The effect of antioxidant vitamins on muscle damage during a 50-mile endurance ride in horses

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THE EFFECT OF ANTIOXIDANT VITAMINS ON MUSCLE DAMAGE DURING A 50-MILE ENDURANCE RIDE IN HORSES

By

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Bachelor of Science
Virginia Polytechnic Institute and State University
1997

A thesis submitted in partial fulfillment of the requirements for the

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Examination Committee Chair

Dean of the Graduate College

Examination Committee Member

Examination Committee Member

Graduate College Faculty Representative

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ABSTRACT

The Effect of Antioxidant Vitamins on Muscle Damage During a 50-Mile Endurance Ride in Horses

By

Danielle Irene Marrone

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Professor of Kinesiology
University of Nevada, Las Vegas

Plasma markers of muscle cell damage, creatine kinase and aspartate aminotransferase, were measured in the serum of 32 endurance exercised horses during a 50-mile endurance ride to determine if supplemental vitamin E singly or in combination with vitamin C could be effective in preventing exercise-induced muscle damage. Blood was collected by jugular venipuncture prior to and at 0, 23, 35, and 50 miles of a 50-mile endurance ride. Both creatine kinase and aspartate aminotransferase increased significantly pre-exercise to post-exercise in both vitamin E and vitamin E plus C groups. Vitamin E plus C was no more effective in preventing muscle damage than vitamin E alone. Additionally, performance times were not different between groups, indicating that although muscle damage did occur during the 50-mile endurance ride, the damage did not have an adverse affect on performance.
ACKNOWLEDGEMENTS

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Finally, thank you to my mother, Marguerite, for serving as an example (again) as she has done throughout my life. I only hope I impress her as much as she impresses me.

And a final thank you to those people who gave me the much-needed push at the end that I needed. I hope you’re as satisfied as I am with the result.
CHAPTER 1

INTRODUCTION

Muscle injury incurred as a result of excessive or intense exercise originates from chemical reactions at the subcellular level. During strenuous or unaccustomed exercise, oxygen supply to skeletal muscle increases dramatically to cope with the increased metabolic demand placed on the tissue (1). While most oxygen is used in the mitochondria for oxidative phosphorylation, a small amount (approximately 2-5%) is converted into highly reactive chemical intermediates called free radicals (1). Unlike stable chemical substances containing paired electrons with opposite spins in their outermost orbital, an unpaired electron in a free radical will strive to balance itself by combining with an electron having an opposite spin in another substance. Because oxygen flux in individual muscle fibers during exercise can increase 100-200-fold (2), it can be assumed that free radical production increases accordingly. The presence of free radicals found after even one bout of exhausting exercise may lead to lipid peroxidation, characterized by free radicals attacking polyunsaturated fatty acids on the cell membrane. Disruption of the lipid bilayer and the structural organization of the cell membrane occur as a result (1). When a radical reacts with a nonradical, another free radical is produced which initiates a chain reaction.
in the tissue. Disintegration of membrane integrity and loss of intracellular contents occurs as a result, and membrane fluidity and permeability become altered (3). Consequently, normal membrane function is lost.

A potential mechanism underlying the loss of membrane function associated with exercise-induced membrane damage is the mechanical disruption of the sarcolemma (4). Disruption of the sarcolemma results in a loss of intracellular proteins down their concentration gradients and into the extracellular space. The excessive force development of eccentric contractions, where muscles are placed on a stretch while contracting, is usually associated with higher specific tensions than isometric or concentric (shortening) contractions (4). As a result, a greater degree of injury is incurred.

The production of free radicals mentioned previously has been proposed as a potential causative agent in exercise induced muscle damage in horses as well as other species. In horses, a condition existing as a result of exercise induced muscle damage is termed rhabdomyolysis, or "tying-up", syndrome commonly characterized by severe cramping, lameness, and loss of performance (5). In performance horses, this syndrome could prove lethal to a horse's riding career.

Antioxidant vitamin supplementation has been found to have a protective effect at the cellular level (4). Historically, most of the research pertaining to cell membrane damage in skeletal muscle has specifically related to vitamin E more often than any other antioxidant. Vitamin E (tocopherol) acts as an antioxidant by quenching free radicals or making them less reactive by reacting with them itself during the increased oxygen flux associated with exercise. The unique
membrane-bound location of vitamin E makes it advantageous in neutralizing free radicals generated in the mitochondrial inner membrane, thus stabilizing the cell membrane, and decreasing cellular damage (4).

Although ample evidence exists in the literature to suggest a role for vitamin E as an antioxidant in muscle, the combined effect of vitamin C in combination with vitamin E has received less attention. This is surprising considering the interaction of the two vitamins. Upon scavenging a free radical, tocopherol is oxidized, becoming a radical itself. In turn, ascorbic acid (Vitamin C) reduces the tocopherol free radical. The stable ascorbate radical is then regenerated as ascorbic acid by a nicotinamide adenine dinucleotide mediated reaction (6), making it reusable by the cell (Figure 1). The regeneration of alpha-tocopherol by ascorbic acid would therefore enable antioxidant function to continue (7-9).

