

Oxidative Stress and Antioxidant Supplementation of Horses During Endurance Exercise

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ABSTRACT: To compare effects of a dietary supplement containing vitamin E alone or vitamin E plus vitamin C on antioxidant status, oxidative stress and membrane leakage during endurance exercise. A nutritional pre-competition survey allowed horses to be paired and randomly assigned to two groups. Three weeks prior to the competition, one group (E) was orally supplemented with 5,000 IU vitamin E/d, the other group (EC) with 5,000 IU vitamin E plus 7 g vitamin C/d. The ride covered 80 km of varying terrain. Blood samples, temperature and heart rate were taken the day before the race, 21 and 56 km during the ride, at completion, and after 20 min of recovery. Plasma lipid hydroperoxides (LPO), α -tocopherol (α -TOC), total ascorbate (ASC), albumin (ALB), creatine kinase (CK), and aspartate aminotransferase (AST), RBC and WBC total glutathione (GSH-T) and glutathione peroxidase (GPx) were analyzed. Thirty-four horses completed the race, 12 horses did not finish for reasons including lameness, metabolic problems, and rider option. Treatment had no significant effect on any of the variables measured, except for ASC, which had a treatment effect ($P = 0.045$). Creatine kinase, AST, RBC GPx, WBC GSH-T, and LPO increased, but RBC GSH-T and WBC GPx decreased with distance ($P < 0.0001$). Positive correlations were found for plasma LPO on CK ($r = 0.25$; $P = 0.001$) and AST ($r = 0.33$; $P < 0.001$). Positive correlations establish an association between muscle leakage and a cumulative index of oxidative stress. No advantage was found for EC over E, but comparison with previous studies suggest that the combination may be beneficial during more strenuous races.

Key Words: Alpha-tocopherol, Ascorbate, Endurance exercise, Equine, Oxidative stress

Introduction

Due to the recent losses of two top endurance horses at the World Equestrian Games 2002 in Jerez, Spain, public interest in the performance and welfare of competitive horses has been provoked. One of these topics of interest is oxidative stress. Oxidative stress occurs when the antioxidant defense system in the body is overwhelmed with reactive oxygen species (ROS). An increase in ROS may occur due to increased exposure to oxidants from the environment, increased production within the body from an increase in oxygen metabolism during exercise, or an imbalance in antioxidants (McBride and Kraemer, 1999). Useful properties of ROS include targeting of bacteria and viruses during respiratory bursts in phagocytes, and serving as special messengers within neurons (McBride and Kraemer, 1999). However, if ROS accumulation becomes too great it can be damaging to the DNA, protein and lipids in cells. Oxidative stress has been implicated in the pathogenesis of certain diseases (e.g. cancer, AIDS, and Alzheimer's disease) and has been linked with the aging process and exercise.

Oxygen consumption in horses increases up to 30 times during exercise, compared to about 20 times in human athletes, and during maximal exercise oxygen flux through exercising muscle is approaching 100-fold (Butler et al., 1993; McBride and Kraemer, 1999). Most of the consumed oxygen forms carbon dioxide and water, however, 4 to 5 % of the oxygen is not completely reduced and instead forms free radicals (chemicals with an odd number of electrons, e.g. HO^\bullet) and reactive oxygen species (e.g. H_2O_2 ; Butler et al., 1993; Clarkson and Thompson, 2000). As exercise increases the oxygen intake, free radical production and lipid peroxidation also increase. Free radicals and ROS may increase enough to overwhelm the antioxidant system and interact with lipids, compromising the integrity of polyunsaturated fatty acids, degrade proteins and promote DNA breakdown, affecting the homeostatic environment of the cell (Clarkson and Thompson, 2000).

Vitamin E is the most commonly supplemented antioxidant in horses. In one study, vitamin E was supplemented above and below current recommendations and plasma thiobarbituric acid reactive substances (TBARS; an indicator of oxidative stress) increased with exercise, especially in horses with low plasma α -tocopherol (McMeniman and Hintz, 1992). Another study found that a single bout of submaximal exercise does not affect plasma α -tocopherol concentration, but horses conditioned for several weeks, may require higher levels of vitamin E supplementation than recommended (Siciliano et al., 1996). It has been found in various species that vitamin C potentiates the effects of vitamin E by reducing the tocopheroxyl radicals and restoring its activity (Chan, 1993). Under maintenance conditions horses have the ability to synthesize sufficient ascorbate, but the demand increases as stress on the body is increased.

Enzyme activity in plasma is used as an indicator of muscle leakage during exercise. Enzymes most useful in evaluating muscular leakage include creatine kinase (CK) and aspartate aminotransferase (AST). They can fluctuate for a number of reasons, including alteration of the membrane permeability, cell necrosis, impaired enzyme clearance, and increased enzyme synthesis (Harris, 1998).

