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Acute phase protein concentrations after limited distance and long distance endurance rides in horses

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ABSTRACT

Acute phase proteins (APP) have been described as useful for assessing health in human and animal patients, as they closely reflect the acute phase reaction (APR). In humans and dogs a reaction analogous to APR has also been described after prolonged or strenuous exercise. The aim of this study was to determine, if similar reactions occur in endurance horses after limited and long distance rides. Seventeen horses that successfully completed various distance competitions were tested. Routine haematological and biochemical tests were performed and the concentrations of serum amyloid A (SAA), C-reactive protein (CRP) and haptoglobin were measured. Typical endurance exercise-induced haematological and biochemical changes were observed in all horses, regardless the distance. After long distance rides, the level of SAA markedly increased, but CRP and haptoglobin concentrations remained unchanged. After limited distance rides no changes in the levels of APPs were noted. Exercise-induced APR in horses occurred only after prolonged, strenuous exertion, and differed from APR in inflammation in that only SAA concentration was increased.

1. Introduction

Acute phase proteins (APP) are produced in the liver during acute phase reaction (APR), the rapid, nonspecific, prominent response to any kind of injury (Petersen et al., 2004; Gruys et al., 2005). APR is induced by pro-inflammatory cytokines: IL-1, TNF-α and especially IL-6 (Tizard, 2009). These cytokines activate receptors on various target cells and promote hormonal and metabolic changes leading to local and systemic effects, including APP synthesis in the liver (Petersen et al., 2004; Gruys et al., 2005; Tizard, 2009). Changes in serum concentrations of acute phase proteins closely reflect the onset of APR and the clinical condition of the patient. Haptoglobin, C-reactive protein (CRP) and serum amyloid A (SAA) are the most strongly reacting APP in humans and animals (Petersen et al., 2004; Gruys et al., 2005).

However, the pattern of APP production varies greatly among species (Eckersall, 2000; Gruys et al., 2005; Pollock et al., 2005; Tizard, 2009). In horses, SAA is defined as the major acute phase protein and CRP and haptoglobin as moderate APPs (Tizard, 2009). In healthy horses SAA is present only at trace levels (Hinchcliff et al., 2004; Petersen et al., 2004), but markedly increases in 6–24 h (Heegaard, 2000) in response to infections (Hulten et al., 1999; Hulten and Demmers, 2002; Petersen et al., 2004), surgical trauma (Hulten et al., 1999; Petersen et al., 2004; Pollock et al., 2005), aseptic experimental and immunological inflammation (Nunokawa et al., 1993; Eckersall, 2000). CRP is the major marker of inflammation in dogs, pigs and humans (Eckersall, 2000; Tizard, 2009). In horses, an increase in CRP concentration has been reported in an induced inflammation and laminitis, pneumonia, enteritis, arthritis and after castration (Takiguchi et al., 1990). Other authors, however, reported that serum CRP concentration was not affected by disease (Eckersall, 2000; Pollock et al., 2005). Haptoglobin prevents the loss of iron by binding free haemoglobin. In the horses, the decrease in serum concentration of haptoglobin in haemolytic diseases and an increase in inflammatory diseases and after surgery have been reported (Taira et al., 1992; Hulten et al., 2002; Pollock et al., 2005).

A reaction analogous to the acute phase response in inflammatory conditions has been reported also after prolonged exercise in human beings (Fallon, 2001; Scharhag et al., 2005; Suzuki et al., 2006; Peeling et al., 2008). This exercise-induced APR was characterized by changes in pro-inflammatory cytokines, CRP, haptoglobin and hepcidin levels (Fallon, 2001; Peeling et al., 2008). It has been proposed that it results from glycogen depletion in working muscles and skeletal muscle damage, as indicated by an increase in creatine phosphokinase (CPK) activity (Fallon, 2001).

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Another pattern of exercise -induced APR has been reported in sled dogs after endurance racing (Wakshlag et al., 2010b). Similarly to humans, the level of CRP increased after the race. The albumin concentration in the dogs decreased. However, in contrast to humans, the concentration of IL-6 remained unchanged. The level of CRP has also been reported to increase slightly after moderate-duration racing events in sled dogs (Wakshlag et al., 2010a).

In horses, changes in haptoglobin concentrations have been reported in standardbred horses after racing and during treadmill training (Pellegrini Masini et al., 2003; Inoue et al., 2005). This may be the result of intravascular haemolysis. Other APP have not been characterized in the context of exercise and acute phase reaction provoked by exertion in horses.

The aim of this study was to determine if exercise-induced acute phase reaction, defined by changes in acute phase proteins: SAA, CRP and haptoglobin occurs in horses after endurance competitions of various intensities.

