

Lipoic Acid as an Antioxidant in Mature Thoroughbred Geldings: A Preliminary Study

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ABSTRACT: Alpha-lipoic acid (LA) has demonstrated antioxidant effects in humans and laboratory animals. The objective of this study was to determine if the effects of LA are similar in horses. Five Thoroughbred geldings were supplemented with 10 mg/kg/d dL- α -lipoic acid in a molasses and sweet feed carrier and five received only the carrier as a placebo (CON). Blood samples were obtained at baseline (0 d), after 7 and 14 d of supplementation, and 48 h post supplementation (16 d). Blood fractions of red and white blood cells (RBC and WBC, respectively), and plasma were analyzed for total glutathione (GSht), glutathione peroxidase (GPx), and total plasma lipid hydroperoxides (LPO). An experienced veterinarian observed no adverse clinical effects. Plasma LPO baselines differed between groups ($P = 0.002$). When covariates were used, there was a decrease over time in the LA group ($P = 0.015$) and concentrations were lower in the LA group than the CON group at 7 and 14 d ($P = 0.022$ and 0.0002 , respectively). At baseline, GSht concentration was 69 ± 7 in WBC and 115 ± 13 mmol/g protein in the RBC with no differences of time or treatment. The GPx activity was 47 ± 4 and 26 ± 5 mU/g protein at baseline WBC and RBC, respectively, with a lower concentration in the LA group's WBC at 7 ($P = 0.019$) and 14 d ($P = 0.013$). The results show that 10 mg/kg LA had no evident adverse effects, and moderately reduced the oxidative stress of horses allowed light pasture activity. These findings encourage studying of LA in horses subjected to strenuous exercise.

Key Words: Dihydrolipoate, Glutathione, Horse, Lipid hydroperoxides, Oxidative stress

Introduction

α -Lipoic acid (LA) and its reduced form, dihydrolipoic acid (DHLA), have received widespread attention as antioxidants with both preventative and therapeutic uses in humans and laboratory animals (Packer, 1995). They have potential value in production and companion animals during periods of oxidative stress. α -Lipoic acid is an eight-carbon structure that contains a disulfide bond as 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. Both LA and DHLA also protect the integrity of cell membranes by interacting with antioxidants—glutathione, and vitamins E and C (Packer, 1995). *In vitro*, intracellular glutathione (GSH) was positively correlated to the concentration of LA, which may play a role with cysteine (Han et al., 1995 & 1997).

Lipoic acid is both water- and fat-soluble, and thus may regenerate water- and fat-soluble antioxidants, such as vitamin C and vitamin E. Rats consuming a vitamin E deficient diet with added LA had no symptoms of vitamin E deficiency, unlike the rats on a vitamin E deficient diet alone (Podda et al., 1994). The low α -tocopherol (α -TOC) concentrations in the tissues of LA supplemented rats suggests that LA eliminates symptoms of vitamin E deficiency by sparing α -TOC.

Another function of LA and DHLA is to scavenge free radicals. Both LA and DHLA eliminate hydroxyl radicals and hypochlorous acid with a potency comparable to glutathione and N-acetylcysteine (Haenen and Bast, 1991; Biewenga et al., 1994). However, only DHLA eliminated superoxide, peroxy, and ascorbyl radicals. Both LA and DHLA did not interact directly with chromanoxyl radicals of vitamin E; however, DHLA did reduce ascorbyl radicals and showed a DHLA/ascorbic acid-mediated reduction of chromanoxyl radicals (Suzuki et al., 1991; Kagan et al., 1992). These studies demonstrate the importance of LA as a protective compound during oxidative metabolism.

Lipoic acid has been shown to have effects during exercise stress, aging and metabolic disease. Khanna et al. (1999) compared rested and exercised rats supplemented with or without LA. The LA supplemented rats, both rested and exercised, had a higher GSH concentration, and a lower lipid peroxidation level measured by thiobarbituric acid

reactive substances as compared to non-supplemented rats. During aging, mitochondria use oxygen inefficiently in ATP synthesis. Old rats supplemented with LA had a higher mitochondrial membrane potential, ambulatory activity, GSH and ascorbate concentration; furthermore, the malondialdehyde concentration was five times higher in the non-supplemented rats (Hagen et al., 1999). Lipoic acid was also used in humans to test its effectiveness on relieving diabetic symptoms (Ziegler et al., 1995). Three different doses of LA were compared to a placebo over a period of 19 d. After 5 d patients dosed with 1200, 600 or 100 mg LA had a greater decrease in the severity of their symptoms than patients given a placebo. Other human tests also show positive results when tested under different diseased states or illness conditions, including Alzheimer-type dementia (Hager et al., 2001).