Evidence of possible oxidative stress in horses has been described in reports dealing with intense exercise (Chiaradia et al., 1998; White et al., 2001) and endurance exercise (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002; Marlin et al., 2002). This study tested two hypotheses: that muscle leakage may be related to oxidative stress during endurance exercise; and that these changes may be decreased by a supplement combining vitamin E and vitamin C versus vitamin E alone.

Materials and Methods

Forty-six trained endurance horses (35 Arabians, 9 part Arabians, 1 Thoroughbred, and 1 grade type), 10.8 ± 0.6 years of age, participated in the Middleburg Research Ride, on April 1st, 2001. The protocol was approved by the Institutional Animal Care and Use

Committee and performed at the Virginia Tech Middleburg Agricultural Research and Extension Center.

Riders responded to a pre-competition survey that detailed nutritional management, training regime, performance and medical history. This survey allowed horses to be paired by grain intake and experience. Participants were then randomly assigned to two groups of electrolyte supplementation (Hess et al., 2002) and 2 groups of vitamin supplementation in a 2x2 factorial design. Three weeks prior to the competition, one group (E; n = 23) was orally supplemented with 5,000 IU vitamin E/d (dl-alpha tocopheryl acetate), the other group (EC; n = 23) with 5,000 IU vitamin E plus 7 g vitamin C/d (98 % ascorbic acid). The ride covered 80 km in northern Virginia with terrain ranging from 121 to 442 m elevation. Ambient temperature ranged from 4.7° C in the morning to 10.7° C in the afternoon and evening, with 100 % humidity. Horses were weighed without tack the day before the race, at the 56-km veterinary check, and after completion of the race on an electronic scale (Tyrel platform, TC-105, Alweights Hamilton Scale Corp, Richmond, VA). *Veterinary checks* were performed according to the American Endurance Ride Conference (AERC) rules (Mackay-Smith et al., 1999) the day before the race (1300 to 1800 hour), at 21, 37, 56, 72 and 80 km during the race (recorded individually on veterinary charts). Blood samples collected in sodium heparin vacuum tubes (Becton Dickinson and Company, Franklin Lakes, NJ) via jugular venous puncture, and were taken the day before the race (PRE), before the veterinary examination at veterinary check 21 and 56 km during the ride, at completion (80 km), and after 20 to 30 min of recovery (REC). Rectal temperature and heart rate were also recorded at these times. Blood samples were collected within 2 minutes after the horse entered a veterinary check and were placed immediately on ice and transported to the laboratory within 15 to 30 minutes to be processed into red blood cell (RBC), white blood cell (WBC), and plasma aliquots.

For assays using RBC lysate, 500 µL of whole blood was transferred to a microcentrifuge tube and centrifuged at 2500 x g for 5 min at 4° C. The plasma was removed and discarded from the sample. The pellet was then frozen at -80°C until analysis, when it was thawed and lysed by 1 mL of ice-cold deionized water. For the determinations using WBC, the buffy coat, was removed after centrifugation of whole

blood at 2500 X g for 5 min at 4° C, and transferred to a tube containing 10 mL of lysis buffer (0.15 M NH₄Cl, 0.01 M NaHCO₃, 0.03 M EDTA free acid.). White blood cells were washed once in the lysis buffer to lyse any residual RBCs, then washed twice in Hank's Balanced Salt Solution (HBSS; Life Technologies, Carlsbad, CA). The pellet was then reconstituted in 1 mL HBSS and mixed thoroughly then 0.5 mL was transferred into micro tubes and frozen at -80° C until sample analysis. Plasma aliquots were prepared by centrifuging the vacutainer tubes at 2500 x g for 5 minutes at 4° C, then transferring the plasma supernatant to micro-tubes, which were frozen at -80° C until sample analysis.

Red blood cell lysate and WBCs were analyzed for total glutathione (GSH-T; Biotech GSH-420, kit #51023; Oxis Health Products, Inc., Portland, OR; inter-assay CV 7.0 %, intra-assay CV 5.6%) and glutathione peroxidase (GPx; Biotech GPx-340, kit #51017; Oxis Health Products, Inc., Portland, OR; inter-assay CV 4.2 %, intra-assay CV 5.0 %) using an OxyScan™ Automated Oxidative Stress Analyzer. Total plasma lipid hydroperoxides (LPO; Biotech LPO-560, kit #21025; Oxis Health Products, Inc., Portland, OR) were analyzed using a spectrophotometer (inter-assay CV 3.0 %, intra-assay CV 4.6 %). Creatine kinase, AST, and ALB were analyzed using spectrophotometric assays (Beckman Instruments Inc., Brea, California, USA). Total ascorbate (ASC; Schiiep et al., 1987) and α -tocopherol (α -TOC; Hargreaves et al., 2002b) were analyzed by high-pressure liquid chromatography methods detailed before. Ascorbate and α -TOC (ASC_{adj} and TOC_{adj}, respectively) were adjusted for changes in fluid redistribution during exercise using albumin (ALB). The equation was as follows:

