Antioxidant status and muscle cell leakage during endurance exercise

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Summary

Antioxidant status of 35 endurance horses was studied during an 80 (OD80) or 160 km (OD160) race. Packed cell volume (PCV), total plasma protein (TPP), plasma ascorbic acid (VIT C), plasma α-tocopherol (VIT E) and erythrocyte glutathione (GSH) concentrations, erythrocyte glutathione peroxidase (GPX), plasma aspartate aminotransferase (AST) and plasma creatine kinase (CK) activities were measured at 0, 40, 80 km and 60 min of recovery (REC) at OD80, and 0, 64, 106, 142, 160 km and REC at OD160. In both races, no changes were found in plasma VIT E concentration, but VIT C and GSH concentrations decreased (P<0.05), and mean GPX, AST and CK activities increased from 0 km (P<0.05). Indices of muscle cell leakage (plasma AST and CK) were correlated (r = 0.36 to 0.67; P<0.03) with indices of antioxidant status (VIT C, GSH and GPX). Associations between increased muscle leakage and decreased antioxidant status may, in part, reflect oxidative stress and suggest the testing of antioxidant supplements in endurance horses to improve performance and welfare.

Introduction

Public interest in animal welfare and competitive interest in equine performance converge on muscle damage and oxidative stress. Increased oxygen consumption during strenuous exercise provides a metabolic advantage for energy production, but paradoxically causes oxidative injury to muscle cells. Oxygen consumption during exercise may increase 10 to 20 times in man, and 30 times in horses (Butler et al. 1993). Oxygen flux through muscle fibres may increase 100 times from rest to maximal exercise (Milnor 1980). Reactive oxygen species (ROS), produced by oxidative reactions, and free radicals, which are molecules that contain an unpaired electron, damage important cellular components resulting in the loss of cellular function (Sjodin et al. 1990; Sen 1999). Free radicals formed during oxygen reduction can trigger a chain reaction of lipid peroxidation so that membrane-bound enzyme and receptor function is lost (Halliwell and Gutteridge 1986). Prolonged strenuous exercise increases the production of free radicals and ROS and may overwhelm antioxidant defences, resulting in oxidative stress. If antioxidant systems become depleted during an exercise bout, the susceptibility of cells and tissues to ROS damage is enhanced. Exercise-induced oxidative damage to cell membranes contributes to muscle damage, fatigue and several pathological conditions (Sjodin et al. 1990; Sen 1999).

Under nonstressed physiological conditions, extensive enzymatic and nonenzymatic antioxidant defence systems in muscle tissue allow effective scavenging of free radicals and ROS before cellular components are damaged. Antioxidants are positioned in specific cellular locations to facilitate a comprehensive protection against oxidant stress. Glutathione peroxidase (GPX), an important enzymatic antioxidant located in both mitochondria and cytosol, removes hydrogen peroxide and organic hydroperoxides. Important nonenzymatic antioxidants include vitamin E, vitamin C and glutathione. Vitamin E (VIT E) is a lipid-soluble, chain-breaking, radical scavenger located in cell membranes. Vitamin C (VIT C) scavenges radicals and recycles VIT E, and is located in the aqueous phase of cells. Erythrocyte glutathione (GSH) is an abundant cellular nonprotein thiol, with multiple antioxidant functions including donating hydrogen atoms to hydroxyl radicals, and removing hydrogen and lipid peroxides in conjunction with glutathione peroxidase (Ji 1995). Glutathione is also postulated to reduce tocopheroxyl radicals of VIT E and is implicated in the reduction of VIT C semidehydroascorbate radicals during VIT E recycling (Packer 1991).

Studies investigating relationships between physical exercises, oxidative stress and antioxidant capacity have been conducted without antioxidant supplementation on Maremmana racehorses (Chiaradia et al. 1998) and Thoroughbred racehorses (Ishida et al. 1999). After 3 months of training, Maremmana racehorses completed a series of exercise tests of increasing intensity. Increased concentrations of malondialdehyde (MDA), an indicator of lipid peroxidation, and plasma glutathione were observed after short duration exercise, and no changes in creatine kinase (CK) activities were observed (Chiaradia et al. 1998). Blood samples collected from Thoroughbred racehorses before and after a race revealed increased lipid peroxide concentrations and superoxide dismutase (SOD) activities (Ishida et al. 1999). Maremmana racehorses were supplemented with vitamin E and selenium and completed a series of exercise tests before and after 70 days training. Horses receiving dietary supplements and training had increased antioxidant defences, demonstrated by increased resistance to erythrocyte peroxidative stress and

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increased GPX activity in lymphocytes, measured in vitro, and decreased plasma MDA concentrations (Avellini et al. 1999). Erythrocyte GPX activity decreased and plasma lipid peroxide concentrations increased in Thoroughbred racehorses during sprint exercise (Ono et al. 1990). When horses were supplemented i.v. with selenium and vitamin E, lipid peroxide concentrations decreased and GPX activity decreases were reduced. Thoroughbred racehorses supplemented with i.v. vitamin C before a race had reduced lipid peroxidation, measured by thiobarbiturite reactive substances (TBARS) after racing. Plasma ascorbate concentrations were unchanged after the race and plasma CK activity increases were similar in supplemented and nonsupplemented horses (White et al. 2001).