Figure 1. Antioxidation Reaction of Vitamin C

\[
\begin{align*}
O_2^* + \text{vitamin E} & \rightarrow \text{vitamin E}^* + O_2 \\
vitamin \text{E}^* + \text{vitamin C} & \rightarrow \text{vitamin E} + \text{vitamin C}^* \\
vitamin \text{C}^* + \text{NADPH}^+ + H^+ & \rightarrow \text{vitamin C} + \text{NADP} + H_2O \\
* \text{Ascorbic acids donates 2 hydrogen electrons to deactivate vitamin E}^*
\end{align*}
\]
Research Question

Vitamin C should enhance antioxidant function when combined with vitamin E in preventing muscle damage. Because vitamin C reduces vitamin E, making vitamin E available to continue neutralizing free radicals, less muscle damage should be observed in muscles treated with both vitamins as opposed to vitamin E alone. Therefore, the purpose of this study was to examine the effect of vitamin E and vitamin C on cellular damage induced by a single prolonged exercise bout in an animal model, specifically the endurance exercised horse.
Free radical production increases after even a single bout of exercise, possibly resulting in damage to the muscle. With the increased energy demand occurring during exercise, there is an accumulation of hydrogen ions, as well as an increased electron flow through the electron transport chain in the mitochondria (9). It is thought that with this rapid movement of electrons through the electron transport chain, an "electron leak" occurs, providing the opportunity for free radicals to form (10). With the increased free radical production that ensues, oxidative damage is likely to occur as the oxidative stress of the activity outweighs the capacity of the body’s antioxidant defense system.

A recent study by Best, et al. (11) investigated changes in the rate of free radical production and antioxidant enzyme activity immediately and 24 h after acute muscle injury in rabbits. After an acute injury to the tibialis anterior muscle, muscle reactive oxygen species (ROS), or free radicals, were measured. No change occurred in injured muscles compared with uninjured muscles immediately after injury was induced. At 24 h post-injury, however, the injured leg had 25% more free radical formation than the control leg (p = 0.005). Malondialdehyde (MDA), a by-product of lipid peroxidation, was measured to
estimate the amount of oxidative damage to the cellular membrane in order to confirm that free radical production was in fact responsible for cellular damage. However, no differences were observed between injured and control muscles. Finally, the antioxidant enzymes glutathione peroxidase (GPX), glutathione reductase and superoxide dismutase (SOD) were measured. While SOD levels exhibited no change during the 24-h post-injury period, activities of the remaining two enzymes changed significantly. GPX activity increased, showing a treatment effect (p = 0.015), while the increase in glutathione reductase was evidence of both a treatment (p = 0.041) and an interactive (p = 0.006) effect. These two enzymes may be activated by increased hydrogen and/or lipid peroxide production, as observed in the increased oxygen flux associated with exercise. The increase in GPX and glutathione reductase suggests a possible role of the glutathione system in defending against oxidative damage from muscle injury. Best et al. concluded that muscle stretch injury results in increased free radical production within 24 h post-injury, accompanied by increases in specific antioxidant enzymes.

Bejma and Ji (12) investigated the effect of an acute bout of exercise on free radical generation in rat skeletal muscle. After performing an exhaustive treadmill run at approximately 75% of VO2max, muscle ROS and malondialdehyde (MDA) were evaluated. A significant increase was found in oxidant production (ROS) after acute exercise (p < 0.01) in the exercise compared with the rested group. A significant increase in muscle lipid peroxidation (MDA) was also observed in the exercised group, as evidenced by an 18-26% increase in muscle MDA (p < 0.05).
The authors concluded that an acute bout of exhaustive exercise promoted generation of free radicals accompanied by increased oxidative stress in working skeletal muscle.

Li et al. (1), ran rats to exhaustion on a treadmill to determine the effect of exhaustive exercise on membrane fluidity and lipid peroxidation. Membrane fluidity, as measured by fluorescence polarization, is indicative of the biophysical and biochemical characteristics of the mitochondrial membrane and is a marker of membrane function (1). When membrane fluidity is high, labeled lipid molecules exhibit less fluorescence polarization when exposed to fluorescent light. Therefore, a low fluorescence polarization indicates high membrane fluidity and vice versa. Lipid peroxidation was determined using thiobarbituric acid-reactive substances (TBARS) to mark cellular damage. Increased TBARS levels indicate greater incidence of lipid peroxidation.

A significantly decreased membrane fluidity of the muscle mitochondrial membrane was found in exercised rats at 0, 24, 48 and 72 h post-exercise compared with control rats (p < 0.05). TBARS contents in the mitochondria were significantly elevated at 0 and 24 h post-exercise compared with control rats, but returned to a level similar to the control group by 48 h.

Based on the above results, the authors concluded that a single period of exhausting exercise led to decreased membrane fluidity and increased lipid peroxidation in rat skeletal muscle mitochondria. They suggested that the presence of free radicals associated with exercise might lead to lipid peroxidation in the membrane causing disruption of the lipid bilayer and ultimately the
structural integrity of the biological membrane (1). Previous studies have shown that this disruption alters membrane fluidity and permeability, resulting in compromised membrane function (3).