$$\text{ASC}_{\text{adj}} = \text{ASC} * (\text{PRE ALB} / \text{sample ALB})$$

Data were summarized as means \pm SE. The effects of treatment, distance, and their interaction were evaluated by ANOVA (SAS Institute Inc., Cary, NC) in a mixed model with repeated measures. No interactions were found between the electrolyte and vitamin treatments, so the electrolyte treatment was removed from the model. Outliers were determined as being > 2 SD's from the mean and then dropped from the analysis using Fisher's normal deviant (z). Data were tested for normality by the Shapiro-Wilk statistic. Significance was inferred when $P < 0.05$. Pearson's product-moment and Spearman's rank order correlations were used to test for an association between measures of muscle

membrane leakage, oxidative stress, and antioxidant status. Horse was included in the model to test for significance, if insignificant then it was removed from the model.

Results

The horses weighed on average 421 ± 5 kg before the race, 400 ± 5 kg at the 56-km veterinary checkpoint, and 406 ± 5 kg at the 80-km completion of the race. Thirty-four horses completed the race (E = 17, EC = 17); reasons for not finishing include lameness (3 horses), metabolic problems (2 had exertional rhabdomyolysis, 1 had insufficient heart rate recovery index, and 1 lacked gut sounds), and rider option (5). Average time of completion for the 80 km was 9 h 15 min, with the first place horse finishing in 7 h 22 min and the last in 11 h 22 min (times include veterinary checks).

For horses completing the race, plasma ASC concentration was higher ($P = 0.045$) in the EC group (4.5 ± 0.1 $\mu\text{g/mL}$) than in the E group (3.8 ± 0.1 $\mu\text{g/mL}$; **Figure 1**). Antioxidant treatment and the interaction with distance had effects ($P > 0.65$) on other variables tested. The α -TOC concentrations (5.4 ± 0.16 $\mu\text{g/mL}$) were similar between groups and did not change over the course of the race; however, 2 horses had extreme high values and 2 had low values (1 high and 1 low from each E and EC group; 11.4 ± 0.05 and 1.9 ± 0.40 $\mu\text{g/mL}$, respectively), which contributed to the large variation. Heart rate, temperature, CK, AST, RBC GPx, WBC GSH-T and LPO increased ($P < 0.0001$), whereas RBC GSH-T and WBC GPx decreased ($P < 0.0001$) with distance, however, WBC GPx increased during recovery (**Table 1**). The pattern throughout exercise of RBC GSH-T and WBC GPx were similar, and those of RBC GPx and WBC GSH-T were also similar.

Plasma CK and AST activity increased during the overall ride and remained high at REC (**Figure 2**). Three horses completing the race had high CK values and one had high AST values (higher than reference range) at 56 km, 80 km, and REC, without showing clinical signs of exertional rhabdomyolysis.

At 21 and 80 km LPO was lower ($P < 0.05$) in the finishers than in the non-finishers (**Figure 3**). A higher concentration of GSH-T was found in finishers at 80-km

than in non-finishers last sample before elimination (**Figure 4**). Plasma CK and AST were also higher in non-finishers that had metabolic problems than in finishers (CK > 5,000 IU/L and AST > 3,000; out of measurement range).

Positive correlations were found for plasma LPO on CK ($r = 0.22$; $P = 0.007$) and AST ($r = 0.32$; $P < 0.0001$).

Discussion

Our results confirm previous reports of oxidative stress and increased muscle leakage during endurance exercise in horses (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002; Marlin et al., 2002). New findings concerning LPO and WBC antioxidants demonstrate possible oxidative stress in these endurance horses. Differences between present results and other studies relate to ASC correlations with muscle enzymes and improvements in antioxidant status with ASC supplementation.