Oxidative stress is evident in horses undergoing exhaustive and endurance exercise (Ji et al., 1990; Ishida et al., 1999; Hargreaves et al., 2001); this warrants the testing of antioxidant supplements. The objective of this study was to evaluate the safety and antioxidant potential of LA in horses undergoing light voluntary pasture exercise.

Materials and Methods

Ten mature Thoroughbred geldings, 7 to 16 y of age, weighing 626.8 ± 8.3 kg, were used in this study. The protocol was approved by the institutional animal care and use committee and performed at the Virginia Tech Middleburg Agricultural Research and Extension Center.

The geldings were maintained on 30 acres of mixed grass/clover pasture during the month of January with *ad libitum* access to orchard grass/alfalfa hay. Five of the geldings were randomly assigned to the treatment group (LA) and received 10 mg/kg body weight dL- α -lipoic acid (Sigma Chemical, St. Louis, MO) mixed into a carrier (70 g of molasses and sweet feed) offered by hand once a day at 1300 h for 14 d. The other 5 geldings were assigned to the control group (CON) and received the carrier as a placebo at the same time as the LA supplement.

Blood samples were collected by venous puncture in 10 mL sodium heparin-containing vacutainer tubes (Becton Dickinson and Company, Franklin Lakes, NJ) prior to supplementation (0 d), after 7 and 14 d of supplementation, and 48 h after termination of the supplement (16 d). Blood samples were taken at 1300 h and immediately processed into red blood cell, white blood cell, and plasma aliquots.

For assays using erythrocyte lysate, 500 μ L of whole blood was transferred into a microcentrifuge tube and centrifuged at 2500 x g for 5 min at 4° C. The plasma was removed and discarded from the sample. Erythrocytes were resuspended in 1 mL ice-cold deionized water, then frozen at -80° C until analysis. For the determinations using white blood cells, the buffy coat, located at the interface of the red cell pellet and the plasma 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 the red blood cells, and then washed twice in Hank's Balanced Salt Solution (HBSS,³ Life Technologies, Carlsbad, CA). After the last wash, 1 mL HBSS was added to the pellet 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 min at 4° C, then transferring the plasma to micro-tubes, which were frozen at -80° C until sample analysis.

Red blood cell lysate and white blood cells were analyzed for total glutathione (GSHt; Oxis Health Products, Inc., Portland, OR, Biotech GSH-420, kit #51023; inter-assay CV 7.0 %, intra-assay CV 5.6%) and glutathione peroxidase [(GPx; Oxis Health Products, Inc., Portland, OR, Biotech GPx-340, kit #51017; inter-assay CV 4.2 %, intra-assay CV 5.0 %) using an OxyScan™ Automated Oxidative Stress Analyzer (Oxis Health Products, Inc., Portland, OR)]. Total plasma lipid hydroperoxides (LPO; Oxis Health Products, Inc., Portland, OR, Biotech LPO-560, kit #21025) were also analyzed using a spectrophotometer (inter-assay CV 3.0 %, intra-assay CV 4.6 %).

Data were summarized as means \pm SEs. Baseline values for the two groups were compared by Student's t-test and when different, were subtracted from values at later times (this assumes a covariance of 1). The effects of time, treatment and time by treatment

interaction were evaluated on the raw data or increments by analysis of variance with repeated measures using the general linear models procedures of SAS (SAS, 1999). Significance was inferred at $P < 0.05$.