The following studies performed in horses during the early 1990's looked at Thoroughbred racehorses during peak training or across one full racing season. The relationship between exercise and serum enzyme levels was studied in 2-3 year old fillies and colts in each study.

In the first study, Rej, et al. (13) studied the effect of exercise on serum aspartate aminotransferase (AST) during a four-week period of training in eight well-conditioned Thoroughbred racehorses. Blood samples were obtained prior to and 15-20 minutes after a 0.6-1km run (considered to be a moderate training for flat racing) at an average speed of 58km/h. Control samples were obtained from unexercised, non-Thoroughbred, retired police horses. Mitochondrial AST activity measurements were performed by electrophoresis after immunochemical precipitation using rabbit anti-human s-AspAT antibodies (13) found to cross-react with homologous equine enzyme. Serum AST increased slightly immediately following exercise from 291±108u/l to 317±112u/l (AST). This change in activity did not vary greatly over the four-week period.

The authors in this study concluded that the modest increases in serum AST activities found in their study was not indicative of rhabdomyolysis, or "tying-up" and were much less than that reported in long-distance endurance-type events (considered to be more extensive/exhaustive). They also attributed the high state of fitness usually seen in Thoroughbred horses stabled at flat tracks as the
primary reason for the only slight increase in AST with no clinical signs of "tying-up."

Williamson, et al. (14) examined the effect of exercise during various stages of a 3-day-event on CK and AST in 36 horses aged 8-16 years of various sexes and breeds. This 3-day-event is considered to be a demanding event requiring speed, jumping ability, and a high degree of fitness for a horse to compete successfully. On Day 2 there is a Speed and Endurance test consisting of four phases: an initials roads and tracks (phase A), followed by steeplechase (phase B), a second roads and tracks (phase C) and cross-country (phase D). Phase C was designed as an endurance test and along with phase A was characterized as an aerobic activity with heart rates well below 150 beats per minute.

Blood samples were collected from the jugular vein 12-16 hours before the Speed and Endurance test (pre-exercise), within 30 seconds after phase B, midway through phase C (at the 4km marker), at the end of phase C, and within 30 seconds after phase D. Serum samples were analyzed for CK and AST on an Abbott Spectrum Analyzer (15).

Both CK and AST changed significantly over time (p<0.01). CK increased from 354.7u/l (pre-exercise) to 378.5u/l (after phase B) to 474.5u/l (4km marker of phase C) to 591.4u/l (after phase C) to 797.7u/l (after phase D). AST increased less dramatically from 356.6u/l (pre-exercise) to 430.3u/l (after phase B), then decreased to 409.7u/l (4km marker of phase C), and increased again to 414.9u/l (after phase C) and 445.5u/l (after phase D). While all increases in CK
were considered to be significant (p<0.01), only the last increase in AST was significant.

The authors in this study concluded that several horses in the study probably had subclinical exertional rhabdomyolysis at the time of pre-exercise sample collection (14). The previous study by Rej, et al. (13) considered a level of 300u/l to be the upper limit for CK and in this study the average CK level taken pre-exercise was 354.7u/l. No signs of clinical soreness or lameness were noted, however. Although CK was increased at each phase in comparison to pre-exercise levels, the highest concentration was noted after phase D. This was contributed to the tendency of CK to peak 6 hours after exertion, which would have correlated with the authors' findings in this case. Additionally, since CK and AST peak at different times, AST peaking at 24h after exertion, this could potentially explain why increases in AST levels did not increase significantly until after phase D (14).

Another study conducted around the same time as the Rej study used 66 Thoroughbred horses (fillies and colts) aged 2-3 years during a complete flat racing season (February to October). Harris, et al. (15) investigated the relationship between age, sex, AST & CK activities and exercise. 24h post-exercise blood samples were obtained after what was considered to be a "hard" workout, which was administered as part of their training program twice a week. AST & CK were measured using standard methods adapted for use on a centrifugal analyzer at 37°C (15).
Sex was found to have an effect with respect to CK and AST activities, with fillies more likely to have CK activities greater than 100u/l (p<0.01) and AST activities greater than 300u/l (p<0.005) than colts. Even when the upper limit was raised to 500u/l, AST activities were considered to be significant (p<0.001). Interestingly, fourteen of the horses showed consistently raised AST activities. Eight of these horses raced and seven won at least one race, showing that at least in this study, performance was not affected (15).

In summary, exercise results in oxidative damage to the muscle as shown by increased levels of various indicators of muscle damage, including intracellular enzymes, ROS, and TBARS. Interestingly, the increase in antioxidant enzymes as a result of muscle stretch injury as seen by Best et al. (11) suggests a possible interaction of muscle injury and antioxidant status.

Antioxidant Vitamin Supplementation and Muscle Injury

The body is equipped with its own antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and xanthine oxidase, to defend against free radical production (16). While these enzymes preserve normal cell function at rest and during mild exercise, they may become overwhelmed during heavy or intense exercise, allowing free radicals to induce cell and tissue damage (16). During nutritional deficiency or pathologic conditions, this defense system is especially weakened, leaving the body more susceptible to oxidative stress as a result (16). Previous studies (17-19) have shown that antioxidants help fight free radical production and prevent the cellular damage that follows by quenching the
unstable radical itself. Recent studies (17, 20, 21) have examined the effect of exogenous vitamin supplementation to augment the positive effect of endogenous antioxidant enzymes in prevention of muscle injury through exercise-induced cellular damage.

