Oxidative Stress in Horses in Three 80 km Races

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ABSTRACT: Oxidative stress and muscle membrane leakage were compared in horses finishing (F; 24) or not finishing (NF; 16) in *Research Ride 2002* (R2). These data were also compared to previous rides—*Old Dominion* (OD; more competitive) and *Research Ride 2001* (R1; less competitive). For R2, blood samples were taken before (PRE), 27, 48 and 80 km, and 170 to 190 min during recovery. Blood was analyzed for plasma lipid hydroperoxides (LPO), α-tocopherol (α-TOC), creatine kinase (CK), aspartate aminotransferase (AST), and red and white blood cell (RBC and WBC, respectively) total glutathione (GSH-T) and glutathione peroxidase (GPx). Activities of CK and AST were higher ($P < 0.05$) before, during and after the ride in NF than in F. In R2, increases were found for RBC and WBC GSH-T, LPO, CK, and AST ($P < 0.001$); decreases for RBC and WBC GPx, and α-TOC ($P < 0.001$) as compared to the PRE sample. Correlations were found between RBC GPx and GSH-T with CK and AST, and these associated oxidative stress and muscle leakage. Values of CK, LPO and RBC GPx and GSH-T were higher ($P < 0.05$) in R2 than R1 or OD. High CK values during and after a race were found in F as well as NF horses. Thus CK was a better indicator of lack of fitness before than during a race. Overall, comparison of races revealed inconsistencies with difficulty and competitiveness, which suggests the need to consider other factors, such as the horse's fitness, terrain and ambient conditions.

Key Words: Antioxidant, Endurance, Equine, Muscle enzymes, Oxidative stress

Introduction

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 lymphocytes, 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 certain diseases (e.g. cancer, AIDS, and Alzheimer's disease) and increases during the aging and exercise.

During exercise, 1 to 2 % of the oxygen consumed is not completely reduced and instead forms ROS (McBride and Kraemer, 1999). 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) or biomarkers oxidative stress (e.g. lipid hydroperoxides, conjugated dienes, and thiobarbituric acid-reactive substances [TBARS]).

A horse's oxygen uptake increases 30 times during maximal exercise (Butler et al., 1993); this makes the horse a good model for studying oxidative stress. Our hypothesis was that during the 80-km *Research Ride 2002* (R2) horses that did not finish (NF) the ride 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; Hargreaves et al., 2002) and *Research Ride 2001* (R1; Williams 2003).

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 or 2001 and 2002, respectively, whereas OD was held in June of 2000. Materials and methods for R1 and OD differed in veterinary check mileage, competiveness, terrain, season of the year, and ambient temperature; they are detailed in Williams et al. (2002) and Hargreaves et al. (2002), 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 yrs 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 (AERC) rules upon arrival the day before the race, and 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), 27, 48, and 80 km during the race, 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 to 30 min for division into red blood cell (RBC), white blood cell (WBC) and plasma aliquots. Methods for erythrocyte lysate and white blood cell separation have been previously described (Williams et al., 2003).

Red blood cell lysate and WBC were analyzed 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 analyzed using a spectrophotometer (Biotech LPO-560, kit #21025). Plasma α-tocopherol (α-TOC) was analyzed by high-pressure liquid chromatography methods (Hargreaves et al., 2002). Plasma α-TOC was the

only measure that showed a difference when adjusted for changes in fluid redistribution during exercise using albumin (ALB). Creatine kinase (CK), aspartate aminotransferase (AST), and ALB were analyzed 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 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 CK and AST with other variables. The natural logarithm of plasma CK was used to allow a normal distribution. 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 (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 (**Figure 1**). All other variables tested were similar between F and NF horses. Higher CK activity possibly indicate more muscle damage in NF compared to F horses, which may have contributed to their elimination (Harris, 1998). Plasma CK activities 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.