The antioxidative status of 5 endurance horses was assessed during a 160 km race (Frankiewicz-Jozko and Szarska 2000). Systemic measurements revealed increased lipid peroxidation measured by TBARS, increased plasma CK activity, decreased total antioxidant status and increased activity of enzymatic antioxidants GPX, SOD and glutathione reductase. Nenzyemtatic antioxidant status, hydration status or changes in bodyweight were not reported. The antioxidant status of 40 horses competing in a 140 km endurance race was studied (Marlin et al. 2002). Blood samples were collected before, immediately after the race and after 16 h recovery. Plasma antioxidants maintained during the race (vitamins C and E), but vitamin C decreased during recovery. Total erythrocyte GSH concentrations increased during the race and recovery.

We propose that the antioxidant defences of endurance horses are severely tested during prolonged and strenuous endurance exercise. The objectives of this study were to determine the antioxidant status and muscle cell leakage of horses during an 80 and 160 km endurance race, and to evaluate the relationship of muscle cell leakage to changes in antioxidant status.

Materials and methods

Endurance races

Horses were studied during the Old Dominion 80 km (OD80) and 160 km (OD160) endurance races in the Blue Ridge Mountains near Front Royal, Virginia in June 2000. The OD80 and OD160 races must be completed in 12 and 24 h, respectively. Mean ambient temperature was 28°C with a range of 18 to 34°C over the 24 h period.
were vortexed for 1 min, placed on a shaker plate for 5 min, followed by centrifugation at 1000 g for 30 to 60 s. The clear top layer containing fat-soluble vitamins removed by Pasteur pipette into a separate tube and dried down over N2. Dried samples were resuspended in 100 µl/ml ethanol, vortexed and placed in autosampler vials, and 40 µl/ml aliquots were injected. A mobile phase of 99% methanol and 1% H2O at a flow rate of 1 ml/min was used. Aliquots were chromatographed using a Microsorb MV 100 Ångstrom pore, reverse phase C18 column and detected at 292 nm.

Erythrocyte GSH concentration and GPX activities were determined in duplicate using Biorad GSH-420 and GPx-340 colorimetric assays, respectively. Plasma aspartate aminotransferase (AST) and CK activities were determined in duplicate using a Beckman CX5 chemical analyzer.

Statistical analyses

Data are summarised as mean ± s.e. Antioxidant variables were adjusted for exercise-induced changes in plasma volume at time points where TPP concentration was significantly increased. Changes with time were evaluated with ANOVA, and a post hoc Fisher’s protected LSD test was performed to test for differences between means (Anon 1999). Logarithmic transformations were applied to CK data, which were not normally distributed. Only data from horses that completed the races were used. Data from OD80 and OD160 were not statistically compared. Simple regressions (y = a + bx) of indices of muscle leakage (y; CK and AST) on indices of antioxidant status (x; VIT E, VIT C, GSH, GPX) were performed using pooled data, because ANOVA revealed that horse had no effect on these variables.

Results

Twelve of 18 horses finished OD80, and 10 of 17 horses finished OD160. Of the nonfinishers of OD80, one was a rider option and 5 were because of lameness. In OD160, 4 of the nonfinishers were because of lameness, one due to a sore back, one rider option and one because of metabolic problems. Mean race time was 9.10 h (range 7.38 to 10.23 h) and mean speed was 8.8 km/h in OD80. Mean race time was 9.43 h (range 18.01–20.54 h) and mean speed was 8.2 km/h in OD160. Mean prerace bwt was 453 ± 12 kg and postrace bwt was 421 ± 12 kg in OD80. Mean prerace bwt was 446 ± 8 kg and postrace bwt was 425 ± 10 kg in OD160.

