Lipoic Acid and Vitamin E Supplementation of Horses Diminishes Endurance Exercise Induced Oxidative Stress, Muscle Enzyme Leakage, and Apoptosis

ABSTRACT: The objective of this study was to compare the effects of a lipoic acid (LA) or vitamin E supplement on oxidative stress, antioxidant status, and apoptosis in horses exercising on a treadmill to simulate an endurance race. Twelve Arabian horses were supplemented with LA (10 mg/kg/d) 2 weeks prior to exercise, vitamin E (5000 IU/d) 3 weeks prior to exercise, or basal diet (140 IU/d). One week before the 55-km endurance exercise test (EET) a heart rate max exercise test was performed to standardize each individual's EET. The EET consisted of 3 exercise bouts or *loops*; first 2 loops were 20 km and the final loop was 15 km. Blood samples, heart rate, ambient temperature, and relative humidity were collected before the start of the EET, and at the start of each loop, halfway through each loop (10, 30, and 47.5 km), near the end of completing each loop (20, 40, and 55 km), and 0.5, 3, and 18 h of recovery. Red and white blood cells were analyzed for glutathione peroxidase, white cells for apoptosis, whole blood for total and oxidized glutathione, plasma for lipid hydroperoxides, creatine kinase (CK), aspartate aminotransferase (AST), ascorbate (ASC), α-tocopherol (TOC), lactate, and albumin. Both the LA and the E groups had higher concentrations of TOC ($P < 0.001$), ASC ($P < 0.020$), and total glutathione ($P < 0.001$), and had lower concentrations of CK and AST ($P < 0.010$) compared to CON. Apoptosis was lower in the E group ($P < 0.050$) and the LA group ($P = 0.063$) than the CON group. A significant correlation was found between apoptosis and ambient temperature ($r = 0.70$; $P < 0.001$). Results indicate that LA supplemented horses increased antioxidant status, and decreased plasma muscle enzyme concentration and white blood cell apoptosis similarly to vitamin E supplemented horses. Thus, horses undergoing endurance exercise in hot temperatures would be better able to handle the oxidative stress produced during the strenuous exercise if supplemented with E or LA.

Key Words: Antioxidants, Apoptosis, Equine, Oxidative stress, Treadmill

Introduction

Exercise increases oxygen consumption and production of reactive oxygen species (ROS), which may damage cell membranes or DNA (Davies et al., 1982; Curtin et al., 2002). Antioxidant supplementation prior to exercise has been shown to alleviate the oxidative stress associated with submaximal and maximal exercise (McMeniman and Hintz, 1992; Siciliano et al., 1996; Khanna et al., 1999). Vitamin E is the most commonly used antioxidant in horses. Horses supplemented with vitamin E above the current NRC recommendations had lower plasma thiobarbituric acid-reactive substances (TBARS; indicator of lipid peroxidation) after a submaximal bout of exercise compared to horses supplemented below recommendations for vitamin E (McMeniman and Hintz, 1992). Siciliano et al. (1996) also supplemented vitamin E and found that a single bout of submaximal exercise does not affect vitamin E status, but if the horse is being conditioned for several weeks, they may require a higher level of vitamin E supplementation.

α-Lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), have received attention as antioxidants in humans and laboratory animals. Both LA and DHLA have potential value in production and companion animals. α-Lipoic acid is an eight-carbon structure that contains a disulfide bond as a part of a dithiolane ring with a five-carbon tail. It is a cofactor in the conversion of pyruvate to acetyl CoA as part of the pyruvate dehydrogenase complex (Reed et al., 1951). It protects membranes by interacting with vitamin C and glutathione, which may also recycle vitamin E. Supplementing LA has found to be beneficial in a number of oxidative stress models, ishemia-reperfusion injury (Serbinova et al., 1992), diabetes (Ziegler and Gries, 1997), cataract formation (Maitra et al.,1994), radiation injury (Ramakrishnan, 1992), aging (Arivazhagan and Panneerselvam, 2000) and exercise (Khanna et al., 1999).

Apoptosis, also called programmed cell death, describes the molecular and morphological processes leading to controlled cellular self-destruction. Apoptosis results partially from an altered redox state in the body during times of oxidative stress. During

exercise, ROS damage important cellular tissues and increase the process of apoptosis. Lymphocytes and skeletal muscle are targets of exercised induced apoptosis due to the extreme oxygen metabolism of their mitochondria under intense conditions (Curtin et al., 2002).

The objective of this study was to compare the effects of a lipoic acid or vitamin E supplement compared to horses receiving a basal diet, on oxidative stress predictors, antioxidant status, muscle membrane leakage, and apoptosis in Arabian horses exercising on a treadmill to simulate an endurance race.

Materials and Methods

This study was performed at the Virginia Tech Middleburg Agricultural Research and Extension Center during the second and third week of April 2003, and was approved by the Institutional Animal Care and Use Committee. Twelve Arabian horses were maintained on mixed grass pasture with orchard grass hay round bales (15.4 IU/kg vitamin E; analyzed by Woodson Tenent Laboratories, Memphis, TN). They were fed 2.0 kg/d of a high fat textured feed (one of the leading commercial sweet feeds fed to endurance horses in the Mid-Atlantic region) to equal 30 % of total diet. Samples were submitted for partial proximate and nutrient analysis (**Table 1**).

