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Effect of a 120 km endurance race on plasma and muscular neutrophil elastase and myeloperoxidase concentrations in horses

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Summary

Reasons for performing study: **Intense physical exercise can induce the degranulation of neutrophils leading to an increase in plasma concentration of the neutrophil marker enzymes myeloperoxidase (MPO) and elastase (ELT). These enzymes have pro-oxidative and pro-inflammatory properties and may play a role in the exercised-induced muscular damage.**

Objectives: **To measure MPO and ELT concentrations in plasma and muscles of endurance horses and to correlate them to the extent of exercise-induced muscular damage.**

Methods: **Seven endurance horses qualified on 120 km races were tested in this study. Neutrophil count, serum creatine kinase (CK), plasmatic and muscular MPO and ELT concentrations were measured before and 2 h after a 120 km endurance race.**

Results: **The race produced a significant increase of neutrophils, CK, and plasma MPO and ELT levels. A significant correlation was observed between the MPO and ELT values in plasma (r^2 = 0.92, P<0.01) and in muscles (r^2 = 0.89, P<0.01) while plasmatic concentrations of MPO and ELT were not significantly correlated to muscular ones. An increase of mean concentrations (± s.e.) of MPO (T0: 9.85 ± 3.9, T1: 228.9 ± 95.9 ng/mg proteins) and ELT (T0: 8.4 ± 2.4, T1: 74.5 ± 39.7 ng/mg proteins) in the muscles were observed after the race. Interestingly, the individual data showed large differences between the horses. Muscular MPO and ELT concentrations were significantly correlated to plasma CK levels. The coefficient of correlation (r^2) was 0.69 (P<0.01) for MPO and 0.66 (P<0.01) for ELT, respectively.**

Conclusions: **Results underline the possible role of MPO and ELT in exercise-induced muscular damage.**

Potential relevance: **Further studies should investigate the effect of exercise type and intensity, as well as the role of the training state on MPO and ELT involvement in muscular damage. The assessment of the intensity of exercise-induced neutrophilic degranulation may have a potential role in the monitoring of the athletic career.**

Introduction

Prolonged strenuous physical exercise attenuates many components of immunity in man (Pedersen and Hoffman-Goetz 2000; Malm 2004; Gleeson 2007). When the integrity of the organism is challenged by vigorous endurance exercise a systemic inflammatory response is induced. Intense physical exercise can induce the degranulation of neutrophils leading to an increase in the plasmatic concentrations of the neutrophil marker enzymes myeloperoxidase (MPO) and elastase (ELT) (Kokot *et al*. 1988; Camus *et al*. 1998; Gleeson *et al*. 1998; Walsh *et al*. 2000; Morozov *et al*. 2001, 2006). The extent of degranulation tends to increase with increasing exercise intensity (Peake *et al*. 2004).

The other aspect of leucocyte responses to exercise is a participation of these cells in aseptic muscle inflammation associated with muscle fibre injury caused by intensive exercise. It has been demonstrated that intense single-bout exercises in man and animals resulted in neutrophil infiltration into skeletal muscles and to an increase of tissue MPO concentrations (Round *et al*. 1987; Michna 1989; Skjeldal *et al*. 1993; MacIntyre *et al*. 1996, 2001; Morozov *et al*. 2001; Tsivitse *et al*. 2003).

It is hypothesised that repetitive skeletal muscle tissue trauma caused by heavy training and competition could result in a persistent systemic cytokine response, which may be associated with a chronic inflammatory state, immune dysfunction and a poorly understood condition of overreaching/overtraining (Halson and Jeukendrup 2004) or underperformance syndrome (Smith 2000; Robson 2003; Robson-Ansley *et al*. 2007).