2. Materials and methods

2.1. Horses and competition

Seventeen endurance-trained horses, participating in limited distance (34 km and 60 km) or long distance: CEI (Concours Endurance International) 2* (120 km) and CEI (Concours Endurance International) 3* (160 km, championships competition) endurance rides in Poland were included in this study (Table 1). Horses were privately owned and were prepared to participate in endurance events by the owners. The owners, Veterinary Commission and the local Ethical Committee agreed to the procedures. The competitions were held in June, July and September under similar, clear weather, temperature 22–24 °C conditions, in similar terrain. All of the horses were dewormed and vaccinated at similar times, did not receive medications or suffer from an infection in the preceding 3 weeks (according to the owner's knowledge). The inclusion criteria included successful completion the distance, all veterinary health checks performed during and following the ride and routine doping control.

2.2. Blood samples

Peripheral blood samples were obtained by jugular venipuncture before and after the competition. Samples were aspirated into 20 ml syringes and immediately transferred into sterile EDTA tubes for haematological tests and into tubes with no anticoagulant for serum analyses. Lactate and glucose concentrations were determined immediately by placing a drop of whole blood onto single-use lactate or glucose strip (Accusport or Accucheck, Roche). The EDTA-3K tubes were kept in refrigerator (+4 °C) and analyzed within 6 h after collection. Routine hematological parameters: haematocrit (HCT), haemoglobin concentration (HGB), red blood cell count (RBC), platelet count (PLT) and white blood cell count (WBC) were performed with an automated haematology analyzer (Abacus, France). Differential counts were determined manually from smears by counting 100 cells, and neutrophil to lymphocyte ratio (N:L) was calculated. The tubes with no anticoagulant were centrifuged at 4380*g* for 5 min, serum was aspirated, immediately frozen, and stored at −20 °C until analyzed. Serum samples were used for measurement of creatine phosphokinase (CPK) activity, total protein concentration (TP), serum amyloid A, haptoglobin and C-reactive protein concentrations. Creatine phosphokinase activity was assayed by the kinetic method (POINTE SCIENTIFIC, USA). Total protein levels were determined by using a Biuret Reagent (POINTE SCIENTIFIC, USA).

Serum haptoglobin levels were measured colorimetrically using PHASE Haptoglobin Assay (TRIDELTA Ltd., Ireland). Serum amyloid A and C-reactive protein levels were measured using a double sandwich ELISA, PHASE Serum Amyloid A (TRIDELTA Ltd., Ireland) and Horse CRP ELISA (KAMIYA BIOMEDICAL COMPANY, USA), respectively.

Serum SAA, haptoglobin and CRP levels were analyzed after correction for serum protein concentration to avoid the influence of haemoconcentration, which resulted in an increase of total protein level and may falsely increase the concentrations of acute phase proteins. To compare the APP concentrations before and after exertion, the values obtained after exertion were recalculated taking into account the changes in total protein concentrations according to the following formulas:

$$\text{Concentration}_{\text{corr}} = \text{concentration} \times \text{TP}_1/\text{TP}_2,$$

where Concentration$_{\text{corr}}$ – corrected serum SAA, haptoglobin or CRP concentration; Concentration – serum SAA, haptoglobin or CRP concentration determined after exertion; TP_1 – total protein level before exertion; TP_2 – total protein level after exertion.

2.3. Statistical analysis

Statistical procedures, means and standard errors of mean were computed using the STATISTICA 6.0 for Windows. Results are

Table 1
Description of the horses included in the study for the assessment of haematological parameters and acute phase proteins concentrations after limited distance and long distance endurance rides.

	Breed	Sex, age	Distance [km]	
Limited distance rides	Arabian	Mare, 5 years	34	*n* = 8
	Half-bred	Stallion, 6 years	34	
	Half-bred	Gelding, 9 years	34	
	Half-bred	Gelding, 5 years	34	
	Arabian	Stallion, 5 years	60	
	Arabian	Stallion, 6 years	60	
	Arabian	Stallion, 6 years	60	
	Thoroughbred	Mare, 7 years	60	
Long distance rides	Arabian	Mare, 8 years	120 (CEI2*)	*n* = 9
	Arabian	Gelding, 11 years	120 (CEI2*)	
	Arabian	Gelding, 10 years	120 (CEI2*)	
	Arabian	Gelding, 8 years	120 (CEI2*)	
	Half-bred	Mare, 12 years	120 (CEI2*)	
	Arabian	Gelding, 12 years	160 (CEI3*)	
	Arabian	Gelding, 9 years	160 (CEI3*)	
	Arabian	Gelding, 11 years	160 (CEI3*)	
	Arabian	Stallion, 10 years	160 (CEI3*)	