In general blood and plasma GSH-T reflect recent changes in muscle cells. The usual pattern during exercise is a progressive increase that continues for some minutes during recovery then declines over 18 h, which was observed here and in previous studies of intense and endurance exercise in horses (Mills et al., 1996; Chiaradia et al., 1998; Hargreaves et al., 2002; Marlin et al., 2002). The 27 % increase in RBC GPx observed in the last two stages of this race likely reflects a response to utilize reduced glutathione during the radical scavenging process (reduced glutathione donates an electron to eliminate free radicals using GPx as a catalyst). A another study in our laboratory observed little change in RBC GSH-T and GPx values that were about 25 to 50 %, respectively, of this study's range (Hargreaves et al., 2002). The comparison is interesting because the horses were competing in major races in the middle of the season, whereas this race was at the start of the season and horses were not as advanced in conditioning. In various breeds of horses racing 160 km, another study revealed that whole blood GPx increased 4 to 5-fold that of resting concentrations about halfway through the race and returned to baseline at 12 h of recovery (Frankiewicz-Jozko and Szarska, 2000). In well-conditioned racing sled dogs, however, RBC GPx did not change during three 1-day stages of 54 km exercise

(Hinchcliff et al., 2000). A likely explanation of the above results is that training improves the antioxidant system as manifested by lesser perturbation of the RBC glutathione/glutathione peroxidase system, even during harder competition.

In contrast to the RBC changes, novel findings here were the changes in the WBC glutathione system. Fluctuations of WBC GPx during exercise and the sharp 41% increase during recovery may reflect replenishment of reduced glutathione, however, the reduced:oxidized ratio was not measured for technical reasons during this field study. Compared to RBC, the higher concentration of WBC GPx and lower WBC GSH-T may affect phagocyte oxidative burst and other immune functions during prolonged exercise. Although moderate endurance training in athletes may enhance the immune system it was found that exhaustive endurance exercise might be detrimental (Vider et al., 2001). It was determined that human endurance athletes undergoing exhaustive endurance exercise have increased lipid peroxidation (increased TBARS) and increased antioxidant status (increased whole blood GSH-T). They also found strong positive correlations between GSH-T and lymphocyte mitogenic response (concanavalin A and phytohemagglutinin) after exercise. Combining the WBC results from the present study with the whole blood and lymphocyte results from the other study suggest that there is a connection between oxidative stress and immune function however, no further conclusion can be made here due to lack of immune function data.

In general, blood and plasma LPO is a measure of cumulative cell membrane lipid peroxidation, similar to the more commonly measured TBARS or malondialdehyde (MDA). In this study the LPO increased (**Figure 3**) and remained high throughout 30 min of recovery regardless of pre-race treatment, although it is unclear from this study how long the elevated LPO might persist. In a simulation of the endurance phase of a 3-day event during intense heat and humidity, an increase in lipid hydroperoxides continued for 30 min of recovery, then decreased to baseline by 24 h (Mills et al., 1996). In other equine studies (Chiaradia et al., 1998; Frankiewicz-Jozko and Szarska, 2000; White et al., 2001; Marlin et al., 2002) TBARS increased at the end of exercise and remained high hours after recovery. A study on Thoroughbreds showed an increase in TBARS (1.7 to 2.2 nmol/L) after a race (White et al., 2001). In another report (Marlin et al., 2002) TBARS had a wide

variation pre-exercise (66 to 1048 nmol/L) and increased post-exercise (150 to 1200 nmol/L). This variation for TBARS in this experiment is greater than the CV of 5 % for LPO in the present study. This variation and overlap makes it hard to compare levels pre- and post-exercise.

Plasma ASC concentrations were lower in the E group than the EC group at rest. This difference progressively diminished during the race as ASC increased in the E group but remained unchanged in the EC group. These findings contrast with a previous result from our laboratory, where a decrease in plasma ASC during a highly competitive and difficult 80 km race (Hargreaves et al., 2002). Plasma ASC also decreased during a race and through the racing season in sled dogs (Donoghue et al., 1993; Hinchcliff et al., 2000), and the season decline was prevented by vitamin C supplementation, 1 g/Mcal ME (Donoghue et al., 1993), compared to about 0.3 g/Mcal ME in the present study.

Plasma ASC ranges were similar here and in other reports from our laboratory (Hargreaves et al., 2002). The range of the current study was almost twice as high as in one previous endurance study (Marlin et al., 2002), and one-half that found in a 1000 m run to maximum velocity (White et al., 2001). These differences between studies could be attributable to dietary vitamin C presence and dosage, assay variation, lack of adjustment for water shift due to dehydration, conditioning of horses, or intensity of effort.

The plasma α -TOC range was wide and unchanged in the present study (1.75 to 12.88 μ g/mL) and in previous reports concerning endurance horses (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002; Marlin et al., 2002). Vitamin E intakes of horses in this study were about 5-times the recommended minimum when on the treatments (NRC, 1989), compared to 1.2- to 5-times in the 46 horses reported in the pre-race survey before the treatments were administered. The 2 horses having high plasma α -TOC during the race were receiving 2,000 to 3,000 IU/d of supplemental vitamin according to the pre-race survey. If protocol was followed they should have terminated their vitamin E containing supplements while feeding our vitamin treatment. Where the horses with low plasma α -TOC were receiving 0 to 10 % grain in their diet and no extra supplements. Thus, it is possible that there was carry-over from the horses' diet 3 wk prior

to initiation of treatments. Vitamin E intakes were not reported in previous studies (Frankiewicz-Jozko and Szarska, 2000; Hargreaves et al., 2002; Marlin et al., 2002), but the similar plasma α -TOC ranges suggest a higher vitamin E content in their hay and grain or aggressive vitamin E supplementation of endurance horses in Spain and England as well as the United States.