The knowledge of exercise-induced inflammatory reaction in horses is sparse. Some studies have demonstrated the effect of strenuous exercise on the innate immune system in horses (Horohov *et al*. 1996, 1999; Robson *et al*. 2003; Donovan *et al*. 2007). These studies demonstrated an impairment in the neutrophil function that could persist for several days after intense exercise. Further, increased levels of circulating MPO and ELT have been shown to occur in the post exercise period (Art *et al*. 2006; Lejeune *et al*. 2010). However, little is known on the effect of the inflammatory response on exercise-induced muscle

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damage. Therefore, better understanding of the exercise-induced inflammatory reaction and its relationship to muscle damage could help to guide appropriate training regimens in horses. The aim of the study was to compare the plasma and muscle ELT and MPO concentrations before and after an endurance race and to correlate them to serum CK levels as a marker of muscle damage (Volfinger *et al*. 1994).

Materials and methods

Horses

Seven trained endurance Arabian horses (mean (± s.d.) age: 10 ± 2 years, qualified on 80–119 km races by the 'Fédération Equestre Internationale' (FEI 1*) were sampled before (T0) and 2 h after (T1) a 120 km endurance race. Samples were collected at 3 different races: Ghlin, Belgium (24/10/2008) for *Horses 1* and *4*, Vittel, France (21/06/2008) for *Horses 2, 5* and *6* and Saint Galmier, France (11/07/2008) for *Horses 3* and *7*. Weather conditions and characteristics of the races were recorded.

Blood samples and analyses

Blood was taken from the jugular vein into 2 EDTA vacuum tubes. An additional vacuum tube for serum collection was taken[1]. Total and differential white blood cell count was performed by a commercial analyser (Medonic)[2] (Roleff *et al*. 2007). The second EDTA tube was centrifuged within 10 min after sampling at 1000 ***g*** for 10 min at room temperature. Plasma was collected and immediately frozen at -80°C until MPO and ELT analysis. Serum creatine kinase concentration (CK) was measured by a spectrophotometric method (Cobas Roche)[3].

Muscle biopsies

Microbiopsies were collected from the *triceps brachii* muscle using a 14 gauge biopsy needle (Pro-Mag ultra biopsy needle)[4] mounted on an automatic instrument (Pro-Mag ultra biopsy instrument)[4]. The sampling site was located at the intersection of a vertical line raised from the tricipital crest and a line running from the scapulohumeral joint (point of the shoulder) to the elbow. Briefly, the sampling site was shaved (one square centimetre), desensitised by injection of 0.5 ml of mepivacain (Scandicaine 2%)[5] and aseptically prepared. Muscle biopsy samples were taken at 50 mm depth in the long head of the *triceps brachii* through a skin incision made with the tip of a scalpel blade No. 11. Closure of the skin stab was not necessary and the whole biopsy procedure was generally completed within 15 min. Samples have been made in the left *triceps brachii* at T0 and in the right muscle at T1.

Extraction of muscular biopsies

Muscular biopsies (20 mg) were immediately frozen in liquid nitrogen and stored at -80°C until use. Biopsies were homogenised with a 2.5 ml glass potter homogeniser in 500 µl of ice cold 20 mmol/l PBS buffer pH 7.4. After homogenisation, the sample protein concentration was measured by using bicinchoninic acid assay[6].

Sandwich ELISA for neutrophil myeloperoxidase

Concentration of MPO in plasma and muscle extracts was determined by a commercial ELISA[7] developed by Franck *et al*. (2005). The primary antibody, rabbit IgG against MPO, was coated onto microplate wells (Cliniplate EB)[8]. Equine MPO standard (0.78–50 ng/ml) and extracts from biopsies diluted 10 times were added (100 µl) into the wells Microplates were incubated overnight at 4°C. After the plates were washed in 0.9% NaCl solution containing 0.1% Tween 20, the immobilised antibody-antigen complexes were incubated for 2 h at 37°C with the secondary antibody, guinea pig IgG, against equine MPO labelled with alkaline phosphatase. After another washing, phosphatase activity was determined by incubation for 30 min at 37°C in the dark with paranitrophenyl phosphate-stabilised solution. The reaction was stopped with 2.5 m NaOH and the absorbance at 405 nm was read with the Multiscan Ascent plate reader[8]. The absorbance was directly proportional to the MPO captured by the primary antibody and therefore to the concentration of MPO in the sample. Each sample was assayed twice and the mean value calculated.

