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Original Research

Training Program Intensity Induces an Acute Phase Response in Clinically Healthy Horses

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ABSTRACT

Physiological and hematochemical changes associated with exercise have been extensively investigated in equine species. It is known that stress elevates circulating levels of acute phase proteins (APPs). This survey evaluated whether horses trained with different training programs exhibit changes in APP levels after exercise event. Twenty Saddle Italian horses (11 geldings and 9 females, 9 ± 1 years old, body weight of 425 ± 35 kg) were divided into two equal groups according to the intensity of training programs they were subjected: group A was subjected to an intense training program, group B was subjected to a moderate training program. At the end of the training period, horses were subjected to a simulated exercise event (show jumping course of 400 m length with 12 obstacles). From horses, blood samples were collected at rest conditions (T_{REST}) and after 12 and 24 hour from the end of exercise ($T_{12\ h}$ and $T_{24\ h}$); the concentration of serum amyloid A (SAA), haptoglobin, albumin, total proteins, iron, and fibrinogen was assessed. The circulating levels of SAA, fibrinogen, and iron were influenced by simulated exercise event ($P < .01$), starting from 12 hour after the end of exercise, suggesting the onset of an acute phase –like response, and it would seem that training program intensity the horses underwent also affected the degree of response, although only SAA values were significantly different between groups ($P < .001$). The findings obtained suggest that jumping exercise induces an acute phase response; however, further studies are advocated to better evaluate mechanisms by which exercise activates this response in the athletic horse.

1. Introduction

Physical exercise is one of the most physiologically stressful stimuli an animal can undergo; the type, intensity, and duration of physical exercise [1] together with athletic fitness and training level [2] have a great influence on the changes occurring in the athlete's body. Several cardiovascular and hematological adaptations are necessary to guarantee the correct supply of oxygen to active muscles during exercise.

Physiological, hematological, and biochemical changes associated with exercise have been extensively analyzed in several types of horses such as thoroughbreds [3,4], horses competing in cross-country events [5,6], show jumpers [7–10], and endurance horses [11,12]. One of the organs affected by exercise is the muscle, which suffers microdamage due to effort employed load [13].

Exercise that results in muscle damage also initiates a well-orchestrated response that ultimately results in the repair of the damaged tissue. Indeed, exercise that may result in a great deal of muscle damage, such as lifting and lowering a heavy weight, also has the capability of producing skeletal muscle hypertrophy, which ultimately decreases the risk of future muscle damage from that particular exercise. Tissue under stress condition and/or damage, similarly to an infection condition, initiates a sequence of defense

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reactions, known as acute phase response (APR) [14]. The onset of an APR is correlated with the duration and intensity of physical exercise. The dynamic process of APR involves systemic metabolic changes, part of systemic nonspecific defense before the triggering of the specific immune response [15].

The complicated but precise regulation network of APR depends on the proinflammatory mediators among which the cytokines play very important roles since initiating the APR cascade through stimulation of several cell types [15,16]. A central pathophysiological step of the APR is hepatic synthesis and, as a result, increased plasma concentrations of some acute phase proteins (APPs) known as positive APPs including haptoglobin, C-reactive protein, serum amyloid A (SAA), ceruloplasmin, fibrinogen, and alpha-1-acid glycoprotein. Other proteins involved in an APR show a decrease in levels in response to challenge and are known as negative APPs which include albumin, the most abundant constitutive plasma protein.

It has been proved that a prolonged physical load on rats resulted in inflammatory response accompanied with increased concentrations of proinflammatory cytokines IL-6 and TNFα, believed to induce APR [17]. A systemic inflammatory response, mild transient endotoxemia, leukocytosis, enhanced leukocyte expression of TNF-α, IL-1β, and IL-6 mRNA, and increased circulating TNF-α and PGF2α concentrations have been found in horses after physical exercise with most pronounced