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Effect of exercise on blood oxidant/antioxidant markers in Standardbred horses: comparison between treadmill and race track tests

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Summary

Reasons for performing study: **Interest in establishing oxidant/antioxidant profiles in competition horses is increasing. Earlier studies performed in horses have mainly been performed under laboratory conditions using a treadmill and it is not known to what extent laboratory results of oxidant/antioxidant studies might be transposed to field conditions.**

Objective: **To compare the impact on the blood oxidant/antioxidant status of a standardised exercise test including a run up to fatigue performed on a treadmill (TM) and on a racetrack (RT) in healthy and trained Standardbred horses.**

Material and methods: **During TM and RT tests the following blood antioxidant markers were analysed in jugular venous blood at rest and 15 mins (E15) after an intense bout of exercise: uric acid (UA), ascorbic acid (AA), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (reduced: GSH and oxidised: GSSG), glutathione redox ratio (GRR) and protein thiol (PSH). Running time to fatigue (RTF), velocity during the last exercise stage (V_{max}), final heart rate (HR_{final}) and venous lactic acid (LA) were also recorded.**

Results: **V_{max} was significantly ($P<0.05$) higher during the RT, whereas LA was significantly lower. HR_{final} and RTF did not differ significantly between TM and RT. Exercise induced a significant increase (R vs. E15) of UA and AA in both tests, whereas GSH and PSH decreased significantly. GPx, SOD, GSSG and GRR remained unchanged. Differences between TM and RT were significant at E15 for UA, AA and PSH.**

Conclusions: **Comparison of oxidant/antioxidant profiles from laboratory and field studies are difficult to standardise and should be interpreted with caution.**

Potential relevance: **For the same RTF and final HR, the TM induced stronger changes in blood lactate and in blood oxidant/antioxidant balance than did RT.**

Introduction

Disequilibrium between endogenous oxidants and antioxidants in favour of oxidants has been defined by Sies (1991) as 'oxidative stress'. Oxidants or reactive oxygen species (ROS) are generated physiologically by mitochondria (electron transfer), several enzymes such as oxidases, as well as during activation of inflammatory cells (respiratory burst). Oxidative processes play an important role in intracellular signal transduction, the so-called redox regulation (Leeuwenburgh and Heinecke 2001). The generation of ROS is naturally counterbalanced by antioxidants, thereby preventing potential cellular damage, such as lipid peroxidation, protein oxidation and DNA damage (Clarkson and Thompson 2000). During exercise, the mitochondria-related ROS release dramatically increases and might lead to exercise-induced oxidative stress (Dillard *et al.* 1978). Although the deleterious repercussions of an exercise-induced oxidant/antioxidant imbalance in healthy individuals remain to be proven (Clarkson and Thompson 2000; Jenkins 2000), there is evidence that antioxidant depletion negatively affects exercise tolerance and performance (Leeuwenburgh and Ji 1995; Sen and Packer 2000). Moreover, oxidative stress might act as a pro-inflammatory stimulus, thereby potentially favouring development of pathological processes (Nieman 1997).

Beside laboratory rodents, the horse is the most investigated animal species in exercise-induced oxidative research. Indeed, for more than 10 years, markers of oxidative stress are used in laboratory and field studies to characterise ever more precisely the effect of ambient conditions (Mills *et al.* 1996), exercise intensity and duration (Mills *et al.* 1996; White *et al.* 2001; Hargreaves *et al.* 2002; Marlin *et al.* 2002; de Moffarts *et al.* 2004a; 2004b), training (de Moffarts *et al.* 2004b), concomitant presence of respiratory disease (Art *et al.* 1999; Kirschvink *et al.* 2002) or antioxidant supplementation (Deaton *et al.* 2002; de Moffarts *et al.* 2005) on exercise-induced oxidative stress. As the maintenance of an oxidant/antioxidant balance seems to favour the maintenance of animal health, well-being and, potentially, performance (Avellini *et al.* 2003), there is increasing interest in establishment of oxidant/antioxidant profiles in competition horses and particularly in race horses. Most of the studies described in horses have been performed under laboratory conditions using a treadmill, with only a few under field conditions. At present, it is not known to what extent laboratory results of oxidant/antioxidant studies might be transposed to field conditions. Therefore, the aim of the present study was to compare, in 6 healthy and trained Standardbred horses, the impact of exercise tests performed on treadmill and racetrack on blood oxidant/antioxidant markers.

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Materials and methods

Horses

Six clinically healthy French Standardbred geldings (mean ± s.d. 4.7 ± 0.8 years, 445 ± 30 kg bwt) that had completed one race season were used. The animals were kept in stalls and were bedded on wood shavings. They were fed grass silage (4 kg) and molassed oats (2 kg) b.i.d. Besides a regular treadmill training for 12 weeks, the horses were allowed to move freely in a paddock for 20 mins/day. The study was approved by the Animal Ethics Committee of the University of Liège.