Sandwich ELISA for neutrophil elastase

A specific ELISA for equine neutrophil elastase was recently developed by De la Rebière de Pouyade *et al*. (2009) and commercialised by BiopTis[7]. Briefly, the microplate wells[9] were coated (overnight, at 4°C) with 150 µl of the rabbit anti-NE IgG solution (primary antibody). After the primary antibody coating, the plates were washed 4 times with 300 µl of the first washing buffer (154 mmol/l NaCl solution with 0.1% Tween 20). Two hundred microlitres of the blocking buffer (coating buffer with 5 g/l BSA) were then added and the plates incubated for 150 min at room temperature (20°C). After 4 washes with the first washing buffer, 100 µl of equine NE standards and extracts from biopsies diluted 5 times were added to the wells and incubated overnight at 4°C. Control (blank) and dilutions of the samples were made with the dilution buffer (blocking buffer added with 0.1% Tween 20). After 4 washes with 300 µl of the second washing buffer (150 mmol/l NaCl, 50 mmol/l Tris–HCl, 0.1% Tween 20, pH 7.5), the plates were incubated (2 h, 37°C) with 100 µl (3 mg/ml) of the secondary antibody conjugated to AP and diluted with the second washing buffer. After washing (second washing buffer), phosphatase activity was detected by incubation (30 min, 37°C, in the dark) with the substrate paranitrophenyl phosphate. The reaction was stopped with 2.5 M NaOH and the absorbance read at 405 nm with the Multiscan Ascent plate reader[8]. A standard curve was generated to allow determination of ELT concentrations in plasma and muscle extract samples.

Statistical analysis

T tests on paired data were used to compare the T0 and T1 values ($P<0.05$). Correlation between MPO and ELT plasma concentrations were calculated between muscle MPO concentration and serum CK levels and between muscle ELT concentration and serum CK levels ($P<0.01$). All tests were performed with MedCalc statistical software[10].

Results

The 7 horses finished the 120 km race at a mean speed of 15.4 ± 1.4 km/h with an adequate recovery period: heart rate <60 beats/min in less than 20 min without lameness or other complications e.g. dehydration or intestinal ileus. While the race characteristics were slightly different, it was not possible to observe an effect of the climatic conditions.

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The 120 km endurance race produced a significant increase ($P<0.05$) of the total number of neutrophils and of the serum CK, and plasma MPO and ELT levels. Mean values (± s.e.) and the results of the statistical analysis are displayed in the Table 1. A significant correlation was observed between the plasma MPO and ELT concentrations ($r^2 = 0.92$; $P<0.01$) while no significant correlations were observed between the plasma and the muscle concentrations for the both enzymes (MPO, ELT).

In the *triceps brachii* muscle, an increase of mean concentrations (± s.e.) of MPO (T0: 9.85 ± 3.9; T1: 228.9 ± 93.9 ng/mg proteins) and ELT (T0: 8.4 ± 2.4; T1: 75.4 ± 39.7 ng/mg proteins) was observed after the race. The individual values of each horse are reported in Table 2. The effects of the 120 km race were different among the horses. Indeed, horses 3, 5, 6 and 7 had a moderate increase of CK, MPO or ELT, while the *Horses 1, 2* and *4* showed a more severe increase of serum CK levels of 4202, 4326 and 5398 iu/l, respectively. This was associated to a severe increase of MPO (120, 358 and 718 ng/mg) and ELT (41, 111, 300 ng/mg) concentrations in the muscles. A significant correlation was observed between the muscle MPO and ELT values ($r^2 = 0.89$; $P<0.01$). Correlation was significant between muscular MPO concentrations and serum CK levels ($r^2 = 0.69$; $P<0.01$) and between muscular ELT concentrations and serum CK levels ($r^2 = 0.66$; $P<0.01$).