The LA supplement consisted of 10 mg/kg body weight (Williams et al., 2001) of dL-α-lipoic acid top dressed on the morning feed at 0700 h starting 2 wk before the 55-km endurance exercise test (EET). The vitamin E group (E) was supplemented with 5000 IU/d of dL-alpha tocopheryl acetate in the morning feed starting 3 wk before the EET. The control group (CON) received their morning feed with the basal amount of vitamin E (approximately 140 IU/d) and no LA.

Horses were trained for 8 wks in an interval-training program before the EET. About one week before the EET a maximum heart rate (HR_{max}) exercise test was performed to standardize each individual's EET (Eaton et al., 1995; Hodgson and Rose, 1994). The EET was conducted on an equine treadmill (EquiGym Products LLC, Paris,

KY) and consisted of 3 exercise bouts or *loops*; first 2 loops were 20 km and the final loop was 15 km (**Figure 1**). The horses were maintained at speeds relative to 30 to 60 % of their HR_{max}, calculated using the following equation:

$$\text{Target HR} = \text{actual } HR_{max} * \% HR_{max}$$

After every loop, the horses were stopped, removed from the treadmill and underwent a veterinary check similar to during an endurance race (AERC; Mackay-Smith et al., 1999). After the loop is completed the time for the HR to reach 64 beats/min was recorded then the horses were given a 20 min hold period before the onset of the next loop. During this hold time they were given access to water and hay, and supplemented with electrolytes (Hess et al., 2002). The horses were weighed before the EET and immediately after completing 55 km. During recovery from the EET the horses were allowed to have access to water and hay, and their afternoon sweet feed meal was fed at 1500 h.

Blood samples were collected from the jugular vein before the start of the EET (1^{st} PRE), and at the start (2^{nd} and 3^{rd} PRE), halfway through (10, 30, and 47.5 km), and near the end of completing each loop (20, 40, and 55 km), and 0.5, 3, and 18 h of recovery (REC; **Figure 1**). Ambient temperature and relative humidity, along with horse's heart rate and temperature, were recorded at each sample time. Horses were catheterized at 0700 h allowed a 30 min acclimation period before the baseline sample was collected. Blood samples were collected into syringes then transferred into 1 EDTA and 2 sodium heparin vacuum tubes (Becton Dickinson and Company, Franklin Lakes, NJ).

Whole blood was analyzed for total and oxidized glutathione (GSHt and GSSG, respectively; Biotech GSH/GSSG-412, kit #21040; Oxis Health Products, Inc., Portland, OR; GSHt inter-assay CV 3.1 %, intra-assay CV 1.0 %; GSSG inter-assay CV 7.6 %, intra-assay CV 6.5 %). The glutathione ratio was calculated in the kit using the equation:

$$\text{Ratio} = (GSHt - 2\ GSSG) / GSSG$$

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, 1 mL of ice-cold deionized water was added to lyse the cells, which was diluted 1:400 then analyzed for 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. This assay refers to the GPx amount in terms of total protein in the sample (Bio-Rad Laboratories, Hercules, CA).

Glutathione peroxidase was also analyzed for in WBC. Here the buffy coat, was removed after centrifugation of whole blood at 2500 X g for 5 minutes at 4° C, and transferred to a tube containing 10 mL of lysis buffer (0.15 M NH_4Cl, 0.01 M NaHCO, 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). 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.

White blood cells were also analyzed by flow cytometry for the number of cells undergoing apoptosis using a propidium iodide staining (PI; Nicolitti et al., 1991). The white blood cells use for the apoptosis analysis were processed similarly only diluted with 2 mL HBSS, then 100 uL was added to 300 uL of PI buffer. They were then refrigerated at 4° C for 2 to 5 d until analysis on the flow cytometer.

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. 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 %). Plasma creatine kinase (CK), aspartate aminotransferase (AST), and albumin (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., 2002) were analyzed by high-pressure liquid chromatography methods. Ascorbate and TOC were adjusted (ASCadj and TOCadj, respectively) for changes in fluid redistribution during exercise using albumin (ALB). The equation was as follows:

$$ASCadj = ASC * (1^{st} \text{ PRE ALB} / \text{sample ALB})$$

Data were summarized as least squared means ± SE, unless otherwise indicated. Analysis of variance with repeated measures using a MIXED model (SAS Institute Inc.,

Cary, NC) were used to evaluate the effects of treatment and stage of EET, and their interaction. 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. Pearson's product-moment and Spearman's rank order correlations were used to test for an association between measures of oxidative stress, antioxidant status, and ambient temperature. Horse was included in the model to test for significance, if insignificant then it was removed from the model.

Results

Horses were 11yr of age (5 – 14 yr 95 % CI), they weighed 450 kg (830 – 1036 kg 95 % CI) at the start of the race and lost 5.0 % (4.25 – 5.73 % 95 % CI) after 55 km. Ambient temperature ranged from 2.5° C and 92 % relative humidity to 30.5° C and 5 % relative humidity. Ambient temperature and humidity showed no effect of treatment (P = 0.51 and P = 0.43, respectively), and temperature only a trend for stage of EET (P = 0.061). Horses completed the 55 km in 5.0 ± 0.05 h including vet checks. From the start of exercise to the 0.5 h recovery horses consumed a total of 0.90 ± 0.2 kg hay and 9.9 ± 1.8 L of water. Information for each horse and treatment group is summarized on **Table 2**. There were no differences between treatments for the above measures.

