136 ± 47.6</td>
<td>142 ± 47.6</td>
<td>98 ± 47.6</td>
<td>188 ± 47.6</td>
<td>97 ± 57.7</td>
<td>135 ± 57.7</td>
</tr>
<tr>
<td>Ex</td>
<td>84 ± 41.6</td>
<td>107 ± 41.6</td>
<td>107 ± 47.2</td>
<td>82 ± 47.2</td>
<td>78 ± 47.2</td>
<td>358 ± 47.2*</td>
<td>199 ± 47.2</td>
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<td><strong>PPGE2 (pg/ml)</strong></td>
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<tr>
<td>Co</td>
<td>48 ± 6.8</td>
<td>16 ± 6.8</td>
<td>30 ± 6.8</td>
<td>59 ± 6.8</td>
<td>58 ± 6.8</td>
<td>54 ± 6.8</td>
<td>54 ± 6.8</td>
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<tr>
<td>Ex</td>
<td>59 ± 4.8</td>
<td>65 ± 4.8*</td>
<td>59 ± 4.8*</td>
<td>62 ± 4.8</td>
<td>65 ± 4.8</td>
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<td><strong>SF PGE2 (pg/ml)</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Co</td>
<td>83 ± 51.5</td>
<td>77 ± 51.5</td>
<td>116 ± 51.5</td>
<td>102 ± 51.5</td>
<td>128 ± 51.5</td>
<td>80 ± 51.5</td>
<td>93 ± 51.5</td>
</tr>
<tr>
<td>Ex</td>
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<td>182 ± 44.7</td>
<td>216 ± 44.7</td>
<td>117 ± 51.0</td>
<td>118 ± 51.3</td>
<td>259 ± 51.3*</td>
<td>126 ± 51.3</td>
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Figure 3.1. Schematic of sampling time course performed in exercised (Ex; n=4) and control (Co; n=3) horses. Co horses underwent the same sampling time course but did not undergo the exercise test.
Figure 3.2. Mean ± SEM plasma nitrite (µM) concentration prior to and at selected time points following high-intensity exercise in exercised (Ex; n=4) and control (Co; n=3) horses. Co horses underwent the same sampling time course but were not exercised. BL = baseline sample taken approximately 24 hr prior to the exercise test.

* Significantly different from Co (p≤0.05).
Figure 3.3. Mean ± SEM of plasma PGE₂ (pg/ml) concentration prior to and at selected time points following high-intensity exercise in exercised (Ex; n=4) and control (Co; n=3) horses. Co horses underwent the same sampling time course but were not exercised. BL = baseline sample take approximately 24 hr prior to the exercise test.

* Significantly different from Co (p≤0.05).
Figure 3.4. Mean ± SEM of synovial fluid (SF) PGE$_2$ (pg/ml) concentration prior to and at selected time points following high-intensity exercise in exercised (Ex; n=4 until 1 hr following exercise after which n=3) and control (Co; n=3) horses. Co horses underwent the same sampling time course but were not exercised. BL = baseline sample take approximately 24 hr prior to the exercise test.

* Significantly different from Co (p≤0.05).
Figure 3.5. Mean ± SEM of synovial fluid (SF) GAG (µg/ml) concentration prior to and at selected time points following high-intensity exercise in exercised (Ex; n=4 until 1 hr following exercise after which point n=3) and control (Co; n=3) horses. Co horses underwent the same sampling time course but were not exercised. BL = baseline sample take approximately 24 hr prior to the exercise test.

* Significantly different from Co (p≤0.05).
CHAPTER 4: DIETARY CUCUMIS MELO REDUCES SYSTEMIC AND ARTICULAR INFLAMMATION FOLLOWING HIGH-INTENSITY EXERCISE IN HORSES

Abstract

Exercise-induced oxidative stress and inflammation are associated with skeletal muscle and articular cartilage damage which can result in discomfort and ensuing poor performance. The objective of this study was to determine the effect of 3 weeks of dietary supplementation with an encapsulated Cucumis melo pulp extract (CME) on biomarkers of local articular and systemic oxidative stress and inflammation resulting from high-intensity exercise in horses. Horses underwent an initial high-intensity exercise test immediately prior to being provided a diet containing CME (0 or 1g; equivalent to 2600 IU SOD/horse/day) once daily for 23 days. On day 22 the exercise test was repeated. Blood and synovial fluid (SF) samples were taken 24 hr prior to exercise (BL), and at 1 and 24 hr following exercise. Plasma and SF were analysed for prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), total antioxidant status (TAS), nitrite, and superoxide dismutase (SOD) activity. In addition, SF was analysed for glycosaminoglycans (GAG), and plasma was analysed for thiobarbituric acid reactive substances (TBARS). Blood biochemistry and haematology analyses were also performed. Comparisons were made using a repeated measures design with the initial exercise test as a covariate. There was a significant upregulation of SF SOD in the CME group (p=0.04). In SF, CME reduced nitrite and GAG at 1 hr compared to unsupplemented controls. Furthermore, CME increased plasma TAS and reduced plasma lymphocytes 1 hr after cessation of exercise compared with controls. At 24 h following exercise, plasma PGE\textsubscript{2} (p=0.04) and creatine kinase (p=0.008) were significantly lower in horses receiving CME than in unsupplemented controls. It is concluded that daily supplementation with dietary CME for 3 weeks in horses reduces articular inflammation and breakdown whilst supporting blood lymphocytes and plasma TAS 1 hr following high-intensity exercise. CME also reduces systemic inflammatory response and muscle cell damage 24 h following intense exercise.
Introduction

Exercise is a known biological stressor, which in horses, has been found to result in an oxidative and inflammatory response (de Moffarts et al., 2004). It is likely that several pathways result in free radical production during and after exercise (Vollaard et al., 2005). In humans, the inflammatory response resulting from 2.5 hr of treadmill running is similar to what occurs following physical trauma (Ostrowski et al., 1998). Repeated sprinting has been found to be a more intense type of exercise in horses when compared to graded or interval exercise regimes (Lamprecht et al., 2008).

Exercise induces systemic inflammation and oxidative stress, but it also influences joint biochemistry in a manner similar to that encountered during articular inflammation (Lamprecht and Williams, 2012). Osteoarthritis (OA) in horses is a chronic joint disease characterized in part by cartilage degradation (Riggs, 2006; Kidd et al., 2001). OA is frequently associated with elevated glycosaminoglycans (GAG) in synovial fluid (SF), serum, and urine (Alwan et al., 1991), and increased biomarkers of oxidative stress, including SF nitric oxide (NO) or protein carbonyl (Dimock et al., 2000; Jang and Murrell, 1998). In addition to biomarkers of catabolism and oxidative stress, strongly inflammatory compounds, such as prostaglandin E2 (PGE2), have been identified as excellent predictors of OA in equine SF (Bertone et al., 2001). It is evident that inflammation and oxidative stress are involved in the pathogenesis of OA.