In OD80, mean PCV and TPP concentrations at 0 km were 41 ± 1% and 57 ± 1 gl, respectively, and no changes in PCV (P = 0.90) or TPP (P = 0.08) were found at 40, 80 km or REC (Table 1). Plasma VIT E concentration at 0 km was 5.0 ± 0.4 µg/ml and no change (P = 0.95) was found at 40, 80 km or REC (Fig 1). Plasma VIT C concentration at 0 km was 4.6 ± 0.1 µg/ml and decreased (P = 0.002) by 15% at REC (Fig 2). Mean erythrocyte GSH concentration at 0 km was 171 ± 29 µmol/g and decreased (P = 0.0001) by 59% at REC (Fig 3). Mean erythrocyte GPX activity at 0 km was 7.5 ± 0.9 µA/mg and increased (P = 0.01) by 185% at 80 km (Fig 4). Mean plasma AST activity at 0 km was 280 ± 14 IU/l and increased (P = 0.01) by 26% at 80 km, and mean CK activity at 0 km was 277 ± 36 IU/l and increased (P = 0.01) by 121% at 80 km (Figs 5, 6). One horse was >3 s.d. from the mean of all other horses and was removed from the analysis as an outlier. Plasma AST activities were correlated with erythrocyte GSH concentrations and GPX activities, but not with plasma VIT E concentrations. Plasma CK activities were correlated with plasma VIT C and erythrocyte GSH concentrations, and erythrocyte GPX activities. The regressions are summarised in Table 3.
In OD160, mean PCV at 0 km was 38 ± 1% and increased (P = 0.04) by 13% at 64 km and 16% at 106 km (Table 2). Mean TPP concentration at 0 km was 60 ± 1 g/l and increased (P<0.0001) by 8% at 64 km and 6% at 106 km (Table 2).

Plasma VIT E concentration at 0 km was 5.6 ± 0.5 µg/ml and no change (P = 0.95) was found at 64, 106, 142, 160 km or REC (Fig 1). One horse was 5 to 7 s.d. from the mean of all other horses, hence removed from the analysis as an outlier. Plasma VIT C concentration at 0 km was 4.4 ± 0.2 µg/ml and decreased (P<0.0001) by 23% at REC (Fig 2). Mean erythrocyte GSH concentration at 0 km was 135 ± 14 µmol/g and decreased (P<0.0001) by 84% at REC (Fig 3). Mean erythrocyte GPX activity at 0 km were 7 ± 1 µM/mg and increased (P = 0.003) by 214% at 160 km (Fig 4). Mean plasma AST activity at 0 km was 294 ± 13 iu/l and increased (P = 0.0003) by 46% at REC, and mean plasma CK activity at 0 km was 237 ± 20 iu/l and increased (P = 0.02) by 327% at 160 km (Figs 5 and 6a). Plasma CK activity was especially increased during exercise in 1 horse during OD80 and 2 horses during OD160. Two horses were >3 s.d. from the mean of all other horses and were removed from the analysis as outliers (Fig 6b).

Plasma AST activities were correlated with plasma VIT C, erythrocyte GSH concentrations and GPX activities, but not with plasma VIT E concentrations. Plasma CK activities were correlated with erythrocyte GSH concentrations. The regressions are summarised in Table 3.

**Discussion**

Evaluating the antioxidant status and muscle cell enzyme leakage of endurance horses competing in the OD80 and OD160 provides valuable information on the ability of horses to cope with oxidative stress. The results reveal associations between muscle cell leakage and diminished antioxidative status in endurance horses. An unexpected finding was the maintenance of plasma α-tocopherol concentration during both endurance races.

Hydration status was maintained in general, except during OD160 at the 64 and 106 km sample collection points (Table 2). These points were between ~10:00 and 14:00 h and between ~14:00 and 20:30 h, respectively. Ambient conditions during these periods were ~32–34°C and may have contributed to the increases in these hydration variables. Further increases in PCV and TPP were not found at the remaining sample collection points during the night.

Plasma VIT E concentrations were maintained during both OD80 and OD160, and similar results were found in an 80 km race over the same terrain conducted 2 months previously (Hargreaves et al. 2002). Similarly, horses competing in a 140 km endurance race had no changes in α-tocopherol concentrations during the race and during 16 h of recovery (Marlin et al. 2002). Maintenance of circulating α-tocopherol may be explained by the concomitant mobilisation of VIT E by fatty acids from adipose tissue stores (Rokitki et al. 1994a), especially as fat is a major energy source during endurance exercise. Plasma VIT C concentrations decreased during both races, presumably through radical scavenging and regeneration of VIT E by GSH (Meister 1994; Winkler et al. 1994). Consequently, concurrent decreases in VIT C and GSH during prolonged strenuous endurance exercise may serve to sustain circulating VIT E. Furthermore, this 'sparing effect' of VIT E may have attenuated muscle CK leakage during the races, as reported in man after intense endurance training (Rokitki et al. 1994b; Itoh et al. 2000), since many horses in OD80 and OD160 had only minimal changes in circulating CK levels during the race. The high plasma CK activities observed in 3 horses (Fig 6b) were not associated with clinically evident exertional rhabdomyolysis, according to the veterinarians' records, but may reflect underlying susceptibility to this disorder.