No interaction was found between treatment and stage, except for apoptosis. Apoptosis in WBC had a significant increase with stage in the CON group (P = 0.002), however in both E and LA groups there was no increase with exercise. Stage was significant for plasma ASCadj (P = 0.014), with increasing values until 47.5 km then a decrease through 18 h of REC. There was also an effect of stage (P = 0.041) for blood GSHt, with increases and decreases similar to ASC. There was a significant increase in plasma CK (P = 0.026) with stage; however, stage was not significant for plasma AST. Plasma lactate had an effect of stage (P < 0.001) with peaks at the most intense sample of each loop (10, 30, and 47.5 km). There was no effect of stage on plasma LPO (P = 0.95).

Compared to the CON group, the LA (27 % higher) and E (49 % higher) group had higher (P < 0.001) plasma TOCadj concentrations (**Figure 2A**). Plasma ASCadj

concentrations (**Figure 2B**) also were higher in the LA group (17 % higher; $P = 0.022$) and tended to be higher in the E group (13 % higher; $P = 0.063$) as compared to the CON group. Compared to CON, LA and E had more than a 40 % greater ($P < 0.001$) blood GSHt concentration. Blood GSH:GSSG ratio in the LA group ($P < 0.001$) was about half that of the CON group; the E group was 16 % than the CON ($P = 0.009$).

Compared to the CON group, RBC GPx was lower in the LA group (12.6 % lower; $P < 0.001$) and the E group (3 % lower; $P = 0.007$). The WBC GPx were about 8 % higher ($P = 0.020$) in the LA and E groups as compared to the CON group.

One horse in the vitamin E group completed the 55 km race, but was diagnosed with exertional rhabdomyolysis, due to stiffness of hindquarter muscles. The CK concentration reached 7717 IU/L at 55 km and AST reached a high of 849 IU/L 18 hr after recovery, therefore he was > 2 SD above the mean and dropped from the analysis. Compared to CON, LA and E were about 1.5-times lower ($P < 0.001$) for plasma CK, but only LA was lower (13.4 % lower; $P < 0.001$) for plasma AST (**Figure 3A**). Plasma lactate (**Figure 3B**) was lower in the LA group (18.5 % lower; $P = 0.082$) and in the E group (32 % lower; $P = 0.004$) as compared to the CON group.

Apoptosis in WBC (**Figure 4**) was about 10-fold lower in the E group ($P < 0.050$) and the LA group ($P = 0.063$) as compared to the CON group. Three horses (1 CON, 1 E, and 1 LA) exercised when the mean ambient temperature during exercise was > 16° C, this allowed them to be removed due to being > 2 SD from the mean.

Correlations between oxidative stress measures, antioxidant status, and other variables are shown in **Table 3**.

Discussion

These results indicate that apoptosis occurs in white blood cells during exercise and it can be moderated by supplementation with vitamin E or lipoic acid. The results have also confirmed an increase in plasma muscle enzyme concentrations during exercise. Correlations with oxidative stress, antioxidant status and muscle enzymes demonstrated that oxidative stress occurs with intense endurance exercise in the horse.

Antioxidant Status. Antioxidants are linked together in various ways; this explains the increase in antioxidant status with supplementation of E and LA. In the present study LA increased the GSHt concentrations in whole blood compared to CON. The LA group also had increased levels of ASC and TOC in the plasma throughout the study. Both the E and LA groups had about 40 % more GSHt, 30 % more TOC, and 15 % more ASC than the CON group. This illustrates recycling and scavenging of antioxidant radicals using the exogenous sources of the vitamin E and LA.

Free radicals are directly scavenged by vitamin E forming a vitamin E radical. Through interplay with vitamin C, the vitamin E radical is regenerated leaving a vitamin C or ascorbyl radical. Two ascorbyl radicals react to form vitamin C and dihydroascorbic acid. Reaction of dihydroascorbic acid and dihydrolipoic acid restores vitamin C and LA (Biewenga et al., 1997). One of the original studies testing this mechanism used rats to determine the effect of LA on vitamin C deficiency (Rosenberg and Culik, 1959). The LA plus ASC supplemented rats had a lower deficiency score compared to each supplemented individually with LA or ASC. The same study observed effects of vitamin E deficiency on reproductive efficiency when supplemented with LA, TOC or both. The combination of LA and TOC was more effective than either one of the compounds alone. Another study fed a vitamin E deficient diet with additional LA. These rats showed no signs of vitamin E deficiency (e.g., weight loss, death, etc.), unlike the rats not supplemented with LA (Podda et al., 1994).

Lipoic acid has also been used to help alleviate oxidative stress in many different scenarios, including aging, disease, and exercise. Exercised rats supplemented with LA had an increase in GSH in liver and blood, and decreased TBARS in the liver, muscle, and heart (Khanna et al., 1999). These results were confirmed in the horse in the present study however; lipid peroxidation was unaffected by treatment or stage of EET.