High-intensity exercise is considered a significant risk factor for the development of OA (Riggs, 2006), as there are multiple physiological parallels between the articular environment following intense exercise and during OA. Exercise instigates articular biochemical changes which mimic those seen in OA. In humans, various forms of exercise increase proteoglycan fragments in SF 30-60 minutes following an exercise bout (Roos et al., 1993). Similarly, studies in horses have identified a transient rise in post-exercise SF PGE2 (Chapter 3; van den Boom et al., 2005). These results demonstrate the occurrence of post-exercise synovial inflammation and cartilage breakdown. Investigating biomarkers of cartilage breakdown, joint inflammation, as well as reactive oxygen species (ROS) within SF are potentially useful in determining joint health and the effects of exercise on cartilage physiology.
Dietary supplements have been investigated as potential ergogenic aids and as interventions for articular maintenance, with some supplements being composed of ingredients known to be high in antioxidants. Antioxidant extracts provided to exercising horses have been found to reduce the inflammatory response to exercise and improve recovery (Streltsova et al., 2006; Liburt et al., 2010). Alaskan sled dogs supplemented with blueberries, a foodstuff known to be high in antioxidant activity, demonstrated improved antioxidant status (Dunlap et al., 2006). These results provide evidence that the provision of antioxidants within the diet can affect parameters of antioxidant activity and oxidative stress when provided to exercising animals. Various nutraceuticals have also been investigated as potential interventions for joint disease (Goggs et al., 2005). A species of spearmint with enhanced rosmarinic acid content significantly reduced the level of GAG and PGE$_2$ in SF when fed to horses prior to the induction of a transient model of arthritis (Pearson et al., 2012). As nutraceuticals can influence both exercise performance and joint biochemistry it is worthwhile to consider parameters of both.

_Cucumis melo_ L.C. extract (CME) is a plant product rich in the antioxidant enzyme superoxide dismutase (SOD). When combined with a biopolymer and fed to mice, CME has protective effects against administered pro-inflammatory compounds (Vouldoukis et al., 2004a). CME has reduced parameters of oxidative stress and enhanced antioxidant activity in a variety of species including humans (Muth et al., 2004), pigs (Kick et al., 2007), felines (Webb et al., 2008), mice (Naito et al., 2005), and horses (Notin et al., 2010). The objective of the current study was to investigate the effects of an encapsulated CME dietary supplement on systemic and local articular biomarkers of oxidative stress and inflammation following an acute bout of high-intensity exercise in horses.

**Materials and Methods**

**Experimental Animals**

All experimental procedures and protocols were approved by the Nutraceutical Alliance Animal Care Committee (Campbellville, ON, Canada) prior to the beginning of this study in accordance with the Ontario Animals for Research Act and the Canadian Council on Animal Care guidelines. Twelve horses of mixed breed, gender (6 mares, 6 geldings), and age (range 5-17,
median 7.5) participated in this study (Table 4.1). Of the 12 horses, 4 participated in the control group (Co), 4 participated in the CME group (CME), and 4 participated in both (Co and CME). All horses were clinically normal and underwent weekly health checks which consisted of checking temperature, pulse, respiration, hydration, menace reflex (a blink reflex in response to rapid movement) and gut sounds. Health checks were also performed on all horses 24 hr prior to a sampling day. The horses had no known history of chronic joint inflammation or lameness. During the study all horses maintained a regular schedule of pleasure riding and light lessons. Horses were randomly assigned to a diet containing 0 (Co; n=8) or 1 g (CME; n=8) of a freeze dried *Cucumis melo* pulp extract encapsulated with palm oil in their morning feed, based on the manufacturer’s instructions. The supplement provided approximately 2600 IU SOD/horse/day according to the manufacturer. All horses were fed a diet which met their nutritional requirements (NRC, 2007). Horses were all maintained at the same facility in a loose-housing system, and had unlimited access to shelter, pasture, hay, and water.

**Study Design**

This study was part of a larger 3-way crossover design (Lindinger et al., 2017). The study was conducted over 3 rounds which consisted of 23 days of supplementation separated by a 3-week washout period to avoid carryover effects. The order of the treatments was randomized across the cohort of horses. Each round consisted of a covariate sampling period, a dosing period, and a treatment sampling period. A high-intensity exercise test was performed on Day 1 (covariate sampling period), followed by 23 days of supplementation provided in the morning meal (dosing period), and a second exercise test on day 22 (treatment sampling period). The first two rounds consisted of 6 horses per round (3 Co, 3 CME), and the last round consisted of 4 horses (2 Co, 2 CME). Refer to Fig. 4.1 for a schematic of the study design.

**Exercise Test**

Horses were matched according to speed and fitness, and exercised under saddle in groups of 2 to 3 at approximately 11 a.m. Heart rate was monitored during galloping by the riders using equine heart rate monitors (Polar Electro Canada, Lachine, QC). Horses were warmed up (at the walk, trot, and canter) for 20 minutes prior to the exercise test. The horses were exercised under
saddle using the exercise test according to Chapter 3. Briefly, the exercise test consisted of repeated single-lap gallops around a ½ mile dirt track at a target heart rate of 180 bpm, separated by approximately 4 minutes of walk, this process was repeated until voluntary exhaustion. The first exercise test sequence performed by each horse was used to determine their maximal heart rate, speed, and ability. The mixed breed horses were able to complete 3 laps while the Thoroughbreds completed 4. The number of laps was conserved for each horse during each exercise test that the horse participated in.

Sample Collection

Samples were obtained at baseline (BL) around 11 am the day prior to the exercise test, then again at 1 and 24 hr following the cessation of exercise. Refer to Fig. 4.1 for a schematic of the sampling time course.

Blood Samples

An area approximately 4 X 5 cm around the sampling area was clipped to reduce hair length to less than 2 mm. Emla cream (2.5% lidocaine, 2.5% prilocaine; AstraZeneca, Mississauga, ON) was applied to the left jugular groove and the left or right intercarpal joint approximately 30 minutes prior to sampling. Blood samples were collected directly into sodium heparin and EDTA vacutainers (Becton-Dickson, Mississauga, ON) from the jugular vein using a 21G 1.5” multiple sample needle. Blood samples were taken immediately prior to arthrocentesis and chilled at 4°C until processing, which occurred within 1-4 hr after sampling.

Synovial Fluid Samples

Aseptic arthrocentesis was performed at each time point, alternating joints between samples to allow sufficient time for replenishment of SF. The left or right intercarpal joint was prepared using a stanhexidine/iodine scrub followed by 99% isopropyl alcohol. Arthrocentesis was performed using aseptic procedures, and approximately 1 mL of fluid was aspirated using a 22 Gx1” needle into a 3 cc sterile syringe. Aspirated fluid was immediately transferred into a
sodium heparin vacutainer tube and chilled at 4°C until processing, which occurred within 1-4 hr after sampling.