Mean values for CK were higher in R2 than in R1 or OD in F groups ($P < 0.05$; **Figure 2A**). Mean plasma LPO ($P = 0.05$; **Figure 2B**), and RBC GPx ($P < 0.01$) and GSH ($P < 0.001$; **Figure 2C**) were also higher in R2. The higher LPO and CK activity observed in R2

could reflect a higher degree of difficulty between the rides. In the post-ride survey, over 80 % of the riders rated R2 easier then 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 in suitable condition to compete, whereas R1 and R2 are early in the competition 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.

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 α-TOC ($P < 0.001$). These data would indicate higher oxidative stress and lower antioxidant capacity. 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 (Harris, 1998). Activities of CK > 5,000 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.

Comparisons of ride effects, as well as correlations between oxidative stress biomarkers, antioxidant status, and muscle enzymes in the rides are shown in **Tables 1** and **2**, respectively. 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 elsewhere. However, a group in Poland found CK correlated with TBARS (Frankiewiez-Jozko and Szarska, 2000). Similar correlations linking lipid peroxidation and muscle enzymes have been found in racing sled dogs (Hinchcliff et al., 2000).

In R1 there was a slight increase in ascorbate concentration throughout the ride, where in OD it decreased ($P < 0.05$; **Figure 2D**). Studies have shown that intense heat and humidity, along with prolonged endurance exercise cause a greater depletion of antioxidants (Mills et al., 1996). Since the horses competing in R1 were not under hot and humid conditions and working at a lower level of intensity, there was no need to use the amount of ASC needed to compete in the OD. Dietary antioxidant supplementation to the individual horses also needs to be taken into consideration.

Implications

These studies confirmed associations between oxidative stress and muscle membrane leakage, which may represent levels of exertion or muscle damage. High plasma CK and AST activity revealed poor fitness before a ride but not necessarily during a race. Comparisons of three races that differed in difficulty and competitiveness revealed inconsistent findings that suggests the need to consider a horse's conditioning, terrain and ambient conditions when interpreting data or coming to conclusions.

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Table 1. Comparisons of changes in oxidative stress measures with distance in the 80-km *Research Ride 2001 and 2002* (R1 and R2, respectively) and *Old Dominion ride* (OD).

	R1	**R2**	**OD**
Month (temp.)	April (5-10° C)	April (25-30° C)	June (25-32° C)
Experienced Horses (%)	~ 60	~ 70	100
LPO	↑	↑	NA[a]
RBC GSH-T	↓	↑	↓
RBC GPx	↑	↓	↑
WBC GSH-T	↑	↓	NA
WBC GPx	↓	↓ (Ex) ↑ (REC)[b]	NA
α-TOC	↔	↓	↔
ASC	↑	NA	↓
CK	↑	↑	↑
AST	↑	↑	↑

[a]NA = Data not available

[b]WBC GPx for R2 decreased during exercise, but increased during recovery

Table 2. Correlations of indices of oxidative stress (X) and muscle membrane leakage (Y) for the Research *Ride 2001 and 2002* (R1 and R2, respectively) and Old Dominion (OD) ride.

R1				R2				OD			
Y	**X**	**R**	**P**	**Y**	**X**	**R**	**P**	**Y**	**X**	**R**	**P**
CK[a]	LPO	0.22	0.007	CK	GPx[b]	-0.21	0.005	CK	ASC	0.40	0.01
AST[a]	LPO	0.32	<0.001	CK	GSH[b]	0.18	0.02	CK	GPx	0.67	<0.001
AST	TOC	0.19	0.02	AST	GPx	-0.15	0.05	CK	GSH	0.36	0.03
AST	ASC	0.18	0.02					AST	GSH	0.45	0.006
								AST	GPx	0.42	0.03

[a]CK and AST are transformed logarithmically.

[b]GPx activity and GSH (total glutathione) content are in RBC.