Muscle Leakage. The lower concentration of muscle enzymes after exercise in the supplemented groups could be due to the amount of stress or work the muscles are undergoing compared to the CON horses. The increase of enzymes in plasma reflects

leakage of proteins and presumably other substances (e.g. enzymes) through muscle membranes (Harris, 1998). Horse 2 from the E group was diagnosed with exertional rhabdomyolysis after he completed the 55-km of exercise. The supplemented horses had 14- to 19-times lower CK levels during the last loop of the EET than horse 2, who also had about 9-times higher CK than the CON group. Horse 2 had TOC and ASC concentrations throughout the study were significantly lower (2- and 1.5-times, respectively) than the other horses in the study including those in the CON group. This horse was being supplemented with 5000 IU of vitamin E per day, which leads to the hypothesis that he has problems with metabolizing the fat in the diet, as well as the vitamin E. Other clinical tests need to be performed to determine the exact origin of the problem. Factors including age, gender, physical fitness, season of year and training can contribute to increased fluctuations in plasma CK and AST activity (Harris, 1998). 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 and not always indicative of exertional rhabdomyolysis.

Other studies have shown increases in muscle enzymes with endurance exercise. Plasma CK increased in previous endurance studies with reported post-ride concentrations ranging from as low as 600 IU/L (Deldar et al., 1982; Ralston and Larson, 1988; Hargreaves et al., 2002) to as high as 2,000 to 3,000 IU/L (Frankiewiez-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.

Apoptosis. We hypothesized that antioxidant supplementation would minimize the exercise induced rise in apoptosis in the white blood cells compared to the CON group. This proved true for the E group (50 % lower) and tended to be true for the LA group (40 % lower) compared to the CON group. The increase in antioxidant status in the E and LA groups aided the white blood cells in scavenging the ROS triggering the apoptosis in these cells. This has been demonstrated in a study where an antioxidant formula containing α-tocopherol, α-lipoic acid, coenzyme Q_{10}, carnitine, and selenomethionine was supplemented to healthy human subjects to determine if antioxidants would decrease

apoptosis in lymphocytes (Mocsa et al., 2002). After 3 wks of supplementation, subjects had an increased antioxidant status, decreased percentage of apoptotic lymphocytes, and decrease in ROS production. Another study found that the addition of metal chelators and radical scavengers rat thymocytes inhibited the manual induction of apoptosis (Wolfe et al., 1994).

The present study failed to find an increase in apoptosis with exercise as found in other species. The authors hypothesize that the lack of exercise-induced apoptosis could have been due to the lack of temperature control during the EET. Three of the 12 horses exercised on days having ambient temperatures over 14° C. These three horses had about a 30-fold higher percentage of apoptosis throughout all the sample times, which overshadowed the increase with exercise in horses with a smaller apoptosis percentage. By referring to figure 4, a numerical increase in the CON group and horse run on hot days may be noted; however, these increases were not significant.

Previous studies have shown that thymocyte apoptosis, measured by DNA fragmentation, was induced in rats that performed two treadmill runs to exhaustion 24 h apart (Concordet and Ferry, 1993). Increased rates of apoptosis were also evident immediately after the first run and continued to be detectable 24 h post exercise. In humans, lymphocyte apoptosis has been documented to occur immediately after and 24 h after an exhaustive exercise bout (Mars et al., 1998). Before exercise ensued, lymphocytes were found in the first two preliminary stages of apoptosis. In the 24 and 48 h samples after exercise, all four of the apoptotic stages were present in lymphocytes, including the single strand DNA breaking and creating a ‘comet tail’. Flow cytometry of these lymphocytes showed an increase in all subjects from before (24.8 ± 20.2 %) to immediately (62.8 ± 17.4 %) and 24 h (86.2 ± 1.7 %) post-exercise.

Oxidative Stress. Higher levels of oxidative stress have been associated with hot and humid conditions. 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). However, in the present study the rise in temperature was inversely related to humidity. The colder days tended to

have rain associated with them and as the temperature increased over a few days the humidity decreased. The correlations with temperature show a strong association between the warmer ambient temperature and higher levels of apoptosis and lipid peroxidation, and lower concentrations of antioxidants. Humidity was inversely related to these same measures. The decrease in antioxidant status on the warmer days is explained by the increased oxidative stress needing to utilize more antioxidants for their scavenging capacity.

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). Correlations with oxidative stress, antioxidant status and muscle enzymes were found to confirm the results found previously (Hargreaves et al., 2002; Marlin et al., 2002). A positive correlation between increasing TBARS and CK was revealed from a previous endurance study (Frankiewiez-Jozko and Szarska, 2000). A positive correlation between plasma isoprostanes and log plasma CK was demonstrated in racing sled dogs over repeated bouts of endurance exercise (Hinchcliff et al., 2000). In another study, a negative correlation was found between ASC and CK (Hargreaves et al., 2002).

The unique finding with the present correlations is that the ASC correlations with AST, CK and apoptosis are in the opposite direction (positive) of those found previously. One explanation could be that the increased apoptosis and muscle leakage caused a mobilization of ASC into the plasma for its transport to other tissues. However, the same was not true for TOC and GSHt. Here negative correlations were found for both with apoptosis indicating a utilization effect of the antioxidant instead of mobilization. This may be due to the location of the antioxidant stores. With TOC as the major protector of cell membranes it may be the first line of defense during stressful times. Many current studies are using assays of total antioxidant capacity or scavenging ability, however, the

present positive correlations with antioxidants (TOC, ASC, and GSHt) solidifies the fact that antioxidant status is increasing across different cellular locations and antioxidant types.