Sample Processing

Fresh blood samples were analyzed for biochemistry [albumin (ALB), albumin/globulin ratio (A:G), aspartate amino transferase (AST), creatinine (Cre), creatine kinase (CK)] (Johnson & Johnson, Ortho Clinical Diagnostics Model 5.1 Fusion) and complete blood count (Siemens Model Advia 2120; Nassagaweya Veterinary Laboratory Services; Campbellville, ON, Canada). Additional aliquots of heparinized blood and SF were centrifuged at 6000 x g for 15 minutes. Supernatant was then transferred into Eppendorf tubes and stored at -20°C until analysis.

Prior to analysis, SF samples were digested via hyaluronidase treatment to improve assay precision (Jayadev et al., 2012). Hyaluronidase (HAase; Sigma-Aldrich, Oakville, ON) was suspended in PBS to a final concentration of 4 mg/ml solution. SF samples were prepared using a 1:1 dilution of HAase solution and sample. Mixed HAase:SF samples were agitated for 1 hour, centrifuged at 1000 x g for 5 minutes, and the supernatant was removed and used in assays.

Sample Analysis

Samples were analysed using colorimetric spectrophotometric assays for nitrite (Griess Reaction; Molecular Probes, Eugene, OR), total antioxidant status (TAS; Cayman Chemical, Ann Arbor, MI), and superoxide dismutase (SOD; Trevigen® Instructions, Gaithersburg, MD). Samples were analysed for prostaglandin E$_2$ using a spectrophotometric enzyme immunoassay (PGE$_2$; DetectX® Prostaglandin E$_2$ Enzyme Immunoassay, Arbor Assays, Ann Arbor, MI). Plasma was analysed using a colorimetric spectrophotometric assay for thiobarbituric acid reactive substances (TBARS; R&D Systems Inc., Minneapolis, MN), and untreated SF was analysed for glycosaminoglycans using a colorimetric spectrophotometric assay (GAG; dimethyl methylene blue; DMBM) (Goldberg and Kolibas, 1990; Chandrasekar et al., 1987).
Statistics

Data are presented as mean ± SEM unless otherwise stated. Marker concentrations (Y_{ijk}) were subject to repeated measures ANOVA using PROC MIXED (University Edition; SAS Institute Inc.) according to the following model:

\[ Y_{ijk} = \mu + \beta \times \text{initial} + \text{trt}_i + \text{time}_j + \text{trt} \times \text{time}_j + \epsilon_{ijk} \]

where \( \mu \) = overall mean, \( \beta \) = the covariate slope, \( \text{initial} \) = marker concentration during the covariate sampling period, \( \text{trt} \) = fixed effect of treatment (\( i = 1 \) or 2), \( \text{time} \) = repeated measure of time (\( j = 1 \) to 3) and \( \epsilon_{ijk} \) = the experimental error. Comparisons were made between treatments at each sampling time using estimate statements. Least square means using a Tukey adjustment were employed to analyse the effects of time within group. Significance was set at \( P \leq 0.05 \).

Results

SF Markers of Oxidative Stress

No effects of time (\( p = 0.2 \)) or treatment (\( p = 0.7 \)) were observed for SF TAS. There was an effect of treatment for SF SOD, with consistently higher SF SOD in the CME group than the Co group (\( p = 0.04 \); Table 4.2, Fig. 4.2).

SF Markers of Inflammation

No treatment effect was observed for SF PGE_{2} (\( p = 0.3 \); Table 4.2) or nitrite (\( p = 0.1 \); Table 4.2). A time effect was observed for SF PGE_{2} (\( p = 0.04 \); Table 4.2) and nitrite (\( p = 0.04 \); Table 4.2). SF nitrite was lower in the CME group compared to Co group at 1 hr (\( p = 0.01 \); Fig. 4.3). No treatment effect was observed for SF GAG (\( p = 0.2 \); Table 4.2). SF GAG was lower in the CME group compared to Co group at 1 hr (\( p = 0.03 \); Fig. 4.4).
Plasma Markers of Oxidative Stress

No effects of time or treatment were observed in plasma TBARs (p = 0.1, 0.3 respectively) or SOD (p = 0.9, 0.3 respectively). There was no overall effect of treatment on plasma TAS (p = 0.1; Table 4.3). However, there was an effect of time on plasma TAS (p = 0.004; Table 4.3). Additionally, plasma TAS was higher in the CME group compared to Co group at 1 hr (p = 0.03; Fig 4.5). In the Co group, plasma TAS was lower at 1 hr compared to BL (p = 0.01).

Plasma Markers of Inflammation

No effects of time (p = 0.6) or treatment (p = 0.4) were observed in plasma nitrite. There was no effect of treatment on plasma PGE₂ (p = 0.09; Table 4.3). However, plasma PGE₂ was reduced in the CME compared to Co at 24 hr (p = 0.04; Table 4.3, Fig 4.6).

Markers of Blood Biochemistry

A time effect was observed for Cre, AST, ALB, A:G ratio, HGB, and RBC (Table 4.3). There was an effect of treatment (p = 0.02) and time (p = 0.02) for CK (Table 4.3). CK was also lower in the CME compared to Co at 24 hr (p=0.008; Fig 4.7). In the Co CK was higher at 24 hr compared to BL (p = 0.03).

Markers of Immunity

No effect of treatment was observed for blood lymphocytes (LYM; p = 0.2; Table 4.3). However, time (p = 0.004) and time by treatment (p = 0.01) effects were observed (Table 4.3). LYM were also lower in CME compared to Co at 1 hr (p = 0.002; Fig 4.8). In the Co, LYM were higher at 1 hr compared to BL (p = 0.0028) and 24 hr (p = 0.006).

Discussion

Based upon our investigation into the use of CME in horses undergoing intense exercise, it appears that dietary encapsulated CME may influence the systemic and articular antioxidant
environment. Horses supplemented with CME displayed reduced post-exercise articular oxidative stress and cartilage breakdown, as well as diminished systemic inflammation and muscle damage.

There is considerable interest in antioxidant products derived from plant sources as a means to improve general health, reduce the risk of chronic diseases, and enhance athletic performance. *Cucumis melo* pulp extract is made from the pulp of melon which has a distinctly high SOD activity (Romao, 2015). CME has produced anti-oxidant and anti-inflammatory properties both *in vitro* and *in vivo* (Vouldoukis et al., 2004a). Ingestion of an unprotected source of SOD results in digestive degradation of the enzyme and displays no influence on antioxidant parameters *in vivo* (Vouldoukis et al., 2004b). However, when it is combined with a dietary delivery system such as encapsulation with liposomes, wheat gliadin, palm oil, shellac, or gum arabic the bioavailability is increased and parameters of antioxidant capacity within the body system are altered following ingestion (Carillon et al., 2013a; Vouldoukis et al., 2004ab; Regnault et al., 1996). Ingestion of encapsulated CME has a number of health benefits including mitigation of diabetic neuropathy (Naito et al., 2005), ischemia/reperfusion injury (Muth et al., 2004), and exercise stress (Skarpanska-Stejnborn et al., 2011). The observed time effects in many of the variables considered in the current study are consistent with physiological changes due to high-intensity exercise (Zobba et al., 2011; Lamprecht et al., 2008; Peake et al., 2005; Smith et al., 1989), indicating that our standardized exercise test was of the desired high-intensity and resulted in significant physiological stress.