Results

An experienced veterinarian observed no adverse clinical signs of toxicity, including gastrointestinal distress, in the group supplemented with LA. Total plasma LPO (**Figure 1**) was $9.32 \pm 0.78 \mu\text{M}$ for CON and $13.56 \pm 0.45 \mu\text{M}$ for LA at baseline ($P = 0.0016$). The concentrations decreased over time in the LA group ($P = 0.015$) and was lower in the LA group than the CON group at 7 and 14 d ($P = 0.022$ and 0.0002 , respectively). There was also an effect of treatment and time by treatment interaction ($P = 0.031$) from 7 to 16 d. Area under the LPO curve was found to be greater ($P = 0.004$) for the LA group ($96.9 \pm 15.7 \mu\text{M} \cdot \text{d}$) as compared to the CON group ($28.2 \pm 7.1 \mu\text{M} \cdot \text{d}$). At baseline, GSHt concentration was 69 ± 7 and $115 \pm 13 \text{ mmol/g protein}$ in the white and red blood cells, respectively. In the LA group white blood cell GSHt was higher at 14 d than 7 d ($P = 0.020$). Red blood cell GSHt tended to increase with time ($P = 0.074$), and white blood cell GSHt increased with time ($P = 0.017$) and tended to increase with treatment ($P = 0.071$). The GPx activity in the white blood cells (**Figure 2**) was 50.6 ± 1.6 for CON and $43.6 \pm 0.33 \text{ mmol/g protein}$ for LA at baseline ($P = 0.041$). The concentrations were higher in the LA group at 14 ($P = 0.019$) and 16 d ($P = 0.013$). The red blood cell GPx showed no differences for time or treatment.

Discussion

The results showed that LA had antioxidant effects on horses, similar to that shown in other species. Our study raises the possibility that LA may have potential benefits in horses during periods of oxidative stress, such as endurance exercise (Hargreaves et al., 2001). Oral supplementation of LA at 10 mg/kg daily did not result in any adverse signs over a 14 d period.

The dose of 10 mg/kg body weight was chosen by referring to LA's effectiveness in other species. It is effective in the rat at a dose of 10 to 100 mg/kg or about 40 to 200 mg/kg^{0.75} (Khanna et al., 1999; Hagen et al., 1999) and in humans at a dose of 600 to 1200 mg (about 8.5 to 17 mg/kg) or about 25 to 50 mg/kg^{0.75} (Ziegler et al., 1995). It caused gastrointestinal distress, however, at the high dosage (Ziegler et al., 1995). Our hypothesis was that a dose of 10 mg/kg or about 50 mg/kg^{0.75} would diminish oxidative stress in horses after being supplemented for 14 d, which is the average duration studied in other species, without causing any clinical signs of toxicity.

In humans LA was used in 260 non-insulin-dependant diabetic patients to determine if it relieved symptoms of diabetic neuropathy including pain, burning, paralysis and numbness (Ziegler et al., 1995). Three different doses of LA were compared to a placebo over a period of 19 d. Patients were tested and assigned a total symptom score by a physician three times during this period for improvements in neuropathic symptoms caused by the diabetes. After 5 d, patients dosed with 1200, 600 and 100 mg LA had a greater decrease ($P < 0.01$) in total symptom score than with the placebo, and after 12 and 19 d the decrease was found in the 1200 and 600 mg dosed patients.

Total plasma LPO may be regarded as a cumulative index of antioxidant status. Even after the 7 d of dosing with LA there was a further decrease in LPO indicating a lower oxidative stress level or higher antioxidant status. In 48 h after the termination of the LA supplement plasma LPO in the LA and CON groups were similar. Other studies using malondialdehyde or thiobarbituric acid reactive substances (indicators of lipid peroxidation) found that after intense, exhaustive exercise lipid peroxidation was lower in the liver ($P < 0.05$), muscle ($P < 0.05$) and heart ($P < 0.001$) tissue of rats supplemented with 100 mg/kg LA for 3 wk (Khanna et al., 1999). In aged rats malondialdehyde was five times higher than in young rats, however with LA supplementation these levels were significantly reduced in the old rats (Hagen et al., 1999).

A series of studies focusing on the LA interaction with GSH performed by Han et al. (1995; 1997) stimulated our interest in this same interaction in red and white blood cells. In vitro, human T-lymphocyte cells, bathed in various concentrations of LA, increased the intracellular level of GSH; this increase had a positive correlation to the

concentration of LA added to the medium (Han et al., 1995). When 100 µM LA was added, GSH increased 45.8 % over the control group after 24 h, and DHLA and GSH levels were positively correlated ($r = 0.57$, $P < 0.01$). This result suggests that DHLA is a contributing factor to the GSH increase after the addition of LA.

In our study, however, the GSht concentration might have changed transiently in response to LA then reverted to normal values after 24 h when the next blood sample was taken. Lipoic acid is rapidly converted to DHLA once inside the cell; it is then transported out of the cell and used as an antioxidant (Packer et al., 1995). Dihydrolipoic acid is very reactive and rapidly used so the effects on GSht may not be long lasting. Further studies are planned to see if the same dose of 10 mg/kg LA would have antioxidant effects on antioxidant status and oxidative stress from immediately after supplementation up to 48 h post-supplementation.