Implications

This study shows evidence that increased antioxidant status is associated with supplementation of vitamin E or lipoic acid. This status is consistently increased for both an oral vitamin E or lipoic acid supplement. The supplementation also decreased the level of apoptosis in the white blood cells, which could be important during hot exercise days or to improve immune function after summer endurance races. Thus, to improve the health and well being of the equine endurance athlete supplementation of antioxidants is warranted.

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Table 1. Nutrient composition of feed on a DM basis as analyzed in the DHI Forage Testing Laboratory (Ithaca, NY).

Nutrient (n = 3)	**g/kg**
DM	879
DE	3.41 (Mcal/kg)
CP	117
ADF	119
NDF	254
NSC	418
Fat	115
Ash	59.6
Ca	9.6
P	5.6
Vitamin E	70 (IU/kg)

Table 2. Information of horses in the control group (CON), vitamin E supplemented group (E), and lipoic acid supplemented group (LA) including horses age, average temperature during exercise, relative humidity during exercise, weight, absolute and relative weight lost during the 55-km of exercise, total time to complete the exercise, water and hay consumed from the first veterinary check through the 30 min recovery sample. Means ± SE are calculated for each group.

Group	Horse #	Age	Avg Temp (C)	Humidity (%)	Total Time (hr)	Water (L)	Hay (kg)	Weight (kg)	Weight Loss (kg)	Weight Loss (%)
CON	**3**	13	4.3	83.5	5.03	8.05	0.9	1032	44	4.26
	8	16	12.9	43.4	4.75	12.31	0.4	976	50	5.12
	10	5	23.2	30.5	5.27	24.14	0.8	786	42	5.34
	12	11	5.6	82.6	4.98	7.57	0.3	910	34	3.70
	Mean	*11.3*	*11.5*	*60.0*	*5.01*	*13.02*	*0.60*	*926*	*42.5*	*4.61*
	SE	2.3	4.3	13.6	0.11	3.86	0.15	53	3.3	0.38
E	**2**	12	4.6	81.8	5.10	4.73	0.7	1188	60	5.10
	4	13	8.7	62.4	5.17	4.73	0.8	830	44	5.30
	6	12	13.3	25.8	4.92	4.73	0.9	920	54	5.87
	11	5	14.0	33.1	4.70	9.47	1.2	942	48	5.10
	Mean	*10.5*	*10.1*	*50.8*	*4.97*	*5.92*	*0.90*	*970*	*51.5*	*5.34*
	SE	1.9	2.2	13.0	0.10	1.18	0.11	77	3.5	0.18
LA	**1**	14	4.2	80.9	4.75	4.73	0.5	1036	44	4.25
	5	8	6.6	84.1	5.12	8.52	0.9	908	52	5.73
	7	11	12.7	27.3	4.98	11.36	2.4	1036	58	5.60
	9	11	17.9	44.4	5.07	18.93	1.1	978	50	5.11
	Mean	*11.0*	*10.3*	*59.2*	*4.98*	*10.89*	*1.23*	*989*	*51.0*	*5.17*
	SE	1.2	3.1	13.9	0.08	3.01	0.41	30	2.89	0.34

Table 3. Least squared means ± SEM for the control group (CON), vitamin E group (E), and lipoic acid supplemented group (LA).

Measure	CON	E	LA	SEM
Apoptosis[g] (%)	16.5[d]	1.52[e]	1.73[e]	1.7
Lactate (mmol/L)	1.35[a]	0.92[b]	1.10[a]	0.10
CK[f] (IU/L)	551.2[d]	341.4[e]	323.9[e]	44.6
AST[f] (IU/L)	261.5[a]	248.0[a]	226.4[b]	7.6
TOCadj[f] (ug/mL)	4.08[d]	6.08[e]	5.19[e]	0.14
ASCadj (ug/mL)	5.42[a]	6.15[a]	6.33[b]	0.27
GSHt (uM)	280.7[d]	401.9[e]	411.2[e]	13.0
GSH:GSSG	24.8[a]	20.9[a]	12.5[b]	2.3
WBC GPx (mU/g protein)	46.1[a]	49.6[b]	49.7[b]	1.1
RBC GPx (mU/g protein)	42.0[d]	40.7[e]	36.7[e]	1.0
LPO (uM)	11.8	11.2	10.8	0.72

[a, b, c] Subscripts within rows differ at $P < 0.05$.

[d, e] Subscripts within rows differ at $P < 0.001$.

[f] Data exclude horse 2 (E group), > 2 SD from mean.

[g] Data exclude horse 9, 10, and 11 (1 CON, 1 E, and 1 LA), > 2 SD from mean.

Table 4. Regressions (Y = B + AX) of indices of antioxidant status, oxidative stress, and other variables for all 12 horses (n = 144) in the control (CON), vitamin E (E) and lipoic acid (LA) supplemented groups combined.

