Comparison of oxidative stress and antioxidant status in endurance horses in three 80-km races

CA Williams1,*, DS Kronfeld1, TM Hess1, KE Saker2, JE Waldron3, KM Crandell1 and PA Harris4

1Department of Animal and Poultry Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
2Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA 24061, USA
3Rectortown Equine Clinic, Rectortown, VA 20118, USA
4Equine Studies Group, WALTHAM Centre for Pet Nutrition, Melton Mowbray, UK

*Corresponding author: Rutgers, The State University of New Jersey, Cook Campus, 84 Lipman Dr., New Brunswick, NJ 08901. cwilliams@aesop.rutgers.edu

Submitted 21 October 2004: Accepted 12 July 2005 Research Paper

Abstract

This study tested our hypothesis that during an 80-km Research Ride in 2002 (R2) horses that did not finish (NF) the ride would have elevated muscle enzyme activities in the blood and changes in biomarkers of oxidative stress as compared to horses that finished (F) the ride. These results were then compared to previous rides – Old Dominion (OD) and the Research Ride 2001 (R1). For R2, 40 mostly Arabian horses competed and had blood samples collected before, at 27, 48 and 80 km, and 170 to 190 min after the 80-km race. Blood was collected similarly in R1 and OD. Blood was analysed for plasma lipid hydroperoxides (LPO), α-tocopherol (TOC), creatine kinase (CK), aspartate aminotransferase (AST), red and white blood cell total glutathione (GSH-T) and glutathione peroxidase (GPx). Data were analysed using a repeated measure ANOVA in SAS. Associations between muscle enzymes and antioxidant status were determined using Pearson’s or Spearman’s correlations. Activities of CK and AST were higher \( (P < 0.05) \) before, during and after the ride in NF than in F; however, TOC, LPO, GSH-T and GPx were not different. In R2, negative correlations were found with GPx and CK \( (r = -0.21; P = 0.005) \), GPx and AST \( (r = -0.15; P = 0.05) \), and a positive correlation was found with GSH-T and CK \( (r = 0.18; P = 0.02) \). Values of CK, LPO, GPx and GSH-T were higher \( (P < 0.05) \) in R2 than in R1 or OD. The overall comparison of 80-km endurance races suggests the importance of considering the horse’s fitness, terrain, ambient conditions and calibre of race when interpreting results from markers of oxidative stress and muscle enzyme leakage.

Keywords: antioxidant; endurance; equine; muscle enzymes; oxidative stress

Introduction

Oxidative stress occurs when the antioxidant defence system in the body is overwhelmed by reactive oxygen species (ROS). The main source of ROS is generated in the electron transport chain in the mitochondria. 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 antioxidants1. Useful properties of ROS include targeting of bacteria and viruses in lymphocytes during respiratory bursts, and serving as special messengers within neurons. 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 a certain diseases (e.g., cancer, AIDS and Alzheimer’s disease) and increases during the ageing process and exercise2.
During exercise, 1 to 2% of the oxygen consumed is not completely reduced in the mitochondria and instead forms ROS\(^1\). Because ROS have a short half-life, the only way to directly determine their production is by using spin trapping or electron spin resonance. These methods are expensive and impractical, so other methods of prediction have been employed including antioxidant status (e.g., glutathione, vitamin E and catalase, etc.) or biomarkers of oxidative stress (e.g., lipid hydroperoxides, conjugated dienes and thiobarbituric acid-reactive substances [TBARS], etc.).

A horse’s oxygen uptake increases 30 times above basal during maximal exercise\(^2\); this makes the horse a good model for studying oxidative stress. Also, to this date there has been no known research including data from horses that failed to complete their endurance race. Our hypothesis was that during the 80-km Research Ride 2002 (R2) the horses that did not finish (NF) the race would have elevated muscle enzyme activities and changes in biomarkers of oxidative stress as compared to horses that finished the ride (F). We also hypothesized that horses participating in R2 would have increased oxidative stress and decreased antioxidant status due to differences in terrain, ride difficulty and ambient temperature when compared to Old Dominion (OD)\(^4\) and Research Ride 2001 (R1)\(^5\).

**Materials and methods**

Each ride covered 80 km in northern Virginia, over terrain ranging from 100 to 400 m of elevation. The R1 and R2 rides took place in early April 2001 and 2002, respectively, whereas OD was held in June 2000. Materials and methods for R1 and OD differed in veterinary check mileage, number of horses finishing the race \((n = 34 \text{ and } 11, \text{ respectively})\), competitiveness, terrain, season of the year and ambient temperature; they are detailed in Williams et al.\(^6\) and Hargreaves et al.\(^4\), respectively. The protocols were approved by the Institutional Animal Care and Use Committee and performed at the Middleburg Agricultural Research and Extension Center.