Discussion

Results of the present study confirm earlier findings of increased post exercise circulating neutrophil numbers and MPO and ELT concentrations in endurance horses (Art *et al*. 2006; Lejeune *et al*. 2010). The finding is not surprising as increased circulating MPO and ELT levels related to exercise-induced neutrophil activation have been described in man and animal models (Morozov *et al*. 2006; Neubauer *et al*. 2008). Exercise may have various effects on the immune system, which has been demonstrated in man and animals, as well as in horses (Wong *et al*. 1992; Horohov *et al*. 1999). A single bout of strenuous exercise temporarily reduces the neutrophilic function (Wong *et al*. 1992), while repeated moderate training may have a beneficial effect on the host's defence mechanism (Hines *et al*. 1996). The mechanisms for regulating the dual effects of exercise are complex, involving a network of neuro-endocrine hormones and cytokines (Hines *et al*. 1996; Horohov *et al*. 1996). A recent study described the exercise-induced neutrophil activation in horses (Donovan *et al*. 2007) by demonstration of increased reactive oxygen species (ROS) production of circulating neutrophils 2 h after exhaustive exercise. The relationship between this systemic inflammatory response and strenuous exercise remains incompletely understood. In particular, its potential role in aggravating muscle lesions remains to be elucidated. In the present study, the *triceps brachii* muscle was selected because of its mixed composition of *type I* and *type II* fibres due to its dual role in posture and locomotion and because it was easily accessible and safe (Snow and Guy 1980; van den Hoven *et al*. 1985).

TABLE 1: Mean (± s.e.) and P values of the total neutrophil counts, serum CK, plasma MPO and ELT in 7 endurance horses before (T0) and 2 h after (T1) a 120 km endurance race

n = 7	T0	T1	P value
Neutrophils (10^6 cells/ml)	4.11 ± 0.27	12.02 ± 1.07	0.001
Serum CK (iu/l)	191 ± 20	2667 ± 734	0.015
Plasma MPO (ng/ml)	158.5 ± 25.6	852.9 ± 197.6	0.011
Plasma ELT (ng/ml)	12.4 ± 4.23	105.7 ± 31.4	0.021

TABLE 2: Individual values of muscular myeloperoxidase (MPO) and elastase (ELT) concentration and serum creatin kinase (CK) before (T0) and 2 h after (T1) a 120 km endurance race

	Muscle MPO (ng/mg)		Muscle ELT (ng/mg)		Serum CK (iu/l)	
	T0	T1	T0	T1	T0	T1
Horse 1	14.00	120.00	9.76	41.76	192	4202
Horse 2	31.75	357.79	11.95	111.17	288	4326
Horse 3	4.31	28.22	19.35	22.27	218	711
Horse 4	3.36	718.07	7.44	300.93	115	5398
Horse 5	4.00	89.20	0.10	23.14	185	2154
Horse 6	7.10	23.90	6.14	8.14	195	777
Horse 7	4.40	265.29	1.92	16.11	147	1106
Mean	9.8	228.9	8.4	75.4	191	2667

Several studies in animal and man have assessed the effect of acute exercise on neutrophilic infiltration into muscles. Neutrophil infiltration as demonstrated by microscopy is not a consistent finding. Some studies proposed a role of neutrophils by demonstrating MPO with biochemical assays although they failed to demonstrate neutrophils infiltrated into the muscle (Schneider *et al*. 2005).

Our study demonstrated an increase of MPO and ELT in the muscular tissue of the horses sampled 2 h after a 120 km endurance race. Further, their increase was significantly correlated to the increase of CK, considered as a marker of muscular damage (Volfinger *et al*. 1994).