X	Y	A	B	Adj R^2	P
ASCadj	AST	10.9	177.8	0.18	< 0.001
ASCadj	Apoptosis	4.37	6.74	0.10	0.001
ASCadj	LPO	-0.54	14.5	0.034	0.012
ASCadj	CK	34.1	199.9	0.034	0.012
TOCadj[a]	GSHt	47.7	133.8	0.31	< 0.001
TOCadj	RBC GPx	-2.27	50.8	0.15	< 0.001
TOCadj	ASCadj	0.49	3.70	0.093	< 0.001
TOCadj	WBC GPx	1.26	42.0	0.037	0.016
TOCadj	Apoptosis	-3.98	41.1	0.025	0.047
GSHt	Apoptosis	-0.08	48.3	0.095	< 0.001
GSHt	AST	-0.08	276.3	0.093	0.032
WBC GPx	Apoptosis	-1.60	96.7	0.21	< 0.001
WBC GPx	GSHt	3.35	204.2	0.048	0.005
RBC GPx	GSHt	-4.84	558.7	0.093	< 0.001
LPO	CK	20.6	192.4	0.077	< 0.001
AST[a]	LAC	0.01	-0.83	0.25	< 0.001
CK[a]	LAC	0.01	0.78	0.11	< 0.001
HRmax	LAC	0.01	0.89	0.031	0.020
HRmax	GSHt	1.51	322.4	0.29	< 0.001

[a]Excluding horse # 2 from E group.

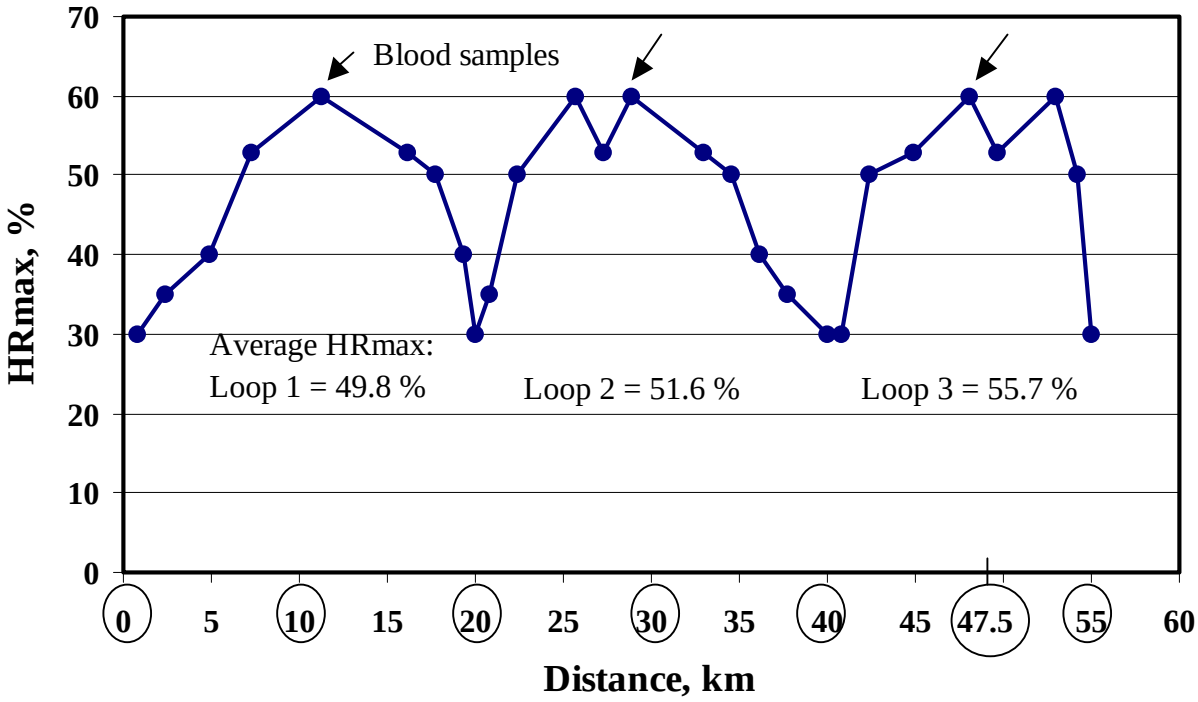

Figure 1. Endurance exercise test (EET) protocol based on heart rate max (HR_{max}). Blood samples were taken at the start of the EET (1st PRE), 10, 20 km, start of 2nd loop (2nd PRE), 30, 40 km, start of 3rd loop (3rd PRE), 47.5, and 55 km, then 0.5, 3, and 18 h of recovery (REC).