For R2, 40 endurance horses (28 Arabians, 10 part-Arabians, and 2 other breeds) averaging 12.2 ± 0.7 years were used. Riders were asked to complete a pre-competition survey that detailed nutritional management, training regime, performance and medical history. A post-ride survey was also given to the riders rating the ride difficulty. Ambient temperature ranged from 25°C in the morning to 30°C in the afternoon and evening. Veterinary checks were performed according to the American Endurance Ride Conference\(^6\) rules upon arrival the day before the race, and at 27, 48, 72 and 80 km during the race. Blood samples, horse weight without tack, heart rate and rectal temperature were taken the day before the race (PRE), at 27, 48, and 80 km during the race, at the veterinary check where the NF horse was eliminated from the race, and after 170 to 190 min of recovery (REC). Blood samples were immediately placed on ice and transported to the lab. within 15–30 min for division into red blood cells (RBC), white blood cells (WBC) and plasma aliquots. Methods for erythrocyte lysate and white blood cell separation have previously been described\(^5\).

Red blood cell lystate and WBC were analysed for total glutathione (GSH-T; Biotech GSH-420, kit #51023) and glutathione peroxidase (GPx; Biotech GPx-340, kit #51017) using an OxyScan™ Automated Oxidative Stress Analyzer (Oxis Health Products, Inc., Portland, OR). Total plasma lipid hydroperoxides (LPO) were analysed using a spectrophotometer (Bio-tech LPO-560, kit #21025). Plasma α-tocopherol (TOC) was analysed by high-pressure liquid chromatography methods\(^4\). Creatine kinase (CK), aspartate aminotransferase (AST) and ALB were analysed using spectrophotometric assays (Beckman Instruments Inc., Brea, California, USA).

Data were summarized as means ± SE. The effects of distance and completion on the variables tested were evaluated by ANOVA in a mixed model with repeated measures using SAS (SAS Inst. Inc., Cary, NC). Outliers were determined as being >2 SDs 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 or Spearman’s rank order correlations were used to test for an association between CK and AST with other variables. The natural logarithm of plasma CK was used to allow a normal distribution. The horse was included in the model to test for significance: if insignificant then it was removed from the model.

**Results and discussion**

There were 24 horses that finished the ride (F) and 16 non-finishers (NF). The NF horses did not finish for reasons including lameness \((n = 6)\), metabolic problems \((n = 5)\), rider option \((n = 2)\) and other \((n = 3)\). The NF horses had higher plasma CK \((P < 0.05)\) and AST \((P < 0.05)\) activity compared to F horses (Fig. 1). These activities were elevated before, during and after the race. Higher CK activity possibly indicates more muscle damage in NF compared to F horses, which may have contributed to their elimination\(^7\). Enzyme activities were not known for these particular horses prior to the race weekend; however, if they were found to be higher than their ‘normal’ at pre-race this may be a good indication of possible metabolic problems during the race. Plasma
CK activities in the future could be part of a pre-race evaluation of the suitability of the horse to enter that race. By determining the ‘normal’ values for a horse, abnormalities may be discovered by collecting that pre-race blood sample. All other variables measured were similar between F and NF horses.

In R2, distance was associated with increases in LPO, RBC and WBC GSH-T, CK and AST ($P < 0.001$), and decreases in RBC and WBC GPx and plasma TOC ($P < 0.001$). These data would indicate higher oxidative stress and lower antioxidant capacity throughout the race. Increased activities of CK and AST indicate an accumulation of these enzymes in the plasma and greater membrane permeability, allowing them to leak out into circulation towards the end of the ride\textsuperscript{7}. Activities of CK $> 5000$ IU/L were found in 1 of 24 F (4.2%) and 4 of 16 NF (25%). The one horse with high enzyme activities finished the race, and showed no clinical signs of muscle injury.

The decline in RBC GPx was found in other studies and suggests a reduction in enzymatic antioxidant activity in RBC\textsuperscript{8,9}. One study reported a 14% decrease in GPx activity after a 1600 km run in sled dogs over the course of 11 days\textsuperscript{8}. Another study found a 25% decrease in GPx over a 12-week period in Thoroughbred racehorses in training that were not supplemented with an antioxidant mixture\textsuperscript{9}. Even though there are differences in distance and total time between the studies, changes in GPx would still not be attributed to changes in amount in RBC. This is because the turn over time is much greater for RBCs than the total period of sampling; however, it is likely that the GPx changes were in activity level and not direct concentrations of the enzyme.

Mean values for CK were higher in R2 than in R1 or OD in F groups ($P < 0.05$; Fig. 2a). Mean plasma LPO ($P = 0.05$; Fig. 2b), and RBC GPx ($P < 0.01$) and

---

**Fig. 1** Plasma creatine kinase (CK) activity for the horses that finished (F; $n = 24$) versus did not finish (NF; $n = 16$) for each distance in km, the veterinary check the day before the ride (PRE) and the recovery (REC). *Significant difference between groups at each distance ($P < 0.05$)

**Fig. 2** Plasma creatine kinase activity (a; CK), RBC total glutathione content (b; GSH), plasma lipid hydroperoxides (c; LPO) and plasma ascorbate content (d; ASC) for horses completing OD ($n = 11$), R1 ($n = 34$) and R2 ($n = 24$). The x-axis denotes distance in km, the veterinary check the day before the ride (PRE) and the recovery (REC).
GSH-T ($P < 0.001$; Fig. 2c) were also higher in R2. The higher LPO and CK activity observed in R2 could reflect a higher degree of difficulty than the other ride. In the post-ride survey, over 80% of the riders rated R2 easier than other rides later in the competitive season, but more difficult than R1. The OD is a championship ride with 100% experienced horses, which would mean that horses were more likely to have sufficient training and be in suitable condition to compete, whereas R1 and R2 are early in the competition season, perhaps not allowing for proper conditioning prior to the race. These survey results indicate that difficulty of the ride and conditioning or experience of the horses could account for the lower LPO and CK activity in R1 and OD, respectively.