Examining the effect of vitamin E intake on indicators of muscle membrane integrity, Siciliano et al. (22) observed 19 horses exercised 5d/wk in one of three dietary treatments for a period of 90 days. The three diets were no supplemental vitamin E (BASAL), BASAL plus 80IU of supplemental vitamin E/kg DM, or BASAL plus 300IU of supplemental vitamin E/kg DM. Nineteen Thoroughbred or Quarter horses ranging in age from 4-15 years were placed on their assigned diet 30 days prior to the beginning of the study to allow time for adaptation to occur. Horses were exercised 5d/wk, 20-30 minutes at a time at a speed of 4-8m/s on a treadmill or free lunging in a round pen. As the horses became more physically conditioned, exercise periods lasted longer and speeds were increased. Venous blood from the jugular vein and muscle samples from the gluteus medius (at four sites) were collected at the beginning of the 90-day treatment period, at day 30, and at day 90 for the determination of serum and muscle alpha-tocopherol concentrations. At the end of the 90-day period horses were subjected to a repeated submaximal exercise test consisting of three consecutive 30-minute bouts at submaximal speeds and heart rates with a 10-15 minute rest period between each bout. Exercise bouts consisted of 5 minutes of trotting at 4 m/s, 10 minutes of cantering at 9 m/s, 5 minutes of trotting at 4 m/s, and 10 minutes of cantering at 9 m/s. Blood samples were taken 30 minutes
before exercise (pre-exercise), immediately following the exercise test, and at 1, 3, 6, 24, 48, and 72 hours after for determination of CK and AST. Muscle biopsies were taken from the gluteus medius immediately before and after the exercise test for determination of alpha-tocopherol, thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) by HPLC and spectrophotometry (22).

Only the treatment group supplemented with 300IU of supplemental vitamin E/kg DM had significantly increased serum and tissue levels of alpha tocopherol (p<0.05) at days 30 and 90 (22). Following the 90-day treatment period, 17 horses completed the repeated submaximal exercise test. Mean serum AST and CK activities were markedly increased in all treatments following the test (p<0.01) (22). No effect of vitamin E supplementation was found on the magnitude of post-exercise increases in serum AST and CK activities. TBARS and CD levels did not change significantly in any of the treatment groups, nor did levels differ between treatments, indicating no effect of vitamin E supplementation. The authors concluded that supplementation of vitamin E had no effect on the integrity of skeletal muscle following repeated submaximal exercise, as measured by changes in serum CK and AST activities (22).

Van Der Meulen, et al. (20) explored the effect of vitamin E on force deficit (a performance measure), muscle fiber damage and serum activity of creatine kinase (CK). The presence of this intracellular enzyme in the serum indicates an increased membrane permeability and cellular damage. After an in-situ pliometric contraction protocol, force deficit did not differ between untreated, vehicle-treated
and vitamin E-treated groups at 0, 30 min, 3 h and 3 days (p<0.05). The percentage of damaged muscle fibers, as observed by light microscopy, were 38 +/- 5, 32 +/- 5 and 29 +/- 5% three days after the injury in the untreated, vehicle-treated and vitamin E-treated groups, respectively. Serum CK activity in control rats exhibited a fourfold increase at 3 h and a twofold increase at 3 days after the injury protocol while the serum CK of vitamin E-treated rats at 3 h and 3 days did not differ from nonexercised rats (p<0.05). Therefore, the authors concluded that an elevation in muscle vitamin E did not decrease force deficit or percentage of damaged muscle fibers. However, they attributed the absence of an increase in serum enzymes for vitamin E-treated rats at 3 h and 3 days to a preventive role of vitamin E in enzyme loss after muscle damage.

Warren et al. (21) found similar results using another eccentric contraction method, downhill walking, to examine the effect of elevated muscle vitamin E on skeletal muscle damage. This study defined damage using force deficit, number of damaged fibers per square millimeter and plasma enzyme levels at 0 and 48 h post-injury. Reductions in force deficit were similar in control and supplemented rats at 0 and 48 hours post-injury (p<0.05). The number of intact muscle fibers per square millimeter decreased in control animals postexercise (68 +/- 8 fibers/mm²) compared to the sedentary animals (101 +/- 10 fibers/mm²) and remained lower at 48 h (70 +/- 5 fibers/mm²). No differences were found between the vitamin E and control dietary conditions (p<0.05). A twofold elevation in CK occurred immediately after exercise in control and vitamin E supplemented animals compared with sedentary animals, but returned to normal levels by 48 h.
It was concluded that vitamin E supplementation in rats did not attenuate muscle injury caused by prolonged downhill walking.

In interpreting the results of the Warren study it is important to take into consideration the exclusive use of female rats. Amelink et al. (23) reported previously that a more pronounced injury was found in male than female rats fed vitamin E-deficient diets before a prolonged bout of treadmill exercise. The authors attributed the difference in degree of injury to the protective effect of estradiol in female rats. Studies by Tiidus et al. (24,25) found similar effects in female rats.