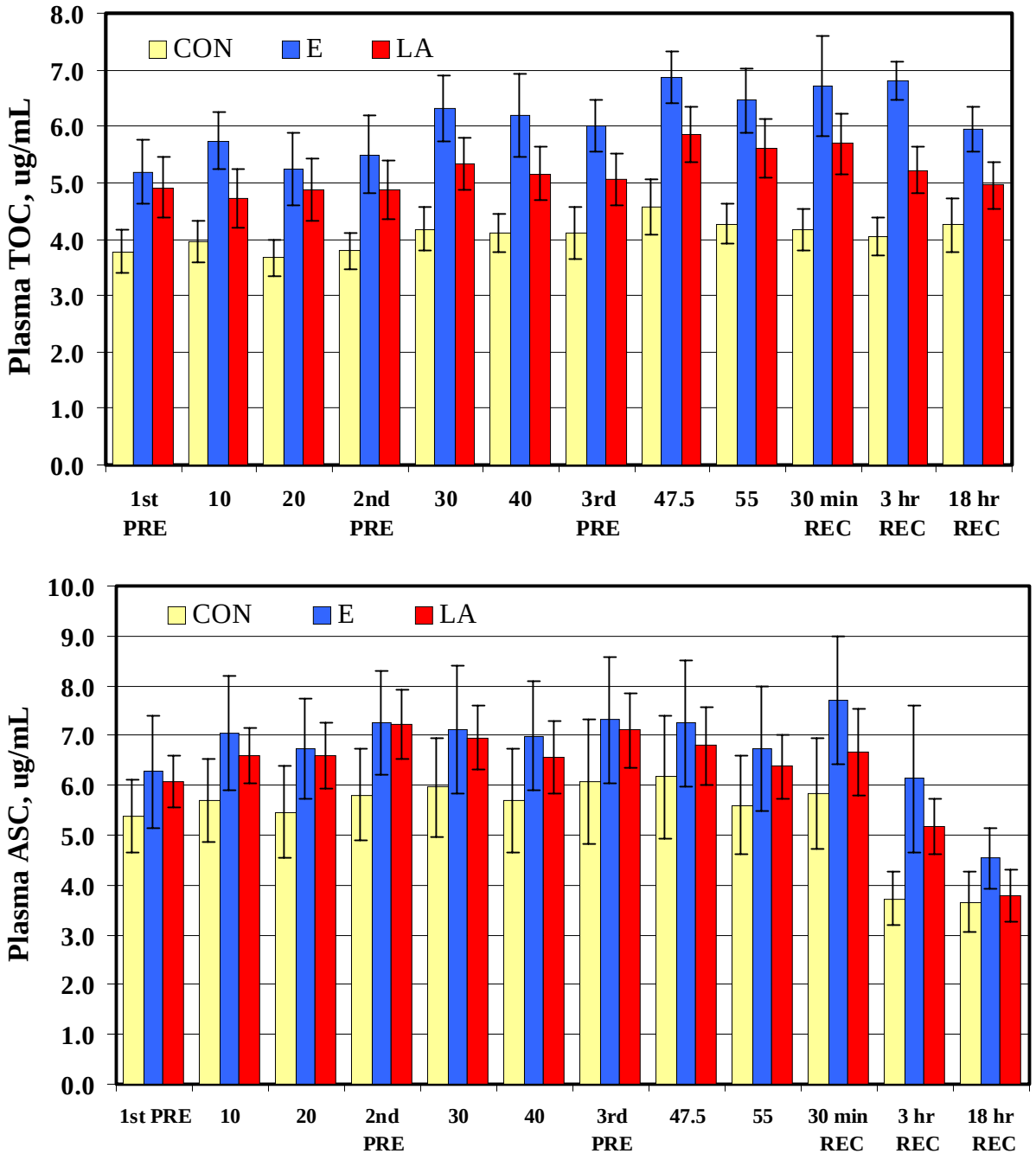

Figure 2. Plasma alpha-tocopherol (TOC; A) and ascorbate (ASC; B) concentrations for the control (CON; n = 4), vitamin E (E; n = 3), and lipoic acid (LA; n = 4) groups. The E group is graphed without concentrations for horse 2 (TOC = 3.45 ug/mL; 2 SD from mean).