In our study, the decrease in white blood cell GPx activity in the CON group, and slightly less marked in the LA group may be due to the weather conditions. During the first week of the study (mid January) the temperature was mild with sunny conditions, into the second week of the study, for the last two sample times, temperatures dropped to near freezing and precipitation increased to freezing rain/snow. It is possible that the weather played a role in level of oxidative stress in these geldings, which are housed outside 24 h a day. This environmental stress would increase activity level and oxygen consumption, thus reactive oxygen species and oxidative stress and explain why the LA group had less of a change in the GPx activity compared to the CON group.

Other studies have used GPx as well as other antioxidant enzymes to measure the effects of oxidative stress. Superoxide dismutase, catalase, and GPx, along with a few non-enzymatic antioxidants, were used to determine if LA decreased lipid peroxidation and increased antioxidant status in the mitochondria of aged rats (Arivazhagan et al., 2001). They found that with LA supplementation there was a lower level of lipid peroxidation and increased level of antioxidant action in the mitochondria, as determined by enzymatic and non-enzymatic antioxidants. In horses, Ji et al. (1990) found that superoxide dismutase, catalase, glutathione reductase, and GPx were numerically increased with an exercise protocol of 12 min of trotting at 4.5 m/s on a grade of 11 %, however, these values were

not significant. They also found that after supplementing with vitamin E before exercise these oxidative stress measures were also unchanged. This could possibly be because these horses were conditioned to the protocol and the amount of work was not great enough to induce oxidative stress.

Implications

The supplementation of α -lipoic acid has been shown to increase antioxidant status and decrease oxidative stress in the horse and in other species. It favors regeneration of antioxidants including vitamin E and C, and glutathione, and thus decreases the need for their supplementation. The present findings encourage testing of lipoic acid supplementation in horses subjected to oxidative stress, for example, during strenuous exercise and prolonged transport.

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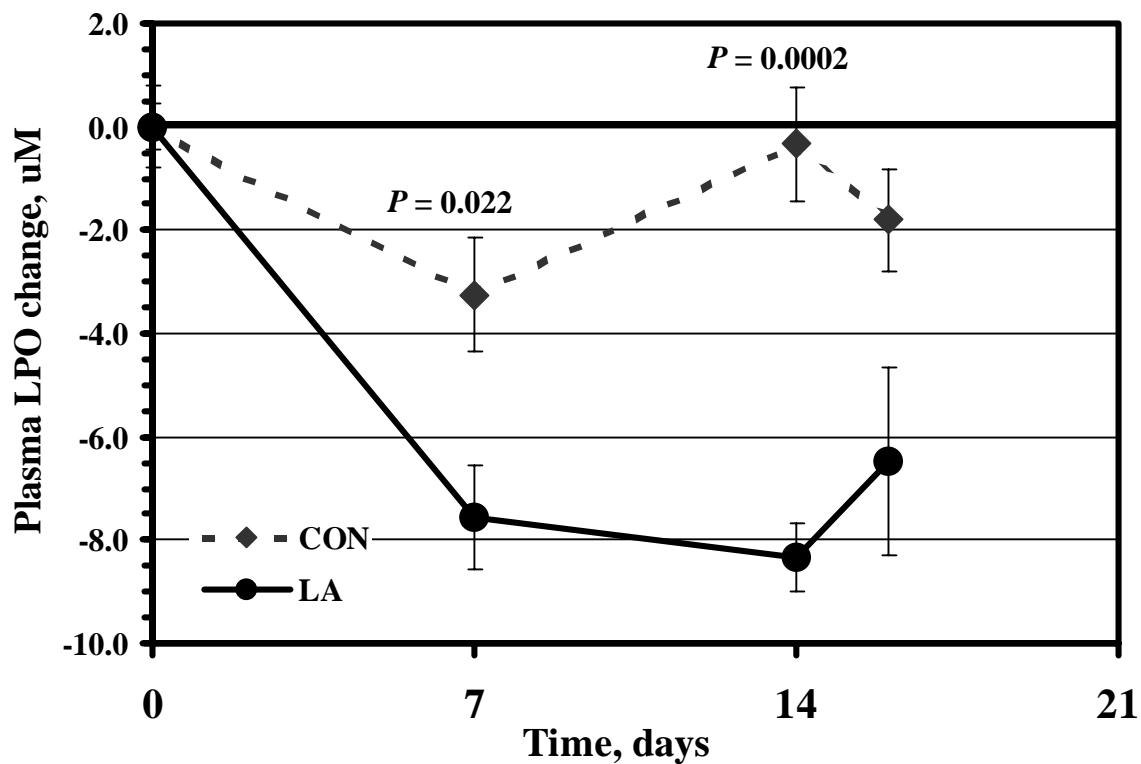