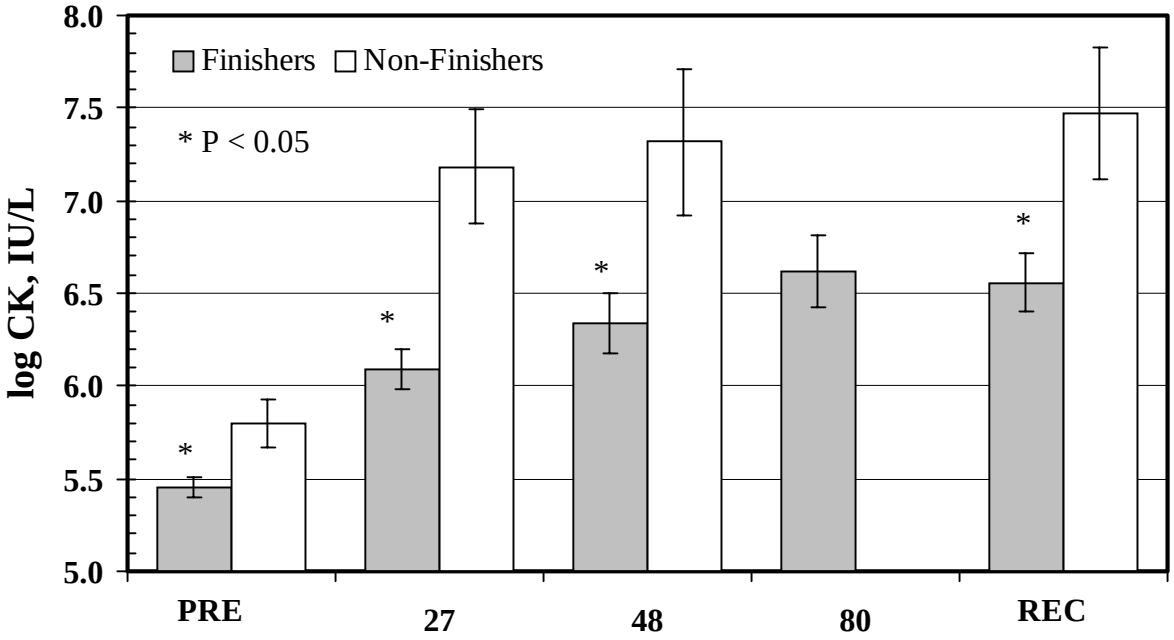
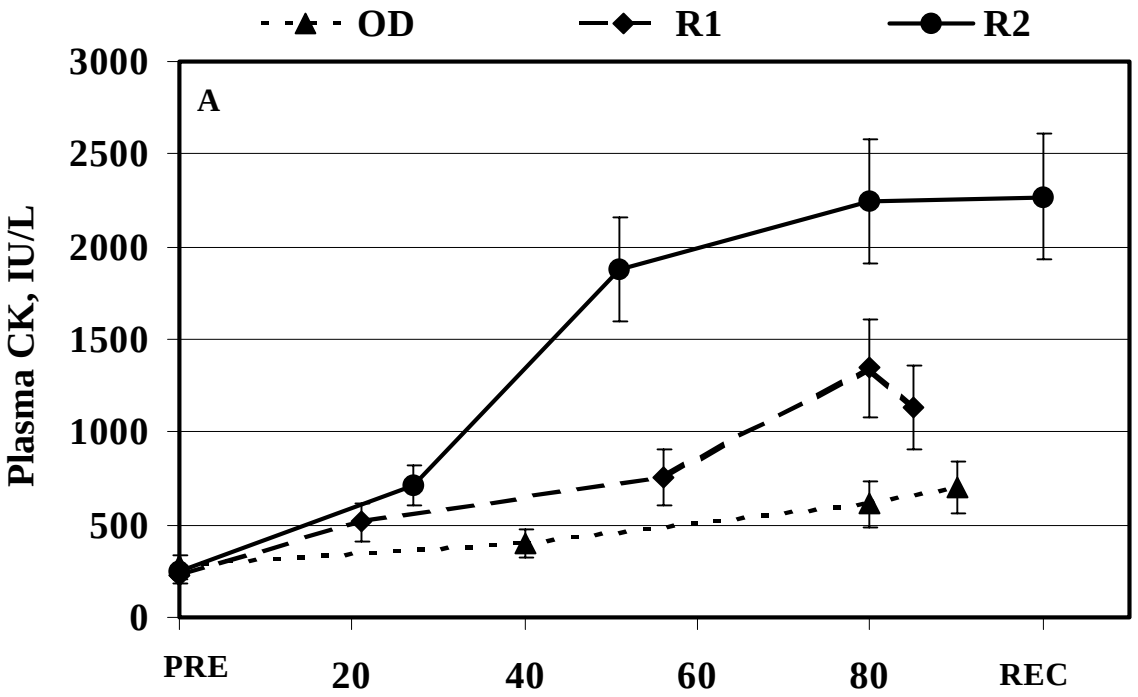