Study design

After a 12-week treadmill interval training protocol (Lovell 1994), the horses performed an exercise test (ET) with run-up to fatigue on the treadmill (TM) followed one week later by an exercise test with run-up to fatigue on a race track (RT). Antioxidant markers were determined in jugular venous blood sampled immediately prior to ET (rest) and 15 min (E15) after the end of each ET.

Treadmill and race track exercise tests

Treadmill test: TM consisted of a warm-up at walk (5 mins, 1.7 m/sec, slope 0%) and slow trot (5 mins, 4 m/sec, slope 0%) followed by a 4 minute high intensity trot of increasing speed (min 1: 7 m/sec, min 2: 8 m/sec, min 3: 9 m/sec, min 4: 10 m/sec) at a slope of 6%. A final stage at constant speed of 11 m/sec and a slope of 6% until the horse showed signs of fatigue. A cool down of 5 mins walking (1.7 m/sec, slope 0%) finished TM.

Race track test: RT included the same stages as TM but was performed on a sandy race track with horses pulling a sulky. The driver controlled speed using a tachymeter.

Exercise monitoring

Running time to fatigue: The duration of the last stage at constant speed was carefully recorded during both tests. Running time to fatigue (RTF) was defined as the total time during which the horses were trotting at high speed (4 min + duration of the last stage). The horses were considered as fatigued when they were unable to maintain a constant speed either on the treadmill or on the racetrack.

Heart rate monitoring: Heart rate (HR) was determined at each ET using a telemetric electro-cardiogram recorder (Life Scope 8)[1]. During the RT the heart rate recorder was placed along the racetrack in order to assess final HR at each stage for at least 20 sec.

Venous blood lactate: Venous blood was sampled from an indwelling jugular catheter at each ET at rest and 1 min after the last exercise stage for analysis of plasma lactate (LA) (Accusport)[2].

Body temperature determination: Rectal temperature was measured after the cool-down walk of each ET.

Blood sample collection

Venous blood collection was performed by jugular puncture for antioxidant marker analysis when the horses were at rest (R) before each ET and 15 min (E15) after the end of the ET. Blood was aliquoted either into EDTA tubes and prepared for analysis of ascorbic acid (AA), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione (GSH), protein thiols (PSH) and haemoglobin (Hb), or into heparin tubes for analysis of uric acid (UA), packed cell volume (PCV) and total protein.

TABLE 1: Blood marker concentrations determined in venous jugular blood of 6 healthy Standardbred horses at rest and 15 min (E15) after an exercise test with run-up to fatigue performed on treadmill (TM) or racetrack (RT). Data are shown as mean ± s.e.

Marker (unit)	Time	Exercise test with run-up to fatigue		Test-related effect	Exercise-related effect
		TM	RT		
GSH (µmol/l)	Rest	1065 ± 34.7	937 ± 39.6	ns	*
	E15	837 ± 47*	881 ± 56*		
GSSG (µmol/l)	Rest	39 ± 10.4	41.3 ± 25.1	ns	ns
	E15	20.6 ± 14.6	17.5 ± 8.1		
GRR (%)	Rest	3.47 ± 0.86	2.63 ± 1.39	ns	ns
	E15	2.14 ± 1.36	1.55 ± 0.74		
GPx (u/gHb)	Rest	257 ± 15	228.3 ± 17	ns	ns
	E15	220.3 ± 14	221.6 ± 13.7		
SOD (u/gHb)	Rest	1785 ± 106	1883 ± 75	ns	ns
	E15	1467 ± 133	1677 ± 104		

GSH = reduced glutathione; GSSG = oxidised glutathione; GRR = glutathione redox ratio (GSSG/[GSH+GSSG]); GPx = glutathion peroxidase; SOD = superoxide dismutase. *Significantly different from respective R value, $P<0.05$; ns = not significant.

Blood sample processing

The blood samples were processed immediately after collection as follows. One part of whole blood (EDTA) was kept on ice for analysis of GPx, SOD and Hb within 8 h of collection; further samples of whole blood (100 µl + 10 µl of 1-methyl-2-vinyl-pyridinium-trifluoro-methane-sulphonate for GSSG analysis; 50 µl for GSH analysis) were frozen immediately on dry ice and stored at -80°C until analysis. After centrifugation of EDTA blood (15 min, 900 ***g***), one part of the plasma was stabilised with 10% metaphosphoric acid (MPA, 1:1 plasma:MPA) and was kept on dry ice to be analysed for ascorbic acid within 6 h of collection. Another part of plasma was kept on dry ice for PSH analysis within 6 h of collection. Blood sampled into heparin tubes was centrifuged (15 mins, 900 ***g***) and plasma was frozen on dry ice and stored at -80°C until analysis of uric acid. Analyses of frozen samples were performed within days of collection.