Figure 1. Total plasma lipid hydroperoxides (LPO) for horses receiving the control supplement (CON; n = 5) or lipoic acid-containing supplement (LA; n = 5). The LPO baselines are subtracted from 7, 14 and 16 d samples assuming a covariance of 1. Results for ANOVA found time ($P = 0.015$), treatment and time by treatment interaction ($P = 0.031$) significant. Data are shown as mean \pm SE.

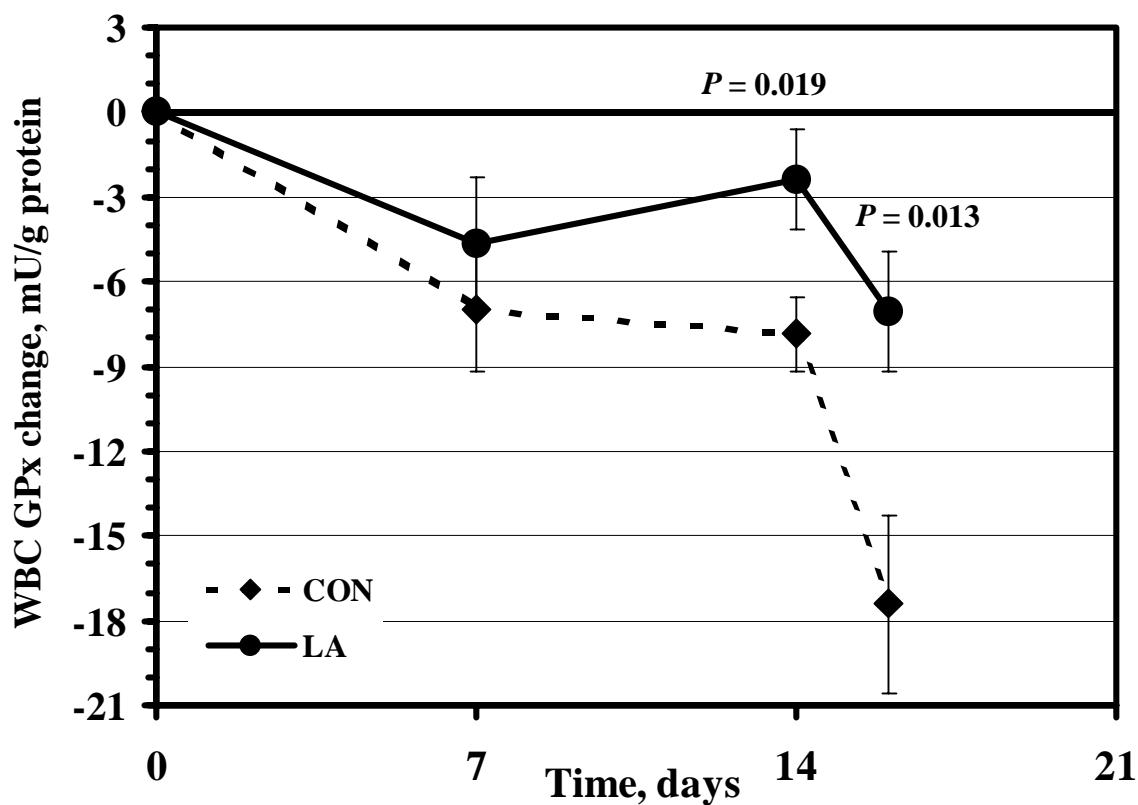


Figure 2. White blood cell (WBC) glutathione peroxidase (GPx) for horses receiving the control supplement (CON; n = 5) or lipoic acid-containing supplement (LA; n = 5). The white blood cell GPx baselines are subtracted from 7, 14 and 16 d samples assuming a covariance of 1. Data are shown as mean \pm SE.