A 1993 study conducted by Kanter et al. (26) considered the effect of vitamin E in combination with vitamin C and beta-carotene on exercise-induced lipid peroxidation after treadmill running in humans. Lipid peroxidation as a result of the rise in O$_2$ consumption associated with exercise is positively correlated ($r=0.85$) with increases in skeletal muscle damage (26). Subjects ingested a daily vitamin mixture (592 mg alpha-tocopherol equivalents, 1,000 mg ascorbic acid and 30 mg beta-carotene) or placebo for 6 weeks. After the dietary intervention they were subjected to 30 min of treadmill running at 60% of VO$_2$ max followed by an additional 5 min of running during which the speed and elevation were increased to elicit 90% of their VO$_2$ max. Evidence of lipid peroxidation was determined by expired pentane production and serum malondialdehyde (MDA) concentration. Exercise resulted in significant increases in expired pentane and serum MDA levels ($p<0.05$). Expired pentane rose from 12.4 pmol/kg$^{-1}$/min$^{-1}$ to 24.6 pmol/kg$^{-1}$/min$^{-1}$ after 60% VO$_2$ max and to 33.4 pmol/kg$^{-1}$/min$^{-1}$ after 90%
VO₂ max in the supplemented group. Subjects exhibited a significantly (p<0.05) lower rate of pentane production (-36%) at rest after the vitamin treatment. Although the increase in pentane production caused by exercise was not prevented by the antioxidant vitamin treatment, absolute pentane production after exercise at 60 and 90% of VO₂ max was significantly lower than that prior to the 6 wk supplementation (p<0.05). Serum MDA concentrations at rest and after exercise at both 60 and 90% VO₂ max were also significantly lower (-17%) than before vitamin supplementation (p<0.05). Pentane production and serum MDA concentrations in the placebo group did not change at rest or after exercise at either intensity following the 6 wk treatment period (p<0.05). The authors concluded that daily ingestion of 592 mg alpha-tocopherol equivalents, 1,000 mg ascorbate and 30 mg beta-carotene for 6 weeks resulted in significantly less expired pentane and serum MDA, at rest and after moderate or heavy exercise. They could not conclude, however, that antioxidant supplementation prevented exercise-induced lipid peroxidation since pentane and serum MDA increased after exercise in both groups.

Two important considerations in the above study are training status of the subjects and the difficulty of compliance when using human subjects in dietary studies. With respect to training status, five of the twenty subjects were considered well trained (VO₂ max > 60 ml/kg⁻¹/min⁻¹), five moderately trained (VO₂ max = 50-60 ml/kg⁻¹/min⁻¹) and 10 untrained (VO₂ max < 50 ml/kg⁻¹/min⁻¹). It has been shown previously that training results in increased activity of several major antioxidant enzymes and overall antioxidant status (27). Also, in the above
study, the use of humans makes it difficult to accurately assess the amount of antioxidant vitamins ingested without sequestering the subjects in a metabolic ward. It is unlikely that all subjects used in the study were fully compliant with the dietary instructions, or that they did not have different antioxidant levels in their diets to begin with. The latter would make it difficult to attribute any effect solely to the vitamin supplementation, as the subjects' total antioxidant levels would differ.

Overall, antioxidant vitamin supplementation has been found to prevent increases in exercise-induced lipid peroxidation. However, it is still unclear whether muscle damage is actually prevented. For example, while serum enzyme activity was affected in the studies by Van Der Meulen and Warren, force deficit and number of damaged fibers did not differ between control and treatment groups in either study. Warren et al. found an increase in serum CK in both control and treatment groups in this study, while the Van Der Meulen et al. study failed to find an difference in serum CK activity between vitamin E supplemented/exercised and non-exercised control rats. They attributed the similar levels of intracellular enzymes in the supplemented/exercised and non-exercised control group to the protective effect of the vitamin supplementation. It appears that antioxidant supplementation may prevent lipid peroxidation and loss of cellular contents, but there is still a question as to whether antioxidants actually prevent muscle injury or not. Therefore, further research is needed to determine the specific mechanism by which vitamin supplementation may prevent muscle injury.
Interaction of Vitamin E and Vitamin C

Vitamin C acts as an antioxidant alone or in combination with other vitamins. Vitamin C is thought to reduce the vitamin E radical formed when vitamin E reacts with a peroxyl radical therefore recycling it back to the intact vitamin E and making it available to be reusable by the cell. In turn, the vitamin C radical, which is stable and almost unreactive, is enzymatically reduced to ascorbic acid (6, 9). Vitamin E, a lipid-soluble membrane bound antioxidant, is oxidized first to prevent a chain reaction or limit extensive damage by free radicals. Clearly if vitamin E is regenerated by vitamin C, vitamin E's availability in the membrane should protect the membrane from lipid peroxidation to a greater extent than when administered alone.