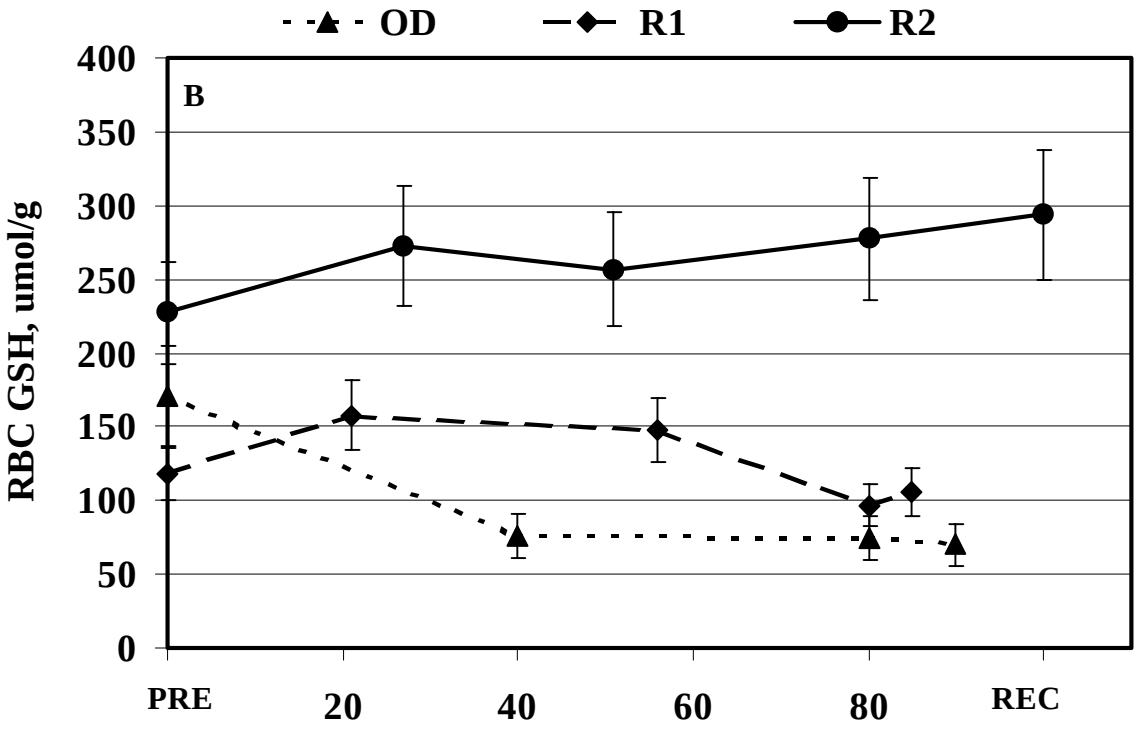