expressed as means ± standard errors of mean (SEM). The Kolmogorov–Smirnov test indicated that the data was not normally distributed. The results before and after the rides were compared with Wilcoxon signed rank test with Bonferroni correction in case of analysis of acute phase reaction on the basis of APP concentrations. Statistical comparisons using Mann–Whitney U test were also performed between the results obtained before the rides in male and female horses, Arabians and non Arabians and the horses participating in limited distance (34 *vs*. 60 km) and long distance (120 *vs*. 160 km) competitions. $p \leqslant 0.05$ ($p \leqslant 0.017$ after Bonferroni correction) was considered significant.

3. Results

The average time for completion of the events were 3.26 ± 0.07 h and 5.7 ± 0.25 h for limited distance rides (34 and 60 km, respectively) and 8.15 ± 0.01 h and 10.94 ± 0.12 h for long distance rides (120 and 160 km, respectively). The average speeds at the distances were 9.8 ± 0.36, 11.43 ± 0.41, 15.45 ± 0.52 and 15.14 ± 0.45 km/h, respectively.

All haematological and biochemical (Table 2) parameters determined before the competition varied within normal ranges for equine species (Hinchcliff et al., 2004; Smith, 2008) in all horses. No significant differences were noted between male and female horses, Arabians and non Arabians and the horses prepared for various distances, the values were similar, with only slight variations among individuals, so data were analyzed in two groups: the horses taking part in limited distance rides and in long distance rides.

After limited distance and long distance rides, erythrogram values, WBC, neutrophil numbers and N:L ratio increased significantly ($p \leqslant 0.05$). Following long distance rides WBC and neutrophil numbers even exceeded physiological values in all horses. Glucose concentration and CPK activity changed significantly ($p \leqslant 0.05$) after limited distance ride. After long distance ride only CPK activity increased significantly ($p \leqslant 0.01$) and glucose concentration did not change. Lactate concentration remained unchanged after both limited and long distance rides.

Before the rides no significant differences in the concentrations of serum haptoglobin, CRP and SAA between male and female horses, Arabians and non Arabians and the horses prepared for various distances were noted. The concentrations of serum haptoglobin and SAA were similar to the values reported previously by other authors in healthy horses. CRP concentration in 10 horses were higher than reported in healthy horses, so that mean values for the horses participating in limited distance and long distance endurance rides were relatively high. After limited distance rides, no significant changes in SAA, haptoglobin and CRP levels were found (Fig. 1a,b). After long distance rides the levels of haptoglobin and CRP remained unchanged, but SAA concentrations increased significantly ($p \leqslant 0.017$), 10-fold or more.

Table 2
Haematological and biochemical parameters in the horses before and after limited distance and long distance rides.

Parameter	Limited distance rides		Long distance rides	
	Before	After	Before	After
RBC [$\times 10^{12}$/l]	8.5 ± 0.5	9.4 ± 0.8*	8.3 ± 0.2	10.4 ± 0.5*
HCT [l/l]	0.36 ± 0.02	0.40 ± 0.02*	0.36 ± 0.01	0.45 ± 0.02*
HGB [g/l]	120 ± 5.6	135 ± 4.9*	126 ± 2.1	157 ± 4.6*
PLT [$\times 10^9$/l]	172.3 ± 24.3	193.2 ± 20.8	163.2 ± 22.1	206.6 ± 18.9
WBC [$\times 10^9$/l]	9.6 ± 2.0	14.4 ± 1.5*	10.4 ± 1.1	19.6 ± 1.8*
N:L	3.2 ± 0.4	6.9 ± 0.3*	3.5 ± 0.5	11.8 ± 1.9*
TP [g/l]	67.0 ± 2.0	68.7 ± 1.4	63.4 ± 1.5	66.8 ± 2.2
Glu [mmol/l]	5.3 ± 0.2	4.7 ± 0.3*	5.4 ± 0.3	5.0 ± 0.2
LA [mmol/l]	2.0 ± 0.1	2.1 ± 0.1	1.8 ± 0.2	2.5 ± 0.5
CPK [U/l]	179.9 ± 9.5	334.7 ± 59.6**	166.5 ± 17.6	2959.4 ± 697.0***

RBC – total number of red blood cells; HCT – packed cell volume; HGB – haemoglobin concentration; PLT – platelets number; WBC – total leukocyte number; N:L – neutrophil to lymphocyte ratio; TP – total protein concentration; Glu – glucose concentration; LA – lactate concentration; CPK – creatine phosphokinase activity.
* $p \leqslant 0.05$.
** $p \leqslant 0.01$.
*** $p \leqslant 0.001$.