Tanaka et al. (28) investigated the interaction of vitamins C and E to determine the possibility of a sparing effect of vitamin C on vitamin E. Interaction between the two vitamins was evaluated by studying changes in tissue levels of these vitamins after depletion of either vitamin C, vitamin E, or both. This study differs from previous studies reviewed in that rather than providing a supplement, a deficiency state was implemented. After a 1-week acclimation diet, four groups of rats were given a treatment of a control, a vitamin E deficient, a vitamin C deficient or both vitamin E and C deficient diet for 21 days. Tissue levels of the vitamins were measured at 0, 14 and 21 d. Evidence of oxidative stress was determined through measurement of thiobarbituric acid reactive substances (TBARS) at 0, 14 and 21 d.
Plasma vitamin E concentrations in tissues of the E deficient and E/C deficient groups were significantly lower than the control and C deficient rats at both days 14 and 21 (p < 0.05). No significant differences were found between the E deficient and E/C deficient groups, however. At day 21, the C deficient group had significantly lower plasma vitamin E concentrations than the control group. The authors concluded that the results demonstrated that vitamin C spared vitamin E, consistent with previous reports (29, 30). Plasma TBARS concentrations of the C deficient and C/E deficient groups were significantly higher than the control and E deficient group at day 21 (p < 0.05), indicating that oxidative stress was higher in the C deficient condition than in the E deficient condition.

The authors observed an interaction of the two vitamins and a sparing effect of vitamin C on vitamin E, attributing it to the in vivo regeneration of vitamin E by ascorbate as previously suggested. The ability of vitamin C to regenerate vitamin E has practical significance. Not only does it suggest that vitamin C may make vitamin E more available, but it also suggests the role of vitamin C as an effective supplement based on its interaction with vitamin E.
CHAPTER 3

METHODS

Subjects
Thirty-two endurance trained horses arrived at Virginia Tech's M.A.R.E. (Middleburg Agricultural Research and Extension) Center and were allowed one day to become acclimated to their new environment so as to minimize any differences associated with the stress of transportation on the animal. Animal-use protocol was approved by Virginia Tech's Institutional Animal Care and Use Committee (IACUC) prior to the beginning of the study.

Dietary Manipulations
Animals were randomly assigned to one of two dietary manipulations receiving either an oral supplement of vitamin E (n=16) or vitamin E + C together (n=16), in addition to their normal diet. Both treatment groups received their respective supplements for 5 days before the endurance ride and were tested on the day of the ride at 0, 23, 35, and 50 miles, and at 20 minutes of recovery.
Exercise Protocol

On the morning of the ride all horses arrived at the starting line at 6am for pre-ride screening by a veterinarian to ensure they were of good health to participate in the event. The ride consisted of 50 miles of rolling hills with no jumps.

Plasma CK and AST Activity

Muscle injury from eccentric exercise is associated with the appearance of intracellular enzymes in the plasma (21). Measurement of the intracellular enzymes aspartate aminotransferase (AST) and creatine kinase (CK) in equine serum is an important diagnostic tool utilized routinely in horses as an indicator of exertion myopathy (rhabdomyolysis) (13). Additionally, CK is relatively muscle specific, reaches peak concentrations within a few hours of insult, and is rapidly removed from the blood, while AST is found in most tissues, reaches peak concentrations after approximately 24 hours and has a much slower half-life (approximately 7-8 days)(15). Therefore, AST and CK were measured (within subject) in the present study as indices of muscle damage. Seven milliliters of blood were collected by jugular venipuncture in heparinised vacutainer tubes at 0, 23, 35, and 50 miles, and at 20 minutes of recovery. Samples were not collected from horses under extreme duress, as they would not have been appropriate for the purpose of analysis. Plasma CK and AST levels were determined as in previous studies by enzymatic analysis and assays were run in duplicate (31).
Statistical Analysis

The data for dependent variables of creatine kinase and aspartate aminotransferase were analyzed by 2 (treatment) by 5 (distance) mixed model ANOVA with repeated measures on the last factor. The data for dependent variable of performance time was analyzed by independent t-test. The accepted level of significance for all tests was set at $P = 0.05$. All values are expressed as means +/- SE.
CHAPTER 4

RESULTS

Creatine Kinase

Interaction was not significant. Muscle damage, as evidenced by presence of CK did not differ between Vitamin E and Vitamin EC treated horses. However, CK did increase significantly over time (F=33.768, p<0.0001)(Figure 2). CK increased significantly at each point from start to mile 50 (Table 1).

Table 1. Creatine kinase activity (units/l)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>23</th>
<th>35</th>
<th>50</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E</td>
<td>234*</td>
<td>376*</td>
<td>719*</td>
<td>1069</td>
<td>984</td>
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* Significantly different from successive values, p<0.05.

Aspartate Aminotransferase

Interaction was not significant. Muscle damage, as evidenced by presence of AST, did not differ between Vitamin E and Vitamin EC treated horses (F=1.676, p=0.205), but did change significantly over time (F=41.582, p<0.0001)(Figure 3).
AST increased significantly at each point from start to mile 50 and decreased at 20 minutes of recovery (Table 2).

Table 2. Aspartate Aminotransferase activity (units/l)

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* Significantly different from successive values, p<0.05.