One limitation to this study was found during the pre-race survey. None of the riders would agree to compete in the negative control group (no supplementation of vitamin E or vitamin C). In order to get rider compliance each horse had to be assigned to the E or EC group. This makes it difficult to come to any conclusions regarding antioxidant supplementation in general.

A study of polo ponies used similar E and EC groups (Hoffman et al., 2001). Throughout the polo season plasma levels of α -TOC and ASC were higher in the EC group than the E group in hard-working ponies but not those in light work. These observations may reconcile the endurance findings in our laboratory where changes were observed in the highly competitive, mid-season races but not in this early season race. In a survey taken after the race, riders ranked the exertion level of the endurance ride easier than most of the rides later in the competition season. Also, ambient temperature was cooler in this race than in the summer when the majority of endurance competitions are held.

Plasma increases of CK and AST, especially during the last 2 stages of the race and recovery, reflect leakage of proteins and presumably other substances (e.g. enzymes) through muscle membranes (Harris, 1998). Three finishers developed high values of plasma CK without showing noticeable clinical signs of exertional rhabdomyolysis or dehydration. Interestingly, 2 of the 3 of these horses finished in the top 10. Four non-finishers had metabolic problems and higher plasma CK and AST activities than the upper limit of the assay. These results fit with previous conclusions that elevated CK and AST primarily reflect muscle leakage during exercise and should be used judiciously in the global differential diagnosis of exertional rhabdomyolysis (Noakes, 1987). Plasma CK and AST activities may increase during exercise without observation of clinical signs or histological detection of changes in muscle cell structure (Valberg et al., 1993). Factors including age, gender, physical fitness, season of year and training can contribute to

increased fluctuations in plasma CK and AST activity. Along with these, hypoxia and reduced ATP availability from the intense aerobic exercise may contribute to increased membrane permeability, making the increased permeability more likely in endurance horses (Harris, 1998).

Plasma CK increased in previous endurance studies with reported post-ride concentrations ranging from as low as 600 IU/L (Rose et al., 1977; Deldar et al., 1982; Ralston and Larson, 1988; Hargreaves et al., 2002) to as high as 2,000 to 3,000 IU/L (Sloet van Oldruitenborgh-Oosterbaan et al., 1991; Frankiewicz-Jozko and Szarska, 2000; Marlin et al., 2002). Several of these studies reported data from non-finishers, including horses eliminated for exertional rhabdomyolysis, which may explain the higher mean CK values.

The correlations of increasing cumulative lipid peroxidation and increasing muscle leakage found in the present study may indicate oxidative stress. One study found a positive correlation between increasing TBARS and CK in a previous endurance study (Frankiewicz-Jozko and Szarska, 2000). In another study, a negative correlation was found between ASC and CK (Hargreaves et al., 2002). A positive correlation between plasma isoprostanes and log plasma CK was found in racing sled dogs over repeated bouts of endurance exercise (Hinchcliff et al., 2000). Other equine endurance exercise studies failed to find the same relationship, however less sensitive measures of lipid peroxidation were used (Marlin et al., 2002).

Positive correlations of plasma CK and AST with various measures of antioxidant status, especially LPO, are consistent with the hypothesis that free radicals produced during exercise change membrane permeability of muscle cells (Butler et al., 1993; McBride and Kraemer, 1999). Exaggeration of oxidative stress associated with increased muscle membrane leakage during endurance exercise in certain horses could contribute to a form of oxidative fiber rhabdomyolysis, in contrast to previous reports of damage exclusively in glycolytic fibers (Valberg et al., 1993).

Implications

Although the present data showed little encouragement for supplementation with vitamin C in addition to vitamin E, previous studies of harder endurance races (Hargreaves et al., 2002) and the comparison of responses of polo ponies (Hoffman et al., 2001) in hard versus light work suggest the present study might have tended toward the lighter work category. These comparisons encourage further investigation of antioxidant supplements to help improve the performance and welfare of the endurance horse.

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Table 1. Plasma lipid hydroperoxides (LPO), alpha-tocopherol (α -TOC), alpha-tocopherol adjusted for albumin (TOCadj), RBC and WBC total glutathione (GSH-T), and RBC and WBC glutathione peroxidase (GPx) for the horses that finished the race (treatment groups combined; n = 34).