Blood marker analysis

Oxidant/antioxidant blood markers were analysed according to the methods detailed elsewhere (de Moffarts *et al.* 2004b, 2005). Briefly, plasma AA was determined spectrophotometrically according to the method described by Omaye *et al.* (1979). SOD and GPx were analysed spectrophotometrically in whole blood using a RANSOD and RANSEL kit[3], respectively. Whole blood GSH and GSSG were determined spectrophotometrically using the Biooxytech kit GSH/GSSG 412 Assay[4]. Plasma concentration of UA and PSH were analysed spectrophotometrically[5].

Standardisation of blood marker concentration: GPx and SOD were standardised by expressing their activities/g Hb. Concentrations of hydrophilic plasma markers (AA, UA) were standardised by total protein concentration. As GSH and GSSG

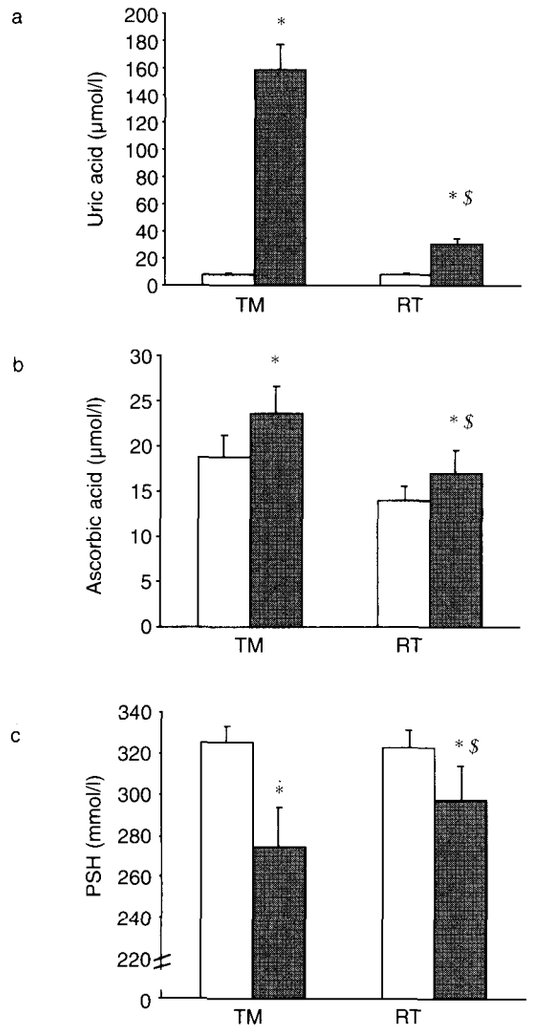

*Fig 1: Plasma concentration of uric acid (a), ascorbic acid (b) and protein thiols (PSH; c) determined in 6 healthy Standardbred horses at rest (□) and 15 minutes after an exercise test (■) performed on a treadmill (TM) or race track (RT). Data are shown as means ± s.e. *Significantly different from respective rest-value; $significantly different from respective TM value, P<0.05.*

were determined in whole blood, their concentrations were adjusted for changes of PCV. Adjustment was performed using the following equation (Hinchcliff *et al.* 2000) :

$$X_{adj} = X_{obs} + (X_{obs} \times [(M_R - M_{obs})/M_R])$$

where (t i) = time point of sampling, X_{adj} = adjusted marker concentration, X_{obs} = measured marker concentration at (t i), M_R = concentration of standardising element determined at rest and M_{obs} = concentration of standardising element determined at (t i).

Statistical analysis

Blood oxidant/antioxidant concentrations measured at R and E15 during both tests were distributed normally and analysed by a mixed linear model for repeated measures (SAS) allowing analysis of the effect of exercise, type of test and exercise-test interaction. Physiological variables were analysed by analysis of variance (ANOVA) for repeated measures and by *post hoc* paired Student's tests (Statview). Differences were considered as significant when $P<0.05$. Data are shown as least square means with associated s.e.m. for antioxidant markers and as mean values ± s.e. for physiological variables.

Results

Comparison of exercise variables

Although HR during warm-up and the first 4 exercise stages tended to be higher during TM (data not shown), final HR recorded during the last 20 sec of the last exercise stage (TM: 223 ± 3 vs. RT: 213 ± 4 beats/min), RTF (TM: 5.49 ± 0.12 min vs. RT: 5.27 ± 0.18 mins) and rectal temperature measured immediately after the cool-down walk (TM: 40.0 ± 0.1 vs. RT: 39.3 ± 0.2°C) did not differ significantly between tests. Maximal velocity during the last stage was significantly higher during RT (11.7 ± 0.25 m/sec) than TM (11.0 ± 0 m/sec, $P<0.05$). Plasma lactate was significantly lower after RT (10.9 ± 1.4 mmol/l) than TM (24.2 ± 0.5 mmol/l, $P<0.05$).