**Articular Effects**

Proteoglycan fragments and GAG release have been observed in SF following injury and as a result of applied irritants (de Grauw et al., 2009; Lohmander et al., 1989). SF GAG concentration is a marker of cartilage degradation and is increased following exercise in horses (Chapter 3; Frisbie et al., 2008), and in human athletes of varying disciplines (treadmill running, road running, and soccer playing) approximately 1 hour following exercise (Roos et al., 1993). While the mechanism of exercise-induced increase in these SF proteoglycan fragments is not fully understood, it has been hypothesized that the increase may result from increased mechanical
loading during exercise (Roos et al., 1993). Thus, the blunted increase in post-exercise SF GAG noted in the CME group in the current study indicates a favourable influence of the CME supplement on articular cartilage structure.

SF and plasma nitrite measured by the Greiss reaction were used as a measures of sample NO (Théron et al., 2000; Moshage et al., 1995). The lower SF NO measured in the CME group at 1 hr mirrored SF GAG in the current study. NO is a signalling molecule (Kingwell, 2000) that is elevated in arthritic joints (Jang and Murrell, 1998). Suppression of NO production via inhibition of NO synthase reduces articular inflammation and tissue damage in rats (McCartney-Francis et al., 1993). Our results implicate a potential targeted effect of CME supplementation on joint inflammation and associated cartilage degradation. Thus, the NO-inhibiting properties of CME may be contributing to the observed decrease in exercise-induced cartilage breakdown. Further exploration into the relationship between encapsulated CME, NO, and cartilage metabolism is required to clarify the sequence of events which leads to reduced cartilage breakdown within the joint. This research is necessary to reveal whether these effects are truly a result of tempered inflammation or whether another process is involved. It is also possible that the increased GAG concentration within SF of the Co is a result of increased breakdown concurrent with increased synthesis of cartilage. Thus, in future studies, an emphasis should be placed on investigating triggers and markers of cartilage synthesis as well as breakdown.

There was an overall upregulation of SOD within the SF of the CME group. This increased SOD activity may augment the antioxidant capacity of the intra-articular space, hence mitigating the increase in SF NO following exercise and contributing to reduced cartilage breakdown. Superoxide radical and other ROS can be produced within the joint by leukocytes, chondrocytes, and synoviocytes (Schneider et al., 2005; Henrotin et al., 1993; Tiku et al., 1990; McCord, 1974). The use of SOD knockout mice or SOD gene overexpression have demonstrated the chondroprotective action of SOD (Ross et al., 2004; Iyama et al., 2001). Thus the upregulation of SF SOD in supplemented horses is a possible safeguard against synovial inflammation and hyaluronic depolymerisation. However, the mechanism of SF SOD upregulation following supplementation with CME is unclear and requires further study. Since SOD is a large enzyme with a molecular weight of 32,600 kDa (McCord and Fridovich, 1969) it is unlikely that ingested
SOD is absorbed to any significant degree from the intestinal tract (Carillon et al., 2013b). Therefore, it has been postulated that the amplified endogenous antioxidant activity which is often observed following encapsulated SOD oral administration may stem from a cascade of events initiated by an upregulation of endogenous antioxidant enzyme transcriptional activity possibly due to the interaction between SOD and the gastric epithelia (Carillon et al., 2013b). The effects of CME supplementation on additional antioxidant levels within SF presents a practical area for future investigation, which will contribute to a better understanding of the role played by SOD in augmenting antioxidant defence systems.

It is currently unclear as to whether increased SOD activity resulting from CME supplementation is universal or restricted to specific tissues. In the current study a treatment effect on SOD activity was only observed in SF and not in plasma. The reasoning for this is unclear, as results in the literature are conflicting. Following CME supplementation, upregulation of erythrocyte SOD was observed in Polish rowers undergoing intensive training (Skarpanska-Stejnborn et al., 2011), whereas in Standardbred trotters it was not (Lamprecht and Williams, 2012; Notin et al., 2010). A difference in species response, dosage, sampling times, exercise protocol, or subject fitness between studies may have contributed to the opposing results. A distinct difference between our study and those previously mentioned was that we investigated extracellular SOD activity as opposed to erythrocyte SOD activity. Within extracellular fluids, such as plasma and SF, SOD is an important enzyme which is present to protect organisms from the production of superoxide in extracellular compartments (Fridovich, 1995; Marklund, 1990). Due to our interest in the influence of CME within the joint as well as systemically, the investigation of extracellular SOD was appropriate. Nevertheless, a detailed description of the cascade of events following encapsulated CME ingestion will aid in improving our understanding of its mechanism of action and why it may influence SOD activity in certain tissues and not others.

**Immune Effects**

At 1 hr post-exercise, blood lymphocyte concentration was maintained at baseline levels in the CME horses, while the Co horses experienced a significant elevation in circulating lymphocytes. Following maximal exertion in eight male oarsmen on a rowing ergometer the concentration of
several populations of WBCs within peripheral blood increased (Nielsen et al., 1996). In Thoroughbred race horses, exercise stress is accompanied by a transient lymphocytosis (Snow et al., 1983; Rossdale et al., 1982). The increase in the lymphocyte population following exercise is mainly composed of CD8+ T-lymphocytes and natural killer (NK) cells (Campbell et al., 2009). It is possible the increased circulating lymphocytes are a product of splenic contraction following exercise onset. Indeed, splenectomised human subjects demonstrated reduced T-lymphocytes and NK cell activity following exercise compared to matched controls (Nielsen et al., 1997). However, in direct contrast to these results, another study demonstrated that the spleen does not meaningfully contribute to post-exercise leukocytosis (Iversen et al., 1994). Similar subject pools were used in both studies. However, different exercise protocols may partially explain the contrasting conclusions. In the study by Iversen et al. (1994) a 5-minute maximal exercise test on an ergometer was employed, whereas the exercise test in the study by Nielsen et al. (1997) consisted of incremental ergometer cycling for 12 minutes followed by superamaximal cycling to exhaustion. Nevertheless, recruited lymphocytes demonstrate reduced telomere length, which indicates maturity, senescence, and reduced faculty for clonal expansion (Simpson et al., 2007). It is likely that multiple organs act as tissue pools of lymphocytes, with the number of cells released being dependent upon the intensity and duration of the exercise undertaken (Pedersen and Hoffman-Goetz, 2000).