Elastase, a serine protease, is widely recognised as a component and marker of inflammatory disorders (Jochum *et al*. 1994; Langhorst *et al*. 2008). Elastase contributes to the tissue remodelling that occurs after injuries (Shapiro 2002; Chua and Laurent 2006). Elastase is able to digest elastin and other extracellular compounds (Shapiro 2002) and may also promote the extravasation of the neutrophils through the endothelial barrier (Scholz *et al*. 2003). This enzyme plays also a role in the ischaemia-reperfusion injury (Bzeizi *et al*. 1996; Okajima *et al*. 2004; Aoki *et al*. 2005). Interestingly, inhibition of ELT attenuates MPO activity in post ischaemic skeletal muscles suggesting a cooperation of ELT and MPO in muscle injuries (Carden and Korthuis 1996). It has been shown that naturally-occurring ELT inhibitors can be inactivated by oxidation by the MPO-mediated production of reactive oxygen species (ROS) (Matheson and Travis 1985; Deby-Dupont *et al*. 1994). Therefore, the generation of ROS may provide a positive environment for ELT activity by inactivating antiproteases. Myeloperoxidase is not the only source of ROS. In recent years it has been suggested that ROS such as superoxide anion and its byproducts hydrogen peroxide and hydroxyl radical are involved in the damage of muscles and other tissues induced by strenuous exercise. The electron transport associated with the mitochondrial respiratory chain is considered the major process leading to ROS production at rest and during exercise (Di Meo and Venditti 2001). Recently, the rate of production of ROS by mitochondria has been estimated to be approximately 0.15% (Brand *et al*. 2005).

Apart from ROS generated by mitochondria, NO represents a major ROS found in the muscle. Its rate of production is increased by contractions (Balon and Nadler 1994). Skeletal muscle normally expresses the neuronal and the endothelial isoforms of NO synthase (NOS). Neuronal NOS is strongly expressed in fast-twitch muscle

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fibres and appears to be the prime source of the NO released from skeletal muscle (Lau *et al*. 2000). Endothelial NOS is central to skeletal muscle metabolic regulation and enzymatic signalling during exercise (Lee-Young *et al*. 2010).

In addition to the exercised-induced production of reactive nitrogen and oxygen species, the increase of plasmatic MPO concentrations could have induced the more severe muscle damage which was observed in some of the horses studied. To exert its oxidant activity, MPO needs chloride anion and hydrogen peroxide (H_2O_2), this latter resulting from the dismutation of superoxide anion, a free radical species derived from O_2. By using H_2O_2 and chloride anion, MPO is able to generate hypochlorous acid and to chlorinate most of the organic molecules (Deby-Dupont *et al*. 1999). MPO also uses as substrate another peroxide, peroxinitrite ($ONOO^-$), which derives from a free radical species, nitric oxide (NO), generated by NOsynthase. The activity of MPO on $ONOO^-$ produces NO_2, a free radical responsible for lipid (O'Donnell *et al*. 1999) and protein nitration (mainly on tyrosine residue) (Eiserich *et al*. 1998; Deby-Dupont *et al*. 1999). Therefore, the accumulation of MPO in equine muscle, as observed in this study, may participate in cell membrane alterations leading to disruption and CK release. Our hypothesis is supported by a study of Liao *et al*. (2010) who reported increasing levels of MPO and lipid oxidation markers 2 h after exercise in rats.

The individual values reported in Table 2 indicate that not all horses showed the same intensity of muscle inflammatory response. This could partly be explained by the fact that all horses are not providing the same effort during the same competition because of their innate capacity or their level of training. It has been shown in man (Pyne 2005) as well as in horses (Wong *et al*. 1992; Hines *et al*. 1996; Horohov *et al*. 1996, 1999) that the effect of exercise on neutrophil function is largely influenced by age, gender, initial fitness levels and the intensity and duration of the exercise protocols used. Therefore, the effect of exercise type and intensity and the impact of training state on exercise-induced systemic and muscular inflammatory response should be investigated further in future studies; especially on a larger number of horses.

Conflicts of interest

The authors have not declared any conflicts.

Manufacturers' addresses

[1]Vacuette, Greiner-Bio, Kremsmünster, Austria.
[2]Medonic CA530, Clinical Diagnostic Solutions, Plantation, Florida, USA.
[3]Roche Diagnostics, Vilvoorde, Belgium.
[4]Angiotech, Gainesville, Florida, USA.
[5]Astra Zeneca, Brussels, Belgium.
[6]Sigma-Aldrich, Bornem, Belgium.
[7]BiopTis, Liège, Belgium.
[8]Fisher Scientific, Tournai, Belgium.
[9]Nunc, MaxiSorp, Roskilde, Danmark.
[10]Medcalc, Merelbeke, Belgium.

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