4. Discussion

It has been reported previously that endurance horses developed a variety of physical and biochemical changes after competition (Szarska, 2003; Hinchcliff et al., 2004). Haematological and biochemical responses to exertion depend on the speed and duration of the endurance ride and the horse's fitness. In our study, the horses developed typical erythrogram and leukogram changes and increased CPK activity after both limited and long distance rides. Erythrograms indicated haemoconcentration, which in endurance horses is believed to result from fluid loss rather then splenic contraction (Hinchcliff et al., 2004). Neutrophilic leukocytosis, lymphopenia and an increase in neutrophil:lymphocyte ratio was consistent with exercise-induced stress. This is well documented in endurance horses and results probably from an increase in circulating corticosteroids, with significant influence of the speed over the course of the ride on neutrophil: lymphocyte ratio (Rossdale et al., 1982; Korhonen et al., 2000; Hinchcliff et al., 2004). Increases in CPK activity have been reported in response to exercise and resulted from either overt damage or a change in the muscle fiber membrane causing a transient increase in permeability (Hinchcliff et al., 2004). The reaction is transient, but CPK activity can easily double without any clinical problems and values in the tens of thousands are routinely seen in endurance horses that successfully complete rides without incident (Marcella, 2008).

In our study, the exertion, regardless of the distance, was intense enough to produce typical exercise-induced leukogram and CPK changes, so we postulated, that exercise-induced acute phase reaction also might have been expected. This hypothesis was supported by marked increases in SAA levels in all horses only after long distance rides. CRP and haptoglobin levels remained unchanged. Limited distance rides did not produce any changes in acute phase proteins levels.

Serum amyloid A is recognized as the most sensitive APP in horses (Petersen et al., 2004; Hobo et al., 2007) and the only APP which concentration increased in our study. It rapidly rises 100–1000 fold in acute inflammation and reaches values over 20,000 ng/ml (Hinchcliff et al., 2004; Hobo et al., 2007). The concentration of SAA has been reported to closely reflect changes in the clinical condition of the horses, and it has been proposed as a useful marker for the onset of bacterial pneumonia (Hobo et al., 2007). In humans, SAA concentration has also been reported to increase after the Ironman triathlon race (Suzuki et al., 2006). Our previous study indicated that in horses, elevated SAA level may serve as a non-specific indicator of the poor condition of endurance horses that results in elimination from long distance competition (Cywinska et al., 2010).

In the present study, we determined, that SAA concentrations increased 10-fold or more after long distance ride and reached values over 12,000 ng/ml. Such high values, although still within reference ranges (Beaufort Cottage Laboratories www.rossdales.com), have never been reported in healthy horses (Hulten et al., 1999; Pollock et al., 2005; Jacobsen et al., 2006; Hobo et al., 2007). Exercise-induced acute phase response occurred after long, but not limited distance ride. This finding was similar to the findings in humans, where exercise-induced APR occurred after prolonged,