Of perhaps more clinical significance than the transient exercise-induced lymphocytosis is the subsequent post-exercise lymphopenia, resulting in a lymphocyte count on average 32-39% lower than baseline values (Hansen et al., 1991). The post-exercise lymphopenia is characterized by reduced CD4+ T-cells during the exercise recovery period (Kurokawa et al., 1995). The pattern of leukocyte alterations indicates a post-exercise period of susceptibility to infection between approximately 2 and 4 hr following exercise cessation (Pedersen et al., 1998). The immune suppression associated with high-intensity exercise is well established (Pedersen and Toft, 2000). Thus, whether the maintenance of lymphocyte concentration in the CME group would be maintained throughout the recovery period and prevent post-exercise lymphopenia would be of great interest. Maintenance of lymphocyte concentration would be particularly significant for horses under extreme exercise stress, such as those in race training. Unfortunately, blood samples were not taken at 2 to 4 hr in this study. Hence, the effect of CME on exercise-
induced lymphopenia remains an unknown. However, this represents an interesting area for future investigation, particularly for horses undergoing race training.

**Systemic Effects**

In addition to maintenance of blood lymphocyte concentrations 1 hr following exercise in the CME group, post-exercise TAS was also maintained at baseline levels in CME horses. Within the Co group, TAS significantly decreased at the 1 hr time point. The influence of exercise on plasma antioxidant capacity is inconsistent. Short intense bursts of physical effort in horses, endurance exercise in dogs, as well as acute bouts of treadmill exercise in rats and cycling exercise in healthy adults result in decreased plasma TAS (Steinberg et al., 2006; Ficiclar et al., 2003; Balogh et al., 2001; Piercy et al., 2000). However, augmentation of plasma TAS has been reported following maximal and submaximal exercise in untrained healthy males (Berzosa et al., 2011) and following a 30 sec Wingate test in football players (Hammouda et al., 2012). In other studies antioxidant capacity has not been observed to change following exercise (Chapter 3; Alessio et al., 1997). It seems likely that exercising below the threshold of an individual’s antioxidant capacity will result in an upregulation of antioxidant activity. However, if an individual exercises beyond the capacity of their antioxidant system, the amount of ROS produced will exceed the neutralizing capacity of the endogenous system, resulting in a reduced antioxidant status. If individuals exercise somewhere within the antioxidant capacity of their system, then the increase in ROS will be balanced by their antioxidant system and result in a net lack of change in general measures of total antioxidant activity, such as TAS. This is in keeping with the hypothesis of exercise-induced hormesis with regards to ROS production during exercise (Ji et al., 2016).

The decline in plasma TAS observed in our study would indicate the exercise protocol employed taxed the antioxidant system of the horses to the extent that a deficit became apparent. Thus, in the CME group it would appear that CME supplementation assisted in maintaining blood antioxidant potential. The reasoning behind this improved antioxidant potential is currently unclear as plasma SOD was unchanged in the CME group, and this was the only antioxidant enzyme investigated. It is possible that an upregulation in the activity of another antioxidant
enzyme, such as glutathione peroxidase or catalase, may have contributed to the increase in plasma TAS. The activity of both glutathione peroxidase and catalase have been found to increase following CME supplementation (Trea et al., 2013; Cloarec et al., 2007; Vouldoukis et al., 2004b). In the future, additional antioxidant enzymes should be investigated to provide a more complete representation of the antioxidant system following supplementation.

Despite the acute changes which manifest soon after exercise cessation, post-exercise muscle soreness, stiffness, reduced muscle force production and joint range of motion do not become apparent until approximately 24 to 48 hr following exercise (Cheung et al., 2003). These clinical signs are likely the result of a combination of metabolic and physiological reactions to exercise including inflammation and muscle damage (Lewis et al., 2012; Cheung et al., 2003). Prostaglandin E$_2$ is an inflammatory mediator which is involved in discomfort and pain following intense exercise. Creatine supplementation for humans, when provided for 5 days preceding a triathlon event, was able to reduce plasma levels of PGE$_2$ at 24 and 48 hr post-exercise compared to the controls (Bassit et al., 2008). The authors proposed that creatine supplementation prior to a strenuous exercise event could reduce the subsequent inflammatory response. Comparably, the mitigation of plasma PGE$_2$ 24 hr following exercise in the CME group suggests CME supplementation may have aided in attenuating post-exercise inflammation.

In the CME group, CK was also reduced compared to controls 24 hr following exercise. Post-exercise elevations in CK are well documented in horses (Hinchcliff et al., 2004; Siciliano et al., 1997; Harris et al., 1990; Kanter et al., 1988). CK is a protein found primarily in equine muscle cells (Thornton and Lohni, 1979), and it assists in the production of ATP via phosphorylation of ADP from creatine phosphate (Kingston, 2008). CK activity in plasma is often used as a marker of muscle cell leakage and damage in humans (Peake et al., 2005; Kanter et al., 1988), dogs (Dunlap et al., 2006; Piercy et al., 2001; Piercy et al., 2000), and horses (Hargreaves et al., 2002a; Chiaradia et al., 1998). The CK activity of plasma can provide a measure of exercise intensity and is influenced by exercise duration (Kingston, 2008). The lower CK observed in the CME group at 24 hr indicates reduced muscle leakage and possibly improved muscle cell membrane integrity. However, concurrent decreases in other associated indicators of muscle damage, such as plasma AST, were not detected. Hence, more targeted investigation of muscle
membrane integrity following CME supplementation and exercise would be required to test this hypothesis.

The reduction in plasma PGE$_2$ and CK observed in supplemented horses 24 hr following exercise may be related to a mitigation in the systemic inflammatory response to exercise. Maintenance of plasma TAS at 1 hr post-exercise may have contributed to reduced oxidative damage, inflammation, and muscle injury. However, the link between maintained plasma TAS and the ensuing reduction in inflammation/muscle leakage is not well-characterized, and further exploration into this relationship will improve our understanding of the connection between oxidative stress, inflammation, and muscle damage (Lewis et al., 2012; Powers et al., 2011a; Reid, 2008). The reductions in plasma CK and PGE$_2$ following supplementation with CME may indicate an improvement in recovery of supplemented horses. A supplement which results in lowered levels of plasma PGE$_2$ and CK 24 hr following high-intensity exercise may be particularly appealing for endurance horses and horses that are required to compete for multiple days. If inflammation and muscle cell damage can be diminished or their recovery enhanced, this may lead to an improvement in overall performance and welfare during multiple day events. However, further work must be done to investigate additional parameters of muscle contractile force and overall performance during the 24-48 hr following intense exercise in supplemented individuals. This additional research will help to determine whether the attenuation of PGE$_2$ and CK at the 24-hr mark translates to any functional changes in parameters related to exercise.

