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Dynamic modulation of platelet aggregation, albumin and nonesterified fatty acids during physical exercise in Thoroughbred horses

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ABSTRACT

The effect of exercise on platelet aggregation, albumin and nonesterified fatty acids (NEFAs) values and the correlation among these parameters were evaluated in ten clinically healthy and regularly trained Thoroughbred horses. All horses were subjected to two simulated races. Blood samples were collected by jugular venipuncture before and after the first simulated race ($T0_{PRE}$ and $T0_{POST}$), every 7 days at rest condition for a month (T_{1R}–T_{2R}–T_{3R}), and before and after the second simulated race ($T4_{PRE}$ and $T4_{POST}$) in order to assess platelet aggregation, albumin and nonesterified fatty acids (NEFAs) levels. One-way analysis of variance showed a significant effect of exercise ($P < 0.01$) on platelet aggregation, albumin and NEFAs values. A negative correlation between platelet aggregation and albumin or NEFAs values, and a positive correlation between albumin and NEFAs values, were found both at $T0_{POST}$ and $T4_{POST}$ ($P < 0.05$). These findings are likely related to dynamic physiological adaptations to exercise that allow re-establishment of the homeostatic equilibrium of the organism.

1. Introduction

Physical exercise induces various stress responses and metabolic adaptations. Similarly to other stressors, it needs adequate responses to re-establish homeostatic equilibrium. Depending on the intensity, duration and type of physical exercise, equine metabolism has to adapt to nervous, cardiovascular, endocrine and respiratory system requirements. In horses, exercise and training are known to have considerable effects on the mechanisms of the hemostatic system involving platelet activity (Sakita et al. 1997; El Sayed et al. 2000; Piccione et al. 2005).

Platelet responses to exercise depend on several factors, including exercise intensity, duration and training condition (Petidis et al. 2008). The exact mechanisms and the regulatory pathways involved in the effects of exercise on platelet function are not completely understood. Studies carried out on the influence of exercise on platelet aggregation produced conflicting results. Some authors reported that strenuous exercise results in increased platelet aggregation (El Sayed et al. 2004; Giordano et al. 2010; Assenza et al. 2013), whereas others reported unchanged or decreased platelet aggregation in response to exercise (Piccione et al. 2014a).

The efficiency of platelets at the sites of vessel wall injury is dependent on the synergistic action of various adhesive and soluble agonist receptors, with the contribution of each of the individual receptors dependent on the prevailing blood flow conditions (Jackson et al. 2003). The activation of platelets is regulated and modulated by several well-characterized factors, including adenosine diphosphate (ADP) (Pelagalli et al. 2002).

Despite the well-characterized factors modulating platelet activity, several studies carried out on experimental animal models and in humans have shown that albumin and nonesterified fatty acids (NEFAs) have an opposite role in the activation of platelets and thrombosis (Dhindsa et al. 2015). Particularly, high levels of NEFAs lead to massive thrombosis and platelet activation (Connor et al. 1963; Burstein et al. 1978), whereas their binding to albumin protects the platelets from activation (Nordoy 1979). Modifications on albumin levels or morphology would potentially result in an impaired binding to NEFAs and an increase in unbound NEFAs and thus to platelet hyperactivity. NEFAs induce oxidative and inflammatory stress and activate monocytes (Tripathy et al. 2003). Activated monocytes express the extrinsic pathway of thrombin generation precipitating thrombosis. Therefore, any condition that leads to an increase in NEFAs or a decrease in plasma albumin concentration or a combination of the two, would contribute to platelet hyperactivity.

It is well demonstrated that albumin and NEFAs levels are influenced by exercise (Hinchcliff et al. 2004; Piccione et al. 2007, 2014b). Albumin is the main protein of mammal serum and it is essential for the regulation and keeping of oncotic pressure or osmotic pressure necessary for the proper distribution of body fluids in the vascular compartment and in tissues (Piccione et al. 2007). This function acquires great importance during exercise, when there are substantial fluid shifts out of plasma as a result of flow and hydrostatic pressure changes

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(Piccione et al. 2007). In addition, albumin serves as a carrier for molecules of low water solubility including lipid-soluble hormones, bile salts, free fatty acids, calcium, ions and some drugs (Kaneko et al. 2008).

Many reports have been published on the effects of physical exercise on hemostasis, protein and lipid profiles in horses; however few studies included in the same experimental protocol the simultaneous evaluation of albumin, NEFAs and platelet aggregation. Moreover, little information is reported in the literature about the effect of albumin and NEFAs on platelet activity as well as the correlation between albumin, NEFAs and platelet aggregation in the athletic horse.

On the basis of such considerations, the aim of this study was to evaluate how the maximum degree of platelet aggregation, albumin and NEFAs values change in response to exercise and training in Thoroughbred horses. In addition, the correlation among these parameters was evaluated in order to clarify if and how platelet aggregation was modulated by albumin and NEFAs in the athletic horse.

2. Material and method

2.1. Animals

All treatments, housing, and animal care were carried out in accordance with the standards recommended by the European Directive 2010/63/EU for animal experiments.