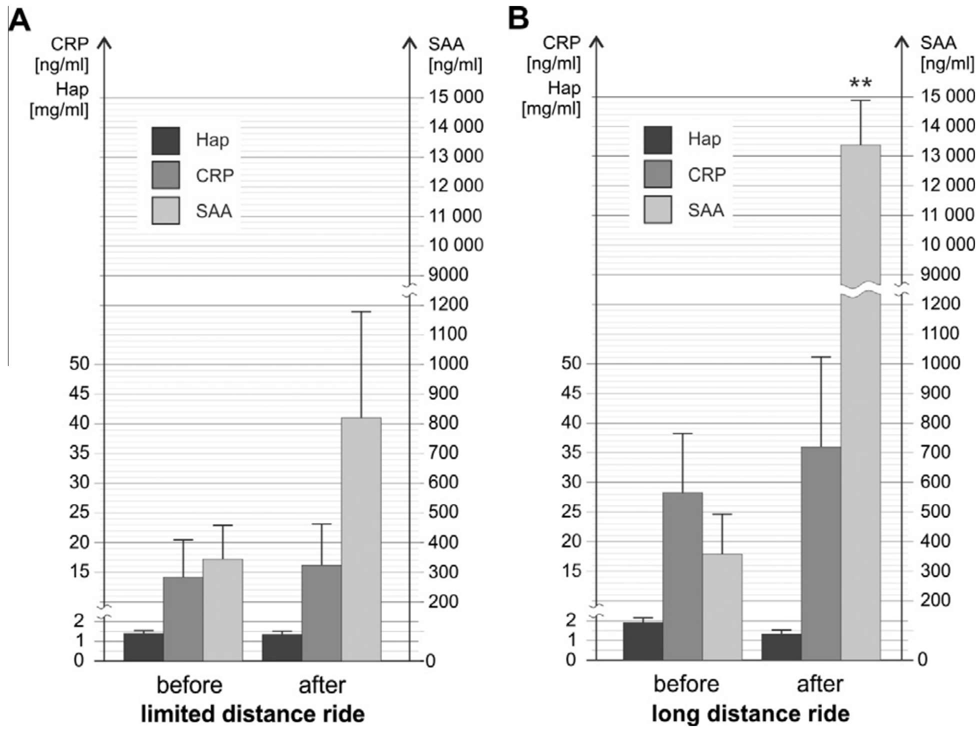

Fig. 1. Acute phase proteins concentrations before and after limited distance and long distance rides. $^{**}p \leqslant 0.017$ (according to Bonferroni correction).

exhausting exercise but not after soccer or netball training (Fallon, 2001; Fallon et al., 2001; Suzuki et al., 2006). In our study, haematological changes and an increase in CPK activity were noted after the rides regardless the distance. They indicated the transient, exercise-induced stress reaction and muscle changes, mentioned above, but not an acute phase reaction.

It has been reported, that SAA correlates well with CRP in horses with clinical inflammation (Nunokawa et al., 1993; Petersen et al., 2004), but it has never been investigated in the context of exercise. Increases in CRP concentrations have been shown in sled dogs (Wakshlag et al., 2010a,b) and in humans after exhausting exercise, but not typical training (Fallon, 2001; Fallon et al., 2001; Scharhag et al., 2005; Neubauer et al., 2008). In humans, CRP concentrations increased 10-fold after marathon and ultramarathon running (Fallon, 2001; Neubauer et al., 2008) and 3-fold after prolonged cycling (Scharhag et al., 2005) and was accompanied by increases in IL-6 level (Neubauer et al., 2008) and CPK activity (Fallon, 2001; Neubauer et al., 2008). In sled dogs, CRP concentration increased 10-fold after a long distance race (Wakshlag et al., 2010b) and about 2-fold after a 2-day (16 miles each day) racing event, defined as high-intensity moderate duration exercise (Wakshlag et al., 2010a). In contrast to humans, IL-6 level did not change, but CPK activity increased (Wakshlag et al., 2010a,b).

Our study showed that in contrast to human and dogs, the increase in CPK activity after limited and long distance rides is not accompanied by any changes in CRP concentration. In the horse, CRP is recognized as a moderate APP and do not elevate as dramatically as in the human or rabbit (Yamashita et al., 1991). Our results indicated that exercise induced APR in horses is different than in human and dogs, which may be explained by the differences in major APP. It also differs from APR during inflammation and it is characterized by an increase in major APP, but not moderate and minor APP. This observation seems supported by results in humans, where exercise-induced APR after an ultramarathon was characterized by increases in CRP and SAA concentrations, but no changes in complement components 3 and 4 and alpha-1 antitrypsin (Fallon, 2001; Suzuki et al., 2006).

In our study there were also no changes in haptoglobin level. Decreases in haptoglobin concentration after exertion have been described in humans and horses as the result of intravascular haemolysis. In this condition, the level of haptoglobin decreased due to the binding of iron released from damaged erythrocytes (Hanzawa and Watanabe, 2000; Pellegrini Masini et al., 2003; Inoue et al., 2005). In the acute phase response, however, the level of haptoglobin increased 2–3-fold after surgery, experimental and natural inflammation in horses (Petersen et al., 2004; Gruys et al., 2005; Pollock et al., 2005). In ultramarathon runners, there is a decrease followed by an increase in haptoglobin concentration (Fallon, 2001) that was associated with haemolysis followed by an acute phase reaction, which fully develops over time. Other authors found no changes in haptoglobin concentration after a 160 km race in a time when APR should be fully operative (Dickson et al., 1982). Haptoglobin is not a major acute phase protein neither in humans nor in horses. Thus, it matches our hypothesis, that moderate and minor APP may or may not be involved in exercise induced APR, depending on the type of exercise and the athletes' fitness.

In conclusion, this study provides evidence that acute phase reaction occurred in endurance horses only after long distance rides and was manifested by the marked increase in SAA, relative to pre-exercise values but not moderate APPs concentration. Exercise-induced APR in horses differs from that in inflammatory condition and seems of limited clinical importance. However, should be taken into consideration to maintain the welfare state of the horse. Limited distance rides produced transient haematological and biochemical changes, but did not result in the induction of acute phase reaction.

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