Performance

To determine a mean difference in performance between the Vitamin E and Vitamin EC treated groups, an independent t-test was performed. No significant difference was found, however, between performance times of the Vitamin E and Vitamin EC treated horses. The mean performance time for the Vitamin E group was 9:18:43.2 (±21.7) (558.73 min) while the Vitamin EC group finished in 9:09:10.8 (±16.3) (549.20min) (Figure 4). Interestingly, some horses showed extremely high levels of CK (i.e. 2008.13u/l) and AST (i.e. 451.5u/l) at the finish of the race, but never exhibited signs of “tying-up,” suggesting that it may be possible to have increased enzyme levels in the blood without clinical signs of damage.
Figure 2. Mean creatine kinase activity at each checkpoint for vitamin E+C and vitamin E groups.
Figure 3. Mean aspartate aminotransferase activity at each checkpoint for vitamin E+C and vitamin E groups.
Figure 4. Mean performance times for vitamin E+C and vitamin E groups.
CHAPTER 6

DISCUSSION

Ultimately it was found that supplemental vitamin C in addition to vitamin E had no beneficial effect on muscle damage or performance in the endurance exercised horse. Both the CK and AST results confirm the idea that a 50-mile endurance ride does, in fact, result in damage to skeletal muscle, however. Although free radical production was not directly measured in this study, by-products of lipid peroxidation at the membrane level (intracellular enzymes CK & AST) were found most likely as a result of free radical attack of polyunsaturated fatty acids in the cell membrane. Therefore, the appearance of increased levels of CK and AST in the blood indicates muscle damage has occurred in this study.

Mechanical disruption of the sarcolemma is another potential mechanism responsible for loss of membrane function associated with exercise-induced membrane damage, and was not directly measured in this study (4). However, since sarcolemmal disruption results in the appearance of intracellular proteins in the extracellular space, the increased presence of CK and AST found post-exercise in this study could also support the notion that disruption of the sarcolemma did, in fact, occur. To determine whether sarcolemma disruption or free radical attack occurred in this case would require further study.
Due to the paucity of literature on vitamin supplementation and exercise in horses, it was necessary to include studies in Thoroughbred, Hunter and Quarter Horses. Because of the differences in training of these breeds, the differences in results from these studies must be considered. For example, Quarter Horses historically were known for their ability to perform short distance races that were a quarter of a mile in length, while the Thoroughbred was developed for longer distances and are noted for their speed and stamina. Because of the differences in training and genetics, differences in muscle fiber type must therefore be considered when interpreting results of studies using various breeds. The present study included horses of various breeds and must be remembered when interpreting the results.

In addition to the difficulty of comparing different breeds, the present study presented challenges in accounting for differences in diet prior to the collection period as each owner/trainer determined the makeup of each diet for each individual horse. Owners/trainers are reluctant to trust a change in diet, fearing that it may affect performance. Therefore, a washout period was not an option and it could not be insured that all horses began the study at the same level of nutrition.

More research is needed to determine whether or not vitamin E in combination with vitamin C has a beneficial effect on preventing skeletal muscle damage in the endurance exercised horse. It would be helpful to have a more controlled environment, if possible, where training and dietary regimens could be more strictly controlled.
### Creatine Kinase Results (u/l)

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Performance Times

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APPENDIX II

RESEARCH RIDE INSTRUCTIONS
WELCOME TO THE MIDDLEBURG RESEARCH RIDE

This Ride is the first dedicated primarily to research. The idea originated in Dr Jeannie Waldron’s interest in the endurance horse. She sought support from the Middleburg Agricultural Research and Extension Center. The first help was provided by a graduate student, Ms Belinda Hargreaves. The results were significant for every measure of oxidative stress. They encouraged the testing of anti-oxidant supplements. Because these supplements might confer a competitive advantage, it was necessary to create a dedicated, experimental race—The Ride!

Animal welfare is the good fit of an animal to its environment. We strive to improve both sides of this interaction by breeding and training the animals, and by improving its environment, including its diet and feeding management. Welfare is assessed in terms of performance and productivity, the absence of disease, and the degree of stress. We evaluate stress in several ways—behavioral, hormonal, and oxidative. In the endurance horse, we also evaluate hydration, electrolytes and acid-base status.

The Middleburg Agricultural Research and Extension Center was the gift of a singular benefactor who was one of the first and most ardent endurance riders, the late Paul Mellon. He loved the land as well as the horse, and he challenged us to improve both at the same time through the development of superior pasture systems.

The endurance horse usually lives on pasture, and we believe that the novel pasture supplement that we have developed for brood mares and foals will serve the pastured performance horse equally well. We expect to test our fiber-and-fat supplement in 2002. For today, April 1, 2001, we are comparing supplementation of vitamin E with or without vitamin C, and electrolyte mixtures with or without potassium. The experimental design uses positive controls, no placebos. Competitors will have either today’s common “best” or something new that we expect might be even better.

These studies should enable the performance horse to perform better with less stress. Our sponsors share our interest in equine welfare: the Virginia Horse Industry Board, the WALTHAM Centre for Pet Nutrition, and the Bernice Barbour Foundation. Their support has enabled this dream come true—this dedicated research ride.

To all riders and volunteers we offer thanks for your help and participation.

Rhonda Hoffman, PhD
The Principal Investigator

Jeannie Waldron, DVM
The Ride Manager
The Middleburg Research Ride
April 1, 2001

Schedule of Events

Friday, March 30

Noon

Base Camp opens.