Comparisons of ride correlations between oxidative stress biomarkers, antioxidant status and muscle enzymes in the rides are shown in Table 1. Correlations were observed for RBC GPx and GSH-T, and muscle enzymes in R2 and OD. It is interesting to point out that correlations with GPx and muscle enzymes in R2 and OD are in opposing directions. Reasons for this discrepancy are thought to occur due to the differences in rides described earlier. Correlations between LPO and muscle enzymes found in R1 were not observed in the other ride (R2). However, a group in Poland found a positive correlation with CK and TBARS, another measure of lipid peroxidation. A positive correlation between plasma isoprostanes and log plasma CK was found in racing sled dogs after repeated bouts of endurance exercise. Positive correlations of plasma CK and AST with various measures of oxidative stress and antioxidant status, especially LPO, are consistent with the hypothesis that free radicals produced during exercise cause membrane permeability of muscle cells. A recent study also found muscle enzymes (CK) to be negatively correlated with TOC in Thoroughbreds before supplementation with an antioxidant mixture. This same correlation has been found with other studies in our lab; however, the current study found TOC to be correlated with AST only in R1. Even though most of the correlations presented could be used to point out that measures of oxidative stress and antioxidant status are a poor predictor of muscle enzyme leakage (explaining only about 5% of the variance), the authors feel there was enough evidence in all three endurance races and other studies to warrant this discussion.

In R1 there was a slight increase in ascorbate concentration throughout the ride, whereas in OD it decreased ($P < 0.05$; Fig. 2d). Studies have shown that intense heat and humidity, along with prolonged endurance exercise, cause a greater depletion of antioxidants. Since the horses competing in R1 were not under hot and humid conditions and potentially working at a lower level of intensity (due to lower LPO, CK and AST values along with verbal comparison of the rides from riders), there was no need to use the amount of ASC needed to compete in the OD. Dietary antioxidant supplementation of the horses was not available for the OD, but should also to be taken into consideration when making any conclusions.

In a study using Thoroughbreds in a 1000 m race at maximal intensity, ASC remained the same before and after exercise. In another study, utilizing endurance horses in a competitive race, Marlin et al. found plasma ASC concentrations to increase, decrease or remain the same in individual horses. The group as a whole did not change between pre- and post-race samples; however, after 16 h of recovery ASC concentrations were significantly lower than in both the pre- and post-race samples. The R1 results from the present study are in agreement with these results in that plasma ASC showed a trend to increase after exercise, and began to decline within 20 min of recovery.

These studies confirmed associations between oxidative stress and muscle membrane leakage, which may represent levels of exertion or muscle damage. High plasma creatine kinase and aspartate aminotransferase activity revealed increased muscle membrane permeability before a ride but not necessarily during a race. The overall comparison of 80-km endurance races suggests the importance of considering a horse’s fitness, terrain, ambient conditions and calibre of race when interpreting results from markers of oxidative stress and muscle enzyme leakage.

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>R2</th>
<th>OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>X</td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>CK</td>
<td>LPO 0.22</td>
<td>0.007</td>
<td>$-0.21$</td>
</tr>
<tr>
<td>AST</td>
<td>LPO 0.32</td>
<td>$&lt;0.001$</td>
<td>0.18</td>
</tr>
<tr>
<td>AST</td>
<td>TOC 0.19</td>
<td>0.02</td>
<td>$-0.15$</td>
</tr>
<tr>
<td>AST</td>
<td>ASC 0.18</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Correlations of indices of oxidative stress (X) and muscle membrane leakage (Y) for the Research Ride 2001 (R1; $n = 170$ total points) and 2002 (R2; $n = 120$ total points) and Old Dominion (OD; $n = 44$ total points) ride$^a$

$^a$ Horse was included in the model to test for significance; if insignificant then it was removed from the model. $^b$ CK and AST are transformed logarithmically. $^c$ GPx activity and GSH (total glutathione) content are in RBC.
Oxidative stress and antioxidant status in endurance horses

Acknowledgements

This project was partially supported by the late Paul Mellon, the John Lee Pratt Program in Animal Nutrition and the Waltham Centre for Equine Nutrition and Care (Melton Mowbray, UK). The technical assistance and encouragement of the staff at the Virginia Tech MARE Center is gratefully acknowledged, along with everyone who helped make the Middleburg Research Rides possible.

References