Mean erythrocyte GPX activities increased during both OD80 and OD160, reflecting the high demand on the antioxidant system to scavenge free radicals and ROS. Similarly, GPX activities increased in horses during a 160 km endurance race in Poland.

### TABLE 3: Regressions (y = a + bx) of indices of muscle leakage (y) on indices of antioxidant status (x) during the 80 and 160 km endurance races

<table>
<thead>
<tr>
<th>Race</th>
<th>Muscle</th>
<th>Stress</th>
<th>n</th>
<th>a</th>
<th>b</th>
<th>r</th>
<th>P</th>
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<tbody>
<tr>
<td>OD160</td>
<td>AST VIT C 28 518.4 -42.89 0.44 0.0194</td>
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<tr>
<td>AST GSH 38 390.8 -0.48 0.44 0.0056</td>
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<tr>
<td>AST GPX 32 310.9 +4.18 0.48 0.0055</td>
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<tr>
<td>CK* GSH 42 6.4 -0.005 0.55 0.0009</td>
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<tr>
<td>OD80</td>
<td>AST VIT C 35 324.4 -0.27 0.46 0.0060</td>
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<tr>
<td>AST GPX 28 271.0 +1.60 0.42 0.0276</td>
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<tr>
<td>CK* VIT C 38 7.4 -0.37 0.40 0.0140</td>
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<tr>
<td>CK* GSH 38 6.1 -0.002 0.36 0.0268</td>
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<tr>
<td>CK* GPX 30 5.4 +0.03 0.67 &lt;0.0001</td>
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*Data for CK are logarithmically transformed.

### TABLE 2: Packed cell volume (PCV) and total plasma protein (TPP) concentration at 0, 64, 106, 142, 160 km and after 60 min recovery (REC) during the 160 km endurance race

<table>
<thead>
<tr>
<th>Distance (km)</th>
<th>0</th>
<th>64</th>
<th>106</th>
<th>142</th>
<th>160</th>
<th>REC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV (%)*</td>
<td>38 ± 1.4</td>
<td>43± ± 1.4</td>
<td>44± ± 2.5</td>
<td>39 ± 1.5</td>
<td>38 ± 1.3</td>
<td>39 ± 1.6</td>
</tr>
<tr>
<td>TPP (g/l)*</td>
<td>60 ± 1</td>
<td>63± ± 1</td>
<td>63± ± 1</td>
<td>58 ± 1</td>
<td>59 ± 1</td>
<td>59 ± 1</td>
</tr>
</tbody>
</table>

*Mean ± s.e. (n = 10); *Mean PCV (P = 0.04) and TPP (P<0.0001) concentrations are different from values within same row.

**Fig 5:** Plasma aspartate aminotransferase (iu/l) activity for horses that completed the 80 km (n = 12) endurance race (OD80, black bars) at 0, 40, 80 km and 60 min recovery (REC), and 160 km (n = 10) endurance race (OD160, hatched bars) at 0, 64, 106, 142, 160 km and REC. Bars are means, flags are s.e. of mean. Means within a race with unlike letters are significantly different (P<0.05).
Exercise-induced increases in plasma creatine kinase (CK) activities during short duration intense exercise (Chiaradia et al. 1995). The large variability in plasma CK activity remained within the normal range for horses at rest (150–400 iu/l). In this study, elevated plasma AST and CK activities demonstrated muscle cell leakage and plasma antioxidant status. Interestingly, 5 of the 10 horses that completed the OD160 received 20 g/day of ascorbic acid 3 days prior to the start, but no differences were found between supplemented and nonsupplemented groups in plasma ascorbate concentration, in other plasma or erythrocyte antioxidant concentrations or muscle cell enzyme activities, before or during the race. Associations between increased muscle cell leakage and decreased antioxidant status emphasise the demands on the delicate oxidative-antioxidative balance and encourage the testing of antioxidant supplements, especially ascorbic acid supplementation during the race, to improve the performance and welfare of endurance horses.

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Manufacturers’ addresses

1 Alwights Hamilton Scale Corp., Richmond, Virginia, USA.
2 Becton Dickinson and Co., Rutherford, New Jersey, USA.
3 Baker Instruments Corporation, Pennsylvania, USA.
4 Varian Instruments Inc., Walnut Creek, California, USA.
References