Saturday, March 31

10:00 AM to 4:30 PM
Check in with Ride Secretary in the HQ Building (Sales Barn)

10:00 AM to 11:00 AM
Veterinary Staff Meeting in the Conference Room of the MARE Center Administration Building (Includes Vet Recorders)

10:00 AM to 11:00 AM
Research Staff Meeting in the Clinic Hall of the MARE Center Administration Building

10:00 AM to 11:00 AM
Meeting of P&R Staff in the HQ Building (Sales Barn)

11:00 AM to Noon
Luncheon/Briefing for Veterinarians, Research Staff and Vet Support Staff including Vet Recorders and P&R Staff in the HQ Building (Sales Barn)

Noon to 1:00 PM
Demonstration/Review Vet Check procedures for Veterinarians, Research Staff and Vet Support Staff including Vet Recorders and P&R Staff in the field just west of the HQ Building (Sales Barn). Riders are welcome but not required to attend.

1:00 PM to 5:00 PM
Pre-ride Veterinary Inspection in the field just to the west of the HQ Building (Sales Barn)

5:30 PM to 6:30 PM
Dinner for Staff and Participants in the HQ Building (Sales Barn)

6:30 PM to 7:30 PM
Ride Briefing in the HQ Building (Sales Barn)

Sunday, April 1 - RIDE DAY!

5:00 AM
Wake up call!

5:15 AM to 7:15 AM
Coffee and donuts served in the HQ Building (Sales Barn)

6:15 AM
Present mounts with tack for final pre-ride veterinary inspection — same location as vet in. Temperature, pulse and respiration will be checked and a blood sample taken. Then Proceed to the starting area and check in with the Timer.

7:00 AM
Ride starts.

5:00 PM to 6:00 PM
Reception for Staff and Participants hosted by Friends of Bull Run Mountain in the HQ Building (Sales Barn)

6:00 PM to 7:00 PM
Awards Dinner in HQ Building (Sales Barn)

Monday, April 2

Noon
Base Camp closes
Protocol for Research Samples at the Middleburg Research Ride, April 1, 2001

Rhonda M. Hoffman, Principal Investigator

Blood samples will be taken and other data collected 8 times as follows:

- Pre-ride (day before the ride, during the Vet-In, March 31);
- 0 miles (morning of the ride, April 1);
- 13, 23, 35 and 45 miles during the ride;
- 50 miles at finish, immediately upon arrival;
- At 20 minutes of recovery.

Selected horses may also be sampled at 24 hours on April 2.

Blood sampling procedures:

1. Immediately ASAP on arrival at stop, record the pulse of the horse. This may be done using a stethoscope, or use the rider’s heart rate monitor.
2. As soon as the HR is recorded, collect a blood sample from a jugular vein. Insist that the rider dismounts for their and your safety. Please alternate between left and right sides of the horse with alternate stops. Collect 4 Vacutainer tubes full (heparinized, green tops).
3. Swipe the area on the neck with an alcohol swab before and after blood collection. Clean off any blood. (This is more for the rider than for the horse.)
4. Do not forget to label the tubes with the horse number. After the sample is collected, invert the tubes to mix the anticoagulant.
5. Puncture through the rubber top of one tube and collect a sample into the blood gas syringe. "Burp" the syringe, expire any air, and place the rubber stopper over the needle. Label the blood gas syringe.
6. Record the rectal temperature of the horse.
7. Place all samples and the blood gas syringe on ice.

NOTE: If the horse is not cooperative, use your best attempt at collecting the sample, but do not insist on getting the sample at any cost. It will do no good for the horse, the rider, or us, and a sample collected under extreme duress will not meet our purposes for analysis.
Protocol for Riders at the Middleburg Research Ride, April 1, 2001
Rhonda M. Hoffman, Principal Investigator

At the VET IN on March 31, the day before the race, record:
1. Pulse, respiration and rectal temperature.
2. Blood sample collected.
4. Body weight of the horse without tack (except for bridle/headstall worn during the race); body weight of the rider and tack; body condition score of the horse recorded.

At intermediate stops (13, 23, 35 and 45 miles) and finish (50 miles) of the race:
1. Immediately ASAP on arrival at stop, a blood sample will be taken and heart rate and rectal temperature recorded.
2. The rider gets the horse ready for entry to P & R and follows usual P & R procedures.
3. Vet check procedures—the usual.
4. Only at 23 miles and 50 miles: Record body weight of the horse without tack (except for bridle/headstall); body weight of the rider and tack; and body condition score of the horse.
5. Before leaving each vet check, the rider or other person presenting the horse should inform the Research Recorder about urination, defecation, and consumption of food, water and electrolytes. Information is needed about such activities between stops as well as during the stop.
6. Complete hold time and (if not at finish) leave.

Blood samples will be taken and other data collected 8 times as follows:
Pre-ride (day before the ride, March 31);
0 miles (morning of the ride, April 1);
13, 23, 35 and 45 miles during the ride;
50 miles at finish, at 0 and 20 minutes of recovery.

Selected horses may also be sampled at 24 hours on April 2.
BIBLIOGRAPHY


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