Variable	PRE	21	56	80	REC	SEM
LPO (uM)	8.19 ^a	7.48 ^a	9.55 ^a	14.5 ^b	16.6 ^b	1.3
RBC GSH-T (umol/g protein)	129 ^a	173 ^b	144 ^{ac}	150 ^c	93.8 ^d	4.9
WBC GSH-T (umol/g protein)	18.0 ^a	19.4 ^a	20.8 ^a	30.1 ^b	30.6 ^b	1.3
RBC GPx (mU/mg protein)	46.9 ^{ac}	41.9 ^b	44.5 ^{bc}	49.1 ^{ad}	52.7 ^d	1.3
WBC GPx (mU/mg protein)	114 ^{ab}	130 ^a	104 ^b	117 ^{ab}	165 ^c	5.4
α-TOC (mg/dL)	5.18 ^a	5.47 ^{ab}	5.81 ^b	5.42 ^{ac}	5.36 ^{ac}	0.4
TOCadj (mg/dL)	4.92	5.17	5.50	5.13	5.01	0.4

^{a, b, c, d} Different subscripts within rows differ ($P < 0.05$)

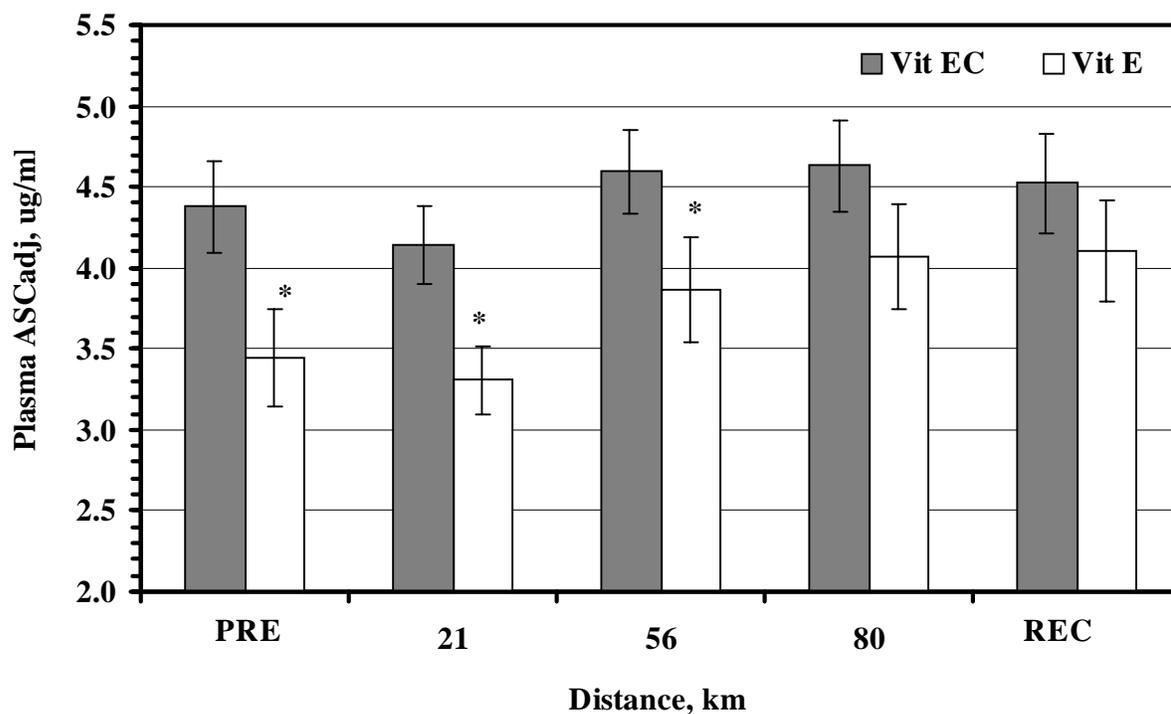


Figure 1. Plasma ascorbate adjusted for albumin (ASCadj) for horses completing the race in the vitamin E group (E; n = 17) and the vitamin E plus vitamin C group (EC; n = 17). ANOVA main effect treatment is significant at $P = 0.045$. * Treatments are significantly different at $P < 0.05$.