Figure 1. Natural logarithm transform of plasma creatine kinase (CK) activity for the horses that finished (F; n = 24) vs. did not finish (NF; n = 16) for each distance in km, the veterinary check the day before the ride (PRE), and the recovery (REC).


OD
R1
R2
A
Plasma CK, IU/L
3000
2500
2000
1500
1000
500
0
PRE
20
40
60
80
REC


OD
R1
R2
B
RBC GSH, umol/g
400
350
300
250
200
150
100
50
0
PRE
20
40
60
80
REC

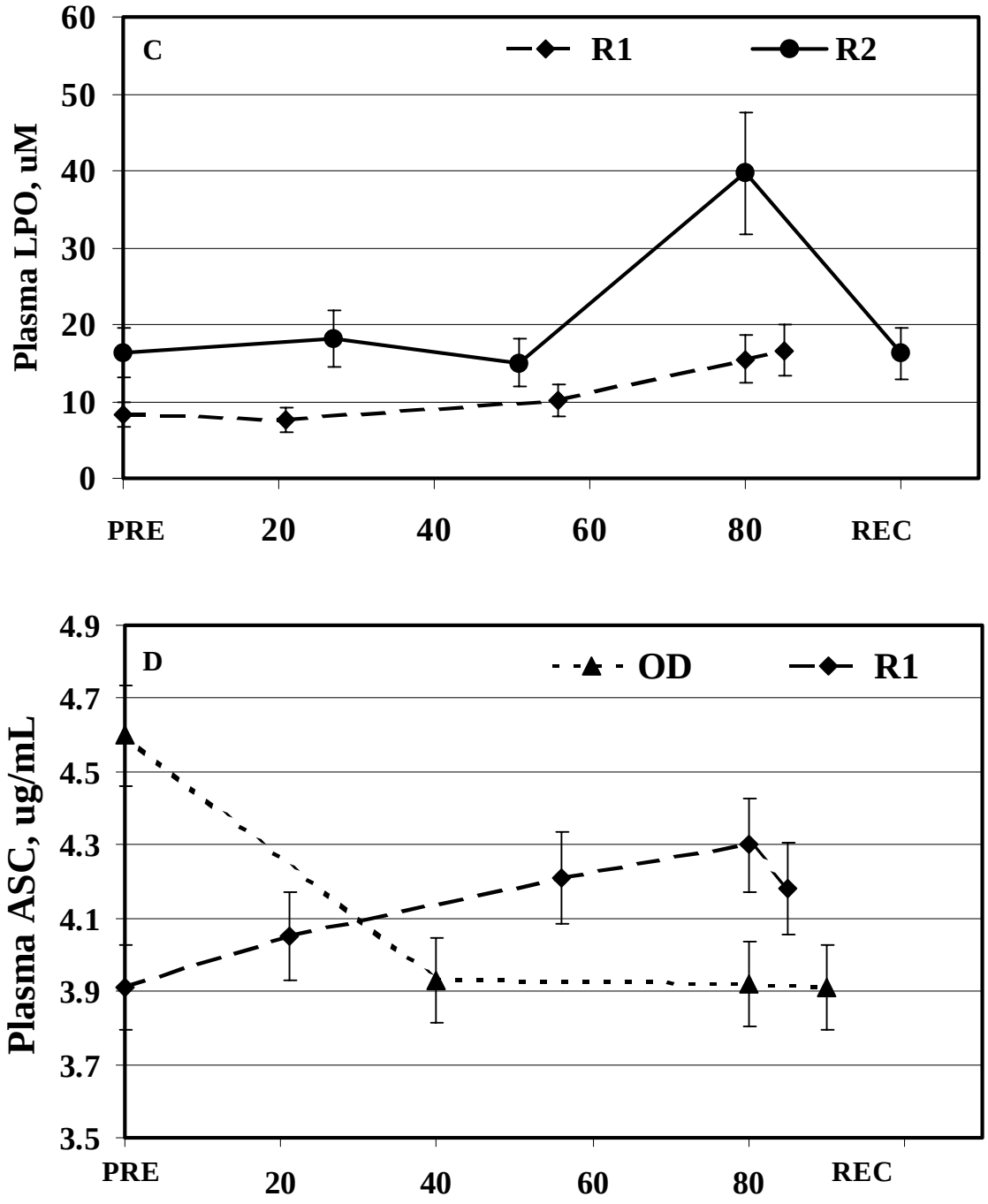


Figure 2. Plasma creatine kinase activity (A; CK), RBC total glutathione content (B; GSH), plasma lipid hydroperoxides (C; LPO), and plasma ascorbate content (C; ASC) during OD, R1, and R2. The x-axis denotes distance in km, the veterinary check the day before the ride (PRE), and the recovery (REC).