**Conclusion**

The results of this study indicate that 3 weeks of supplementation with CME influences articular and systemic responses to high-intensity exercise in horses. There was an overall upregulation in SF SOD activity, as well as reduced SF NO and GAG 1 hr following exercise in supplemented horses. TAS of supplemented horses was maintained 1 hr following exercise while PGE$_2$ and CK were reduced at 24 hr. These data indicate that dietary CME may mitigate oxidative stress and inflammation/muscle damage following intense exercise. Future studies should seek to better characterize the nature of the relationship between these physiological processes, and the role that CME can play within it. The CME-mediated modulation of post-exercise lymphocytosis is
also important to explore in future research as it could represent a response to CME which could be exploited in heavily exercised horses.
Table 4.1. Description of horse breed, age, gender, and group of control (Co; n=8) and supplemented (CME; n=8) horses used in the current study.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Breed</th>
<th>Gender</th>
<th>Age</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thoroughbred</td>
<td>Mare</td>
<td>13</td>
<td>Co</td>
</tr>
<tr>
<td>2</td>
<td>Quarter horse</td>
<td>Mare</td>
<td>5</td>
<td>Co</td>
</tr>
<tr>
<td>3</td>
<td>Dutch warmblood</td>
<td>Mare</td>
<td>5</td>
<td>Co</td>
</tr>
<tr>
<td>4</td>
<td>Thoroughbred</td>
<td>Gelding</td>
<td>8</td>
<td>Co</td>
</tr>
<tr>
<td>5</td>
<td>Morgan</td>
<td>Gelding</td>
<td>16</td>
<td>CME</td>
</tr>
<tr>
<td>6</td>
<td>Oldenburg</td>
<td>Mare</td>
<td>7</td>
<td>CME</td>
</tr>
<tr>
<td>7</td>
<td>Quarter horse</td>
<td>Gelding</td>
<td>7</td>
<td>CME</td>
</tr>
<tr>
<td>8</td>
<td>Thoroughbred</td>
<td>Gelding</td>
<td>13</td>
<td>CME</td>
</tr>
<tr>
<td>9</td>
<td>Morgan</td>
<td>Gelding</td>
<td>17</td>
<td>Co, CME</td>
</tr>
<tr>
<td>10</td>
<td>Quarter horse / Haflinger X</td>
<td>Mare</td>
<td>5</td>
<td>Co, CME</td>
</tr>
<tr>
<td>11</td>
<td>Thoroughbred</td>
<td>Gelding</td>
<td>7</td>
<td>Co, CME</td>
</tr>
<tr>
<td>12</td>
<td>Quarter horse X</td>
<td>Mare</td>
<td>8</td>
<td>Co, CME</td>
</tr>
</tbody>
</table>
Table 4.2. Time, treatment, and treatment by time effects within synovial fluid (SF) markers of oxidative stress and inflammation in response to high-intensity exercise in control (Co; n=8) and supplemented (CME; n=8) horses. Marker concentrations are means ± SEM. BL = baseline sample take approximately 24 hr prior to the exercise test.

* Denotes a significant difference between Co and CME (p≤0.05).

<table>
<thead>
<tr>
<th>SF</th>
<th>BL</th>
<th>1 hr</th>
<th>24 hr</th>
<th>ptime</th>
<th>Put</th>
<th>Put*time</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAG</td>
<td>Co</td>
<td>342 ± 143</td>
<td>820 ± 130 *</td>
<td>298 ± 141</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>CME</td>
<td>345 ± 133</td>
<td>344 ± 155</td>
<td>256 ± 169</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>Co</td>
<td>6 ± 7.8</td>
<td>39 ± 7.7 *</td>
<td>7 ± 8.0</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>CME</td>
<td>5 ± 7.9</td>
<td>9 ± 7.7</td>
<td>6 ± 8.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE₂</td>
<td>Co</td>
<td>196 ± 31.6</td>
<td>227 ± 32.1</td>
<td>163 ± 31.6</td>
<td>0.04</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>CME</td>
<td>171 ± 30.8</td>
<td>144 ± 30.5</td>
<td>128 ± 30.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD</td>
<td>Co</td>
<td>166 ± 13.0</td>
<td>170 ± 13.0</td>
<td>187 ± 13.0</td>
<td>0.4</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>CME</td>
<td>183 ± 13.0</td>
<td>204 ± 13.0</td>
<td>198 ± 13.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>BL</td>
<td>1 hr</td>
<td>24 hr</td>
<td>P_{time}</td>
<td>P_{trt}</td>
<td>P_{trt\times time}</td>
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<td>----------</td>
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<td>-----------------</td>
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</tr>
<tr>
<td>TAS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>1.09 ± 0.023\textsuperscript{a}</td>
<td>0.97 ± 0.023\textsuperscript{b*}</td>
<td>1.06 ± 0.024\textsuperscript{ab}</td>
<td>0.004</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>CME</td>
<td>1.07 ± 0.022</td>
<td>1.04 ± 0.024</td>
<td>1.09 ± 0.023</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>537 ± 210</td>
<td>457 ± 210</td>
<td>789 ± 212\textsuperscript{*}</td>
<td>0.4</td>
<td>0.09</td>
<td>0.5</td>
</tr>
<tr>
<td>CME</td>
<td>138 ± 197</td>
<td>133 ± 198</td>
<td>157 ± 208</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>0.92 ± 0.035</td>
<td>1.04 ± 0.053</td>
<td>0.91 ± 0.044</td>
<td>0.004</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>CME</td>
<td>0.96 ± 0.033</td>
<td>1.04 ± 0.051</td>
<td>0.93 ± 0.043</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Co</td>
<td>31.4 ± 0.54</td>
<td>34.8 ± 1.65</td>
<td>31.9 ± 0.64</td>
<td>0.0062</td>
<td>0.6</td>
<td>0.3</td>
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<td>CME</td>
<td>32.2 ± 0.53\textsuperscript{ab}</td>
<td>36.3 ± 1.62\textsuperscript{a}</td>
<td>31.6 ± 0.65\textsuperscript{b}</td>
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<tr>
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<tr>
<td>Co</td>
<td>342 ± 25.8</td>
<td>399 ± 34.2</td>
<td>490 ± 74.2</td>
<td>0.013</td>
<td>0.7</td>
<td>0.4</td>
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<tr>
<td>CME</td>
<td>401 ± 26.1</td>
<td>417 ± 33.2</td>
<td>468 ± 71.0</td>
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<td></td>
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<tr>
<td>CK</td>
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</tr>
<tr>
<td>Co</td>
<td>295 ± 76.8\textsuperscript{a}</td>
<td>651 ± 79.9\textsuperscript{ab}</td>
<td>665 ± 73.0\textsuperscript{b*}</td>
<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
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<td>460 ± 71.9</td>
<td>353 ± 77.6</td>
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<tr>
<td>Co</td>
<td>117 ± 5.0\textsuperscript{a}</td>
<td>137 ± 5.5\textsuperscript{b}</td>
<td>119 ± 5.0\textsuperscript{a}</td>
<td>&lt;0.0001</td>
<td>0.9</td>
<td>0.7</td>
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<tr>
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<td>139 ± 5.0\textsuperscript{b}</td>
<td>121 ± 5.0\textsuperscript{a}</td>
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<tr>
<td>Co</td>
<td>13.7 ± 0.55\textsuperscript{a}</td>
<td>16.0 ± 0.59\textsuperscript{b}</td>
<td>15.3 ± 0.55\textsuperscript{b}</td>
<td>0.0005</td>
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<td>14.5 ± 0.55\textsuperscript{ab}</td>
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<td>2.9 ± 0.30\textsuperscript{a}</td>
<td>4.8 ± 0.30\textsuperscript{b*}</td>
<td>3.0 ± 0.30\textsuperscript{a}</td>
<td>0.004</td>
<td>0.2</td>
<td>0.1</td>
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<td>3.3 ± 0.30</td>
<td>3.3 ± 0.30</td>
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<tr>
<td>Co</td>
<td>8.1 ± 0.29\textsuperscript{a}</td>
<td>9.6 ± 0.33\textsuperscript{b}</td>
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<td>0.0004</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>CME</td>
<td>8.1 ± 0.30\textsuperscript{a}</td>
<td>9.3 ± 0.29\textsuperscript{b}</td>
<td>8.5 ± 0.30\textsuperscript{ab}</td>
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Table 4.3. Time, treatment, and treatment by time effects within plasma markers of oxidative stress and inflammation, as well as blood biochemistry and hematology parameters in response to high-intensity exercise in control (Co; n=8) and supplemented (CME; n=8) horses. Marker concentrations are means ± SEM. BL = baseline sample take approximately 24 hr prior to the exercise test.