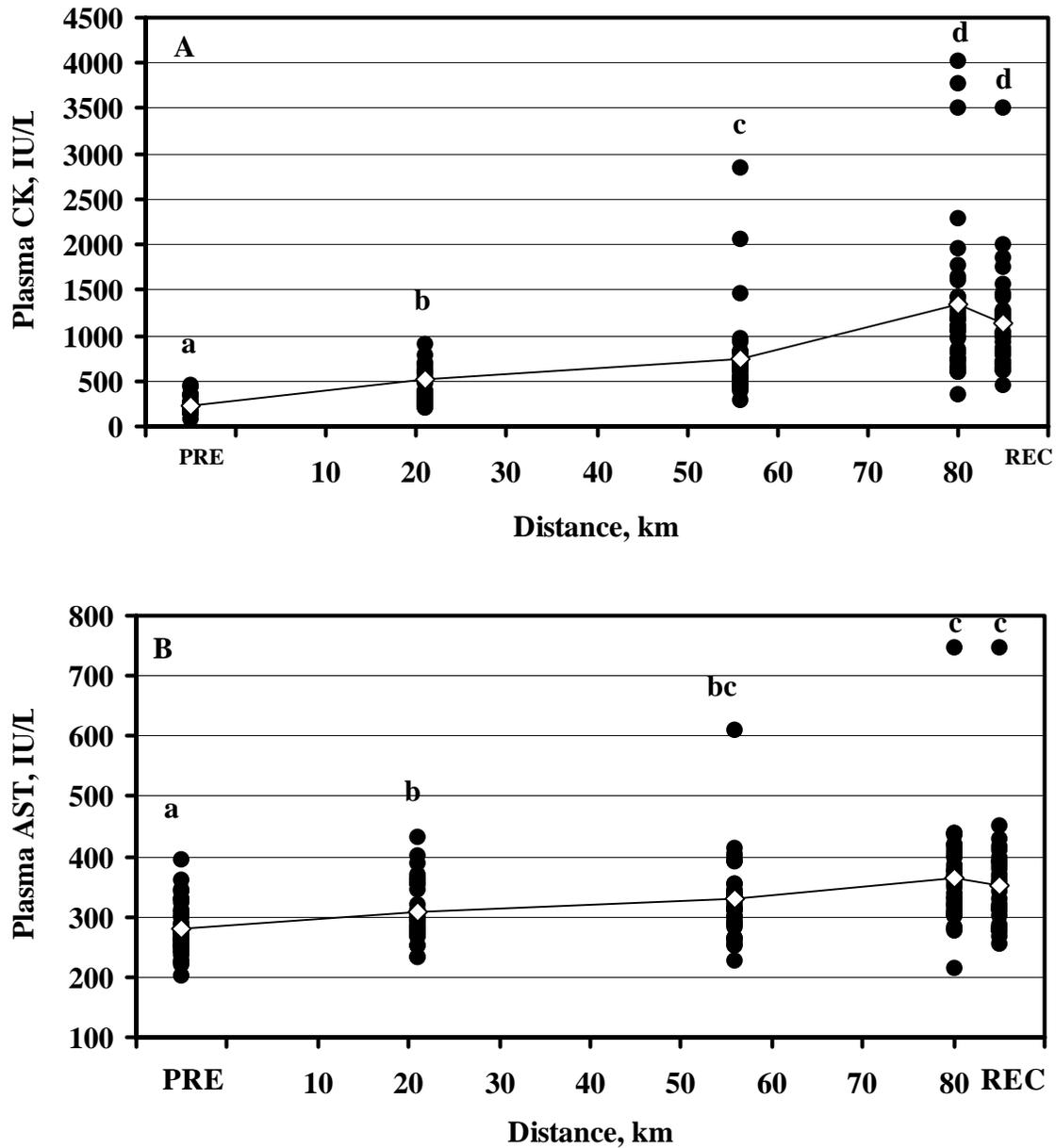


Figure 2. Plasma creatine kinase (CK; upper) and aspartate aminotransferase (AST; lower) for each horse that completed the race ($n = 34$). Individual observations are shown by the closed circles, and the mean is indicated by the open diamonds connected by a line. Different subscripts indicate differences between the mean at each distance ($P < 0.001$).