Ten clinically healthy and regularly trained Thoroughbred horses (five geldings and five males with an age ranging from 3 to 5 years and a mean body weight of 395 ± 16 kg) were enrolled in this study with the informed consent of the owner.

The horses were deemed healthy if they did not have a history of change in hemostatic mechanisms and if their physical examination as well as packed cell volume and platelet count where within normal limits. No pharmacological treatment was administered for one month prior to the study.

The horses enrolled in the study carried out fitness training 6 days a week with a rest day on Sundays. Training included walking (30 min), trotting (20 min) and galloping (5 min).

All animals were housed in individual boxes (3.50 × 3.50 m) under natural spring photoperiod (sunrise at 06:00 h, sunset at 18:00 h), at an indoor temperature of 18–21 °C.

All animals were fed standard rations consisting of hay (first cut meadow hay, sun cured, late cut, average 8 kg/horse/day) and a mix of cereals (oats and barley, 50% each, about 3.5 kg/horse/day) provided three times a day (at 7:00, 12:00 and 19:00). Water was available ad libitum.

2.2. Experimental design

At the beginning and at the end of the experimental period (30 days), all horses were subjected to two simulate 1660-m races with an average speed of 800 m/min (T0 and T4). Throughout the experimental period, animals carried on a specific training program for 6 days a week with a day of rest (Table 1). From each animal, blood samples were collected by jugular venipuncture into: vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) (Terumo Corporation, Tokyo, Japan) in order to verify the number of platelets in whole blood of each animal. Blood samples were also collected in 3.6-mL vacutainer tubes (Terumo Corporation, Tokyo, Japan) containing 3.8% of sodium citrate, maintaining the blood to anticoagulant ratio of 9:1, in order to assess platelet aggregation, and in 9-ml vacutainer tubes containing clot activator (Terumo Corporation, Tokyo, Japan) in order to assess albumin and nonesterified fatty acids (NEFAs) concentration.

Blood collection was performed by the same operator at the same hour of the day, before and after the first simulated race ($T0_{PRE}$ and $T0_{POST}$), every 7 days at rest (T_{1R}–T_{2R}–T_{3R}), and before and after the second simulated race ($T4_{PRE}$ and $T4_{POST}$), within 10 min from the end of exercise.

Table 1
Specific training program of Thoroughbreds horses.

Days of week	Gait	Duration (min)	Speed (m/min)
First and fourth day	Walk	15	100
	Trot	18	300
	Canter	5	100
	Walk	15	100
Second and fifth day	Walk	10	100
	Trot	20	200
	Canter	6	350
	Walk	10	100
Third and sixth day	Walk	15	100
	Trot	20	200
	Galop	3	800
	Walk	10	100

2.3. Laboratory analysis

Blood samples collected in vacutainer tubes containing EDTA were stored at 4 °C pending analyses that were performed within 2 h from blood collection. The number of platelets in each animal was verified by the same operator using an automated hematology analyzer (HeCo Vet C, SEAC, Florence, Italy) and two blood smears for each sample were made and microscopically examined for platelet clumps and visual verification using an optical microscope (Nikon Eclipse e200, Nikon Instruments Europe BV, Amsterdam, Netherlands). A manual platelet count was performed to verify and validate the automated count.

To determine platelet aggregation, platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were obtained by centrifugation. The PRP was obtained by centrifugation at 300 rpm for 20 min at room temperature. About two thirds of the PRP was carefully removed and transferred into coagulation cups (Seac Clot 2, Florence, Italy). After removing the PRP, the remaining blood was centrifuged at 3000 rpm for 10 min to obtain the PPP. The minimal and maximal amplitudes of the aggregometer (Seac Clot 2, Florence, Italy) were adjusted with PRP (0% transmission) and PPP (100% transmission).

The same operator verified the number of platelets in obtained PRP that resulted ranging from 2.8- to 4.5-fold increase over whole blood according to previous studies carried out on equine species (Piccione et al. 2008, 2010, 2014a; Fontenot et al. 2012; Bazzano et al. 2015).

Platelet aggregation was tested with ADP (Mascia Brunelli S.p.a., Milano, Italy) at 1 μM concentration as agonist to promote platelet activation. To perform the test, 15 μL of ADP was added to 290 μL of PRP while stirring.

Platelet aggregation response was evaluated using the following two parameters: the maximum degree of aggregation and the initial velocity of aggregation (slope). The maximum degree of aggregation was determined by measuring the maximum height of the aggregation wave over a 4-min period, beginning at the onset of platelet aggregation. The maximum degree of aggregation was expressed as a percentage (%) of the maximum possible change in light transmission. The slope was determined by drawing a line tangent through the steepest linear part of the aggregation tracing, and determining the slope from one point along the curve. The slope of this tangent was expressed in percentage/min (%/min).

The tubes without anticoagulant agent, after standing at room temperature for 20 min, were centrifuged at 3000 rpm for 10 min and the obtained sera, not lipemic or hemolyzed, were analyzed for the determination of albumin and NEFAs concentration using commercially available kits (albumin, Byosistems, Reagents and Instruments, Barcelona, Spain; NEFAs, Randox, Crumlin, UK) by means of an automated analyzer ultraviolet–visible spectrophotometer (model Slim SEAC, Florence, Italy).