\textsuperscript{a,b} Superscripts which differ denote significantly different values within a row (p\leq0.05).

\* Denotes a significant difference between Co and CME (p\leq0.05).
Figure 4.1. Schematic of study design and sampling time course performed in supplemented (CME; n=8) and control (Co; n=8) horses. BL = baseline sample taken approximately 24 hr prior to the exercise test.
Figure 4.2. Mean ± SEM of synovial fluid (SF) SOD (IU/ml) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test.
Figure 4.3. Mean ± SEM of synovial fluid (SF) nitrite (µM) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test.

* Denotes significant difference between the Co and CME (p≤0.05).
Figure 4.4. Mean ± SEM of synovial fluid (SF) GAG (µg/ml) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test.

* Denotes significant difference between the Co and CME (p≤0.05).
Figure 4.5. Mean ± SEM of plasma TAS (mM) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test.

Lines connect significantly different time points within the Co.

* Denotes significant difference between the Co and CME (p≤0.05).
Figure 4.6. Mean ± SEM of plasma PGE$_2$ (pg/ml) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test.

* Denotes significant difference between the Co and CME (p≤0.05).
Figure 4.7. Mean ± SEM of CK (U/l) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test. Lines connect significantly different time points within the Co. * Denotes significant difference between the Co and CME (p≤0.05).
Figure 4.8. Mean ± SEM of LYM (10^9/L) in control (Co; n=8) and supplemented (CME; n=8) horses 24 hr prior to (BL), at 1 and 24 hr following, a standardized high-intensity exercise test. Lines connect significantly different time points within the Co. * Denotes significant difference between the Co and CME (p≤0.05).
CHAPTER 5: GENERAL CONCLUSION

The first objective of this thesis was to determine the influence of high-intensity exercise in horses on variables of oxidative stress and inflammation, both systemically and within the intercarpal joint. The second objective was to quantify the effects of 3 weeks of dietary encapsulated CME supplementation on systemic and articular oxidative stress and inflammation following high-intensity exercise in horses. These research questions were developed based on the relationship between exercise, oxidative stress, inflammation, and joint disease (Lewis et al., 2012; Powers et al., 2011a; Reid, 2008).

The pilot study revealed elevated NO and PGE$_2$ in exercised horses compared to controls between 0.5 and 2 hr following exercise cessation. This was preceded by increased SF PGE$_2$ and GAG 8 hr after exercise. These results provide evidence that horses undergo a transient inflammatory state following high-intensity exercise. Additionally, this study indicated that synovial inflammation and cartilage breakdown occur after intense exercise in the equine intercarpal joint. Thus, the articular release of PGE$_2$ and GAG documented herein provides evidence of the relationship between intense exercise and joint disease in horses. This study provides preliminary evidence for the oxidative and inflammatory responses to intense exercise within a 24-hr period which have not previously been established in the horse.

The second trial, in which horses were provided with encapsulated CME for 23 days prior to an exercise challenge, demonstrated improved SF antioxidant activity and post-exercise recovery. The SF of CME-supplemented horses had augmented SOD activity, as well as reduced SF NO and GAG 1 hr post-exercise. Horses that were supplemented with CME also maintained plasma TAS and blood LYM levels 1 hr post-exercise, compared with control horses which demonstrated a reduction in plasma TAS and an increase in blood LYM. By 24 hr post-exercise, CME-supplemented horses had significantly reduced CK and plasma PGE$_2$. These results indicate supplementation may provide articular protection, improve recovery from strenuous exercise, and that CME supplementation may modulate the immune response to exercise. Additional research is required to provide further support for the use of encapsulated CME as a supplement to support optimal athletic performance.
Limitations

These studies utilized a small sample size of horses, and included a wide variety of ages/breeds, without specific criteria to establish the relative joint health of each subject. Chondrocytes from older horses are more sensitive to LPS stimulation and release significantly more PGE$_2$ and newly synthesized GAGs upon stimulation (Briston et al., 2010). SF concentrations of GAG, hydroxyl proline, and MMP-1 are also related to equine age (van den Boom et al., 2004a; Brama et al., 2004). Furthermore, conformation faults and increased age are risk factors for OA in horses (Schlueter and Orth, 2004). Thus, the wide age range (5-17 yrs) and variety of breeds included in the studies may have affected study outcomes. Furthermore, we did not block treatment groups according to specific criteria characterizing joint health status, which may also have impacted the interpretation of results. Although the horses used in our studies had no history of chronic intercarpal joint lameness, it is possible that a horse with mild joint disease may have been recruited. Future studies could use arthroscopic evidence of cartilage integrity and radiographic evidence of subchondral bone integrity as inclusion criteria to avoid this limitation. Using horses as their own controls, as was initially intended in the CME study, may have helped to correct for potential individuals with compromised cartilage health. However, the high levels of attrition due to lameness and owner discretion prevented a true crossover design. Additional horses were not placed in each group prior to the start of the study to account for the potential loss of subjects as this practice would have been cost prohibitive. For similar reasons, control horse 1 was not replaced in the pilot study when he was found to be intractable with the sampling procedure. The small sample sizes employed in these studies reduced their power, and thus the experiments should be repeated with a larger pool of more specifically characterized subjects.