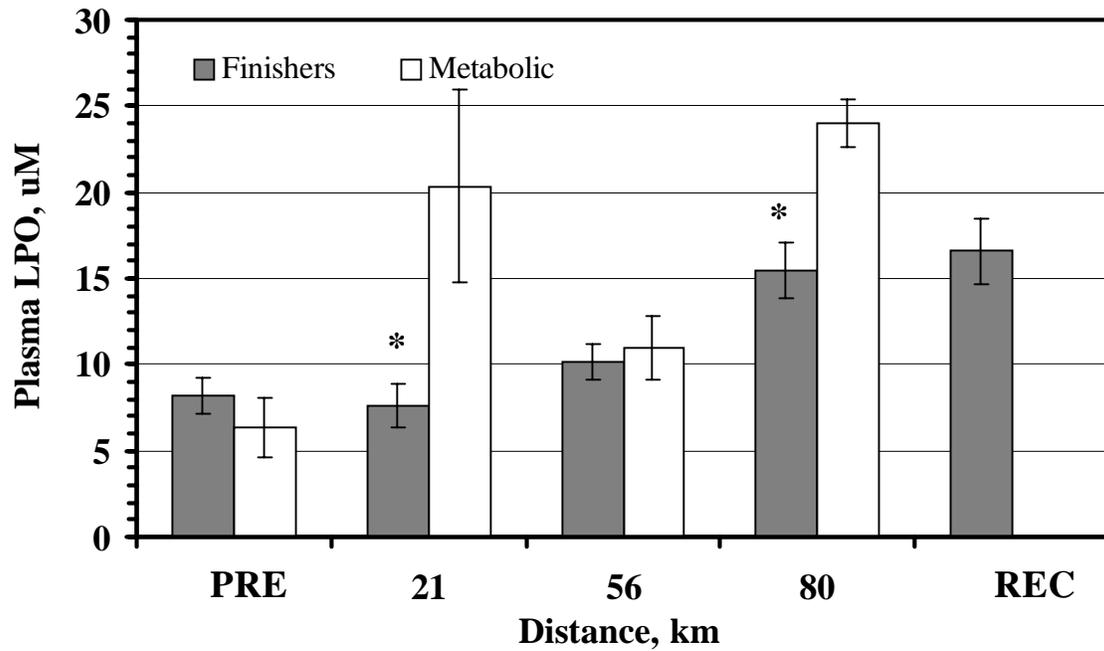


Figure 3. Plasma lipid hydroperoxides (LPO) for the horses that finished the race (treatment groups combined), and horses that pulled because of metabolic problems ($n = 4$). Distance 80 represents 80 km for the finishers and the point in the race where the non-finishers were eliminated. ANOVA main effect of LPO increasing with distance is significant for the finishers at $P = 0.001$. * Finished vs. metabolic different at $P < 0.05$.

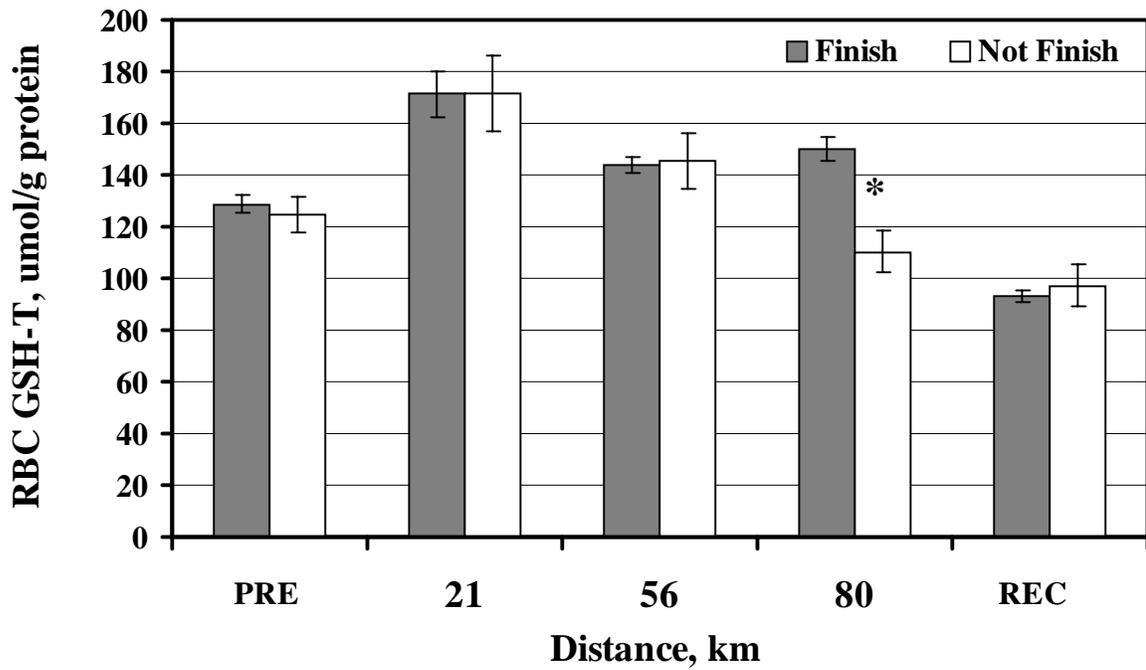


Figure 4. Red blood cell total glutathione (GSH-T) for the horses that finished the race (treatment groups combined; $n = 34$), and horses that pulled from the race (metabolic, lameness, and rider option reasons combined; $n = 12$). Distance 80 represents 80 km for the finishers and the point in the race where the non-finishers were eliminated. * Finished vs. not finished different at $P = 0.005$.