Blood oxidant/antioxidant markers

A significant exercise-induced increase of UA and AA was recorded for TM and RT. Both markers were significantly higher at E15 after TM than after RT (Figs 1a,b). Protein thiols (PSH) were significantly decreased at E15 after both tests, but were significantly lower after TM than RT (Fig 1c). Table 1 summarises test- and exercise-related changes for GSH, GSSG, GRR, GPx and SOD concentrations. Both exercise tests induced a significant decrease of GSH at E15, but no test-related differences were detected. The were no exercise-or test-related changes in GSSG, GRR, GPx and SOD concentrations.

Discussion

Although the exercise tests were designed to be similar (identical velocity and distances to be run during the exercise stages 1–4), a higher maximal velocity during the last exercise stage was obtained during the exercise test on the race track. Final heart rate, running time to fatigue and rectal temperature did not differ significantly between tests, but lower lactate values were recorded during the race track test. These differences of exercise variables suggest that the oxidative challenge induced during both tests might not have been similar. Several reasons could account for this discrepancy. All horses had been familiarised to a race track during the race season preceding this study but the training period for this protocol had been exclusively performed by use of incremental treadmill training. It was noted by the investigators that the horses were more excited during TM than RT (horses appeared more nervous and HR during warm-up and running stages at determined velocity were higher during TM; data not shown), which might have contributed to lower LA values during the field test.

It is unlikely that a difference of fitness influenced the results because both tests were performed within one week. However, a crossover design rather than a sequential design would have been preferable. Another factor that might have affected these results was the 6% slope of the treadmill aiming at compensating the supplemental workload of a sulky. Indeed, a comparison between treadmill tests and sandy race tracks where horses were pulling a sulky suggests that 3% treadmill slope would be more appropriate (Couroucé 1999). This could mean that exercise intensity during the treadmill test was higher and further contributed to increased LA_{max} values. Although the maximal velocity reached during the RT was higher and RTF somewhat shorter than during TM, no

compensation was made for LA_{max}. Consequently, exercise-induced changes of the oxidant/ antioxidant balance have probably been influenced by the test conditions and by a difference of exercise intensity.

During both tests, significant increases of plasma UA and AA were recorded at E15. These changes are in agreement with earlier studies performed in Standardbred and Thoroughbred horses (Räsänen *et al.* 1993; Essen-Gustavson *et al.* 1999; White *et al.* 2001). The exercise-induced increase of both markers was significantly higher during TM than RT. Uric acid increase has been reported to be correlated with exercise length and intensity, as well as with LA (Räsanen *et al.* 1993; Evans *et al.* 2002), which suggests that the workload during TM was effectively higher than RT.

Plasma PSH has been investigated in equine Cushing's disease (Keen *et al.* 2004), but, to the best of the authors' knowledge, not yet with regard to exercise in horses. Both thiol-derivative markers, plasma PSH and erythrocyte GSH, were significantly decreased at E15 after TM and RT. Interestingly, exercise-induced decrease of PSH was significantly greater after TM, whereas no test-related differences occurred for GSH. This suggests that for this type of exercise test plasma PSH could be a more appropriate marker of thiol-depletion than GSH. With regard to GSSG and GSH, no significant changes were detected in the present investigation. Mills *et al.* (1996) found a significant increase of haemolysate GSSG and GRR when horses were exercised under hot and humid conditions, showing that the pro-oxidative burden depends on exercise as well as on ambient conditions. Despite a trend of decrease for GPx and SOD, no significant exercise- or test-related changes were found, which is in agreement with earlier studies (de Moffarts *et al.* 2004b).

In conclusion, it has been shown in this study that treadmill and race track exercise tests might induce changes of the oxidant/antioxidant blood balance. Urid acid, ascorbic acid and protein thiol markers appeared to be most sensitive to exercise-induced changes and differed significantly at post exercise between the tests. As the workload induced by TM was probably higher than that induced by RT, the oxidant/antioxidant results were affected by this difference. Comparisons between laboratory and field oxidant/antioxidant studies are difficult to perform, because similar exercise conditions might not be achieved despite a thorough standardisation. Consequently, such studies lose their strength and provide results that need to be interpreted with caution.

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Manufacturers' addresses

[1]Nihon Kohden corp., Tokyo, Japan.
[2]Boehringer Mannheim, Mannheim, Germany.
[3]Randox Laboratories, Antrim, Co. Antrim, UK.
[4]Oxis, Portland, Oregon, USA.
[5]Roche Hitachi, Mannheim, Germany.

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