The study design of the main nutritional trial, which was developed as a 3-way crossover to allow each horse to undergo each of the 3 treatments throughout 3 rounds, extended over a 5-month period from August to December. While this design would have provided the benefit of each horse acting as their own control, it also resulted in the horses being exposed to varying external temperature, humidity, and wind shear during their exercise test days. Additionally, due to the number of horses, the exercise tests were staggered over a period of 3 days. Therefore it
was not possible to exercise all horses on the same day in each round. Environmental temperature plays a role in influencing basal oxidative stress (Hargreaves et al., 2002b) and exercise in the heat induces significantly more oxidative stress than the same exercise protocol performed in moderate or cool temperatures (Quindry et al., 2013). Thus, the fluctuations in temperature between exercise test days (24°C-4°C) may have influenced the degree of oxidative stress experienced by the horses. If all subjects had participated in all rounds, round could have been included within the statistical model to account for the variability associated with it. Nevertheless, it would have been preferable for all horses to be exercised on the same day such that the standardized exercise test represented a consistent and repeatable work load.

As this study was designed as a field trial, there were several variables, such as the aforementioned environmental conditions, which were beyond our control. A standardized exercise test was used such that each horse’s time around the race track acted as a measure of fatigue. However, the horse’s speed and level of exertion likely differed to some degree between exercise tests. Using a treadmill to deliver an exercise test has the advantage of fixing the speed and angle of incline, ensuring each test is identical in these regards. The exercise test utilized in our studies was performed under saddle. Rider experience and skill level can influence the movement pattern of their mount (Schollhorn et al., 2006; Lagarde et al., 2006; Licka et al., 2004; Peham et al., 2001). Although we used a pool of 4 competent riders, differences in style, weight and comfort may have resulted in variable exertion between horses, with certain horses potentially undergoing a more intense and stressful bout of exercise.

Several of the limitations mentioned herein represent limitations which are common to field trials. Although field trials increase certain measures of variability they also represent realistic situations and provide data which are applicable to horses beyond a narrow research setting.

*Future Research*

The results of the pilot study demonstrated elevations in systemic levels of NO and PGE₂ compared to unexercised controls within the 24 hr immediately following intense exercise. Based on these results, the roles of NO and PGE₂ during recovery from intense exercise require
further attention. In horses NO has been found to be correlated with body temperature following exercise (Alberghina et al., 2015) and its vasodilatory actions (Mills and Higgins, 1997) are likely involved with its role in post-exercise thermoregulation. Inhibition of COX-1 and 2 prostanoids via NSAID treatment prior to exercise results in reduced resolution of inflammation during exercise recovery (Markworth et al., 2013). Thus, it appears post-exercise inflammatory and oxidative compounds, such as NO and PGE₂, may be essential during exercise recovery. The use of specific inhibitors may help to explain the functions of NO and PGE₂ post-exercise.

Exercise-induced hormesis to ROS has been proposed as an important adaptive response (Ji et al., 2016). Thus, it is likely that PGE₂ and NO function on a threshold basis, at which they are essential but beyond which they become detrimental. Identifying these threshold values will enable future investigators to determine whether a response is adaptive or harmful.

Both studies used SF GAGs as a measure of cartilage breakdown following high-intensity exercise. However, no markers of cartilage synthesis were used. It is necessary to look at both breakdown and synthesis of cartilage in order to understand the joint response to a particular stimulus. In future research, biomarkers representing cartilage synthesis, such as CS-846 or carboxypropeptide of type II collagen (Henrotin et al., 2016; Lotz et al., 2013; McIlwraith, 2005), should be included. By determining the presence of both anabolic and catabolic markers, a superior and more inclusive picture of the joint environment can be created.

During the CME trial the reduction in post-exercise PGE₂ and CK identified 24 hr following exercise indicates a potential improvement in recovery of supplemented horses. However, the functional benefits of these reductions during subsequent bouts of exercise require quantification using measures such as speed or time to fatigue. By determining improvements in performance during subsequent bouts of exercise following supplementation, the use of CME as an equine supplement to aid in recovery during multiple day events could be established. Furthermore, investigations into the influence of CME on post-exercise muscle membrane integrity would provide information on the mechanism of the post-exercise reduction in CK. It is possible that improved muscle membrane integrity may have contributed to the reduction in plasma CK. Alternatively, improved CK removal from blood might have been responsible for this result.
Measuring post-supplementation muscle cell membrane integrity will assist in determining the site of action of CME with respect to the muscle cell.

Another opportunity for further research is the influence of CME on the post-exercise immune response. Studies of CME supplementation in cats infected with FIV have demonstrated immune enhancement (Webb et al., 2008) and our results indicate a potential mitigation of post-exercise lymphoid cell fluctuations. A next step would be to establish whether CME supplementation could moderate post-exercise lymphopenia. Lymphopenia following intense exercise represents a window of increased vulnerability of athletes to infection. If CME promotes lymphocyte maintenance it could aid in supporting the health and immune system of athletes and based on our results more research is warranted.

Finally, future research regarding the use of CME as a supplement should explore the mechanism of action following ingestion. There are several reports detailing the specific effects of CME with regards to mitigation of oxidative stress in particular situations such as hyperbaric oxygen related cell damage (Muth et al., 2004), ischemia reperfusion injury (Kick et al., 2007), and following intense exercise in horses as discussed herein and by Notin et al. (2010). Results are equivocal across studies (Romao et al., 2015), mainly due to wide variations in study conditions and subjects. Until a detailed description of the cascade of events following encapsulated CME ingestion is provided, one can only speculate as to why certain parameters are influenced in some circumstances and not in others. It is possible that CME interacts at the gut interface with intestinal epithelial cells. It may act on certain transcription factors upregulating the production of endogenous antioxidants. However, this does not explain the discrepancies observed in the literature and more work must be done to determine which genes may be influenced by the presence of CME in the GIT and what thresholds are required for gene stimulation. It is also possible that CME acts on other gastrointestinal cells, such as those involved in GIT immune surveillance, and thus its effects may be carried out by a different series of events. The mechanism of action of CME must be described in order for it to be employed to its full potential and for targeted studies to be developed.
Concluding Remarks

In consideration of strengths and limitations of the studies described herein, the data presented in this thesis provide a foundation for future investigations. They provide evidence for physiological impacts of high-intensity exercise in horses, and the use of CME as a supplement to protect equine athletes against some of the pro-inflammatory and oxidative impacts of high-intensity exercise.
CHAPTER 6: REFERENCES


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van den Boom, R., Brama, P.A.J., Kiers, G.H., DeGroot, J., Barneveld, A. and Weeren, P.R., 2004b. The influence of repeated arthrocentesis and exercise on matrix metalloproteinase and

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