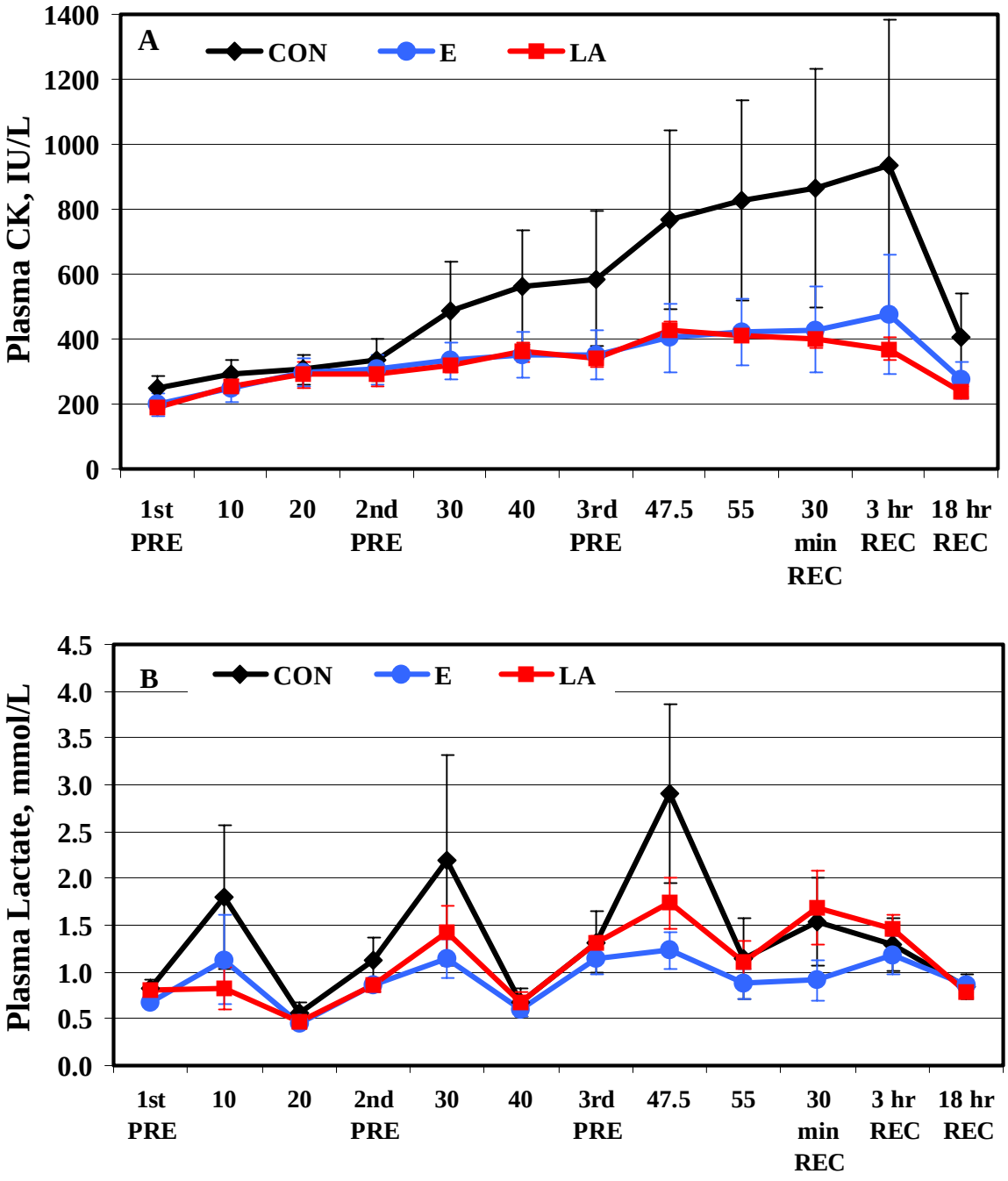

Figure 3. Plasma creatine kinase (CK; A) and lactate (B) concentrations for the control (CON; n = 4), vitamin E (E; n = 3), and lipoic acid (LA; n = 4) groups. The E group is graphed without concentrations for horse 2.

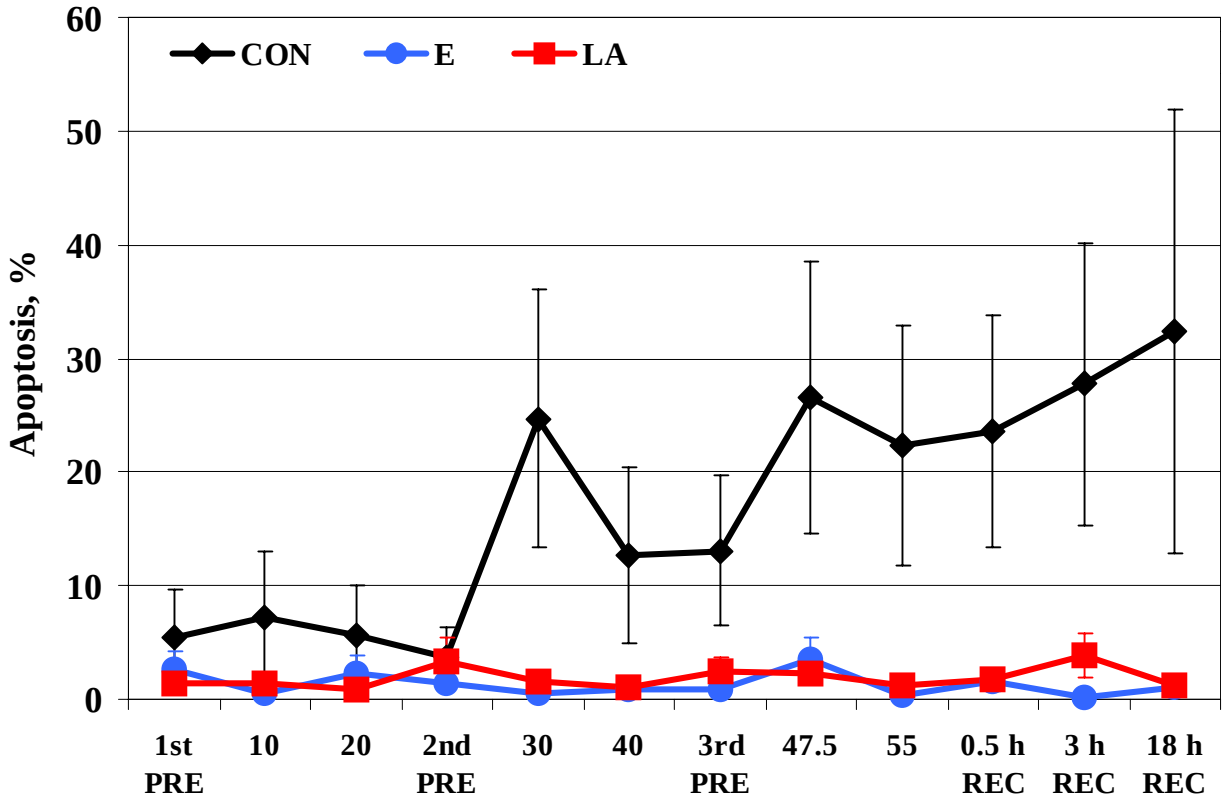

Figure 4. White blood cell apoptosis for the control (CON; n = 3), vitamin E (E; n = 3), and lipoic acid (LA; n = 3) groups. Horses 9, 10, and 11 were removed from their groups (1 CON, 1 E, 1 LA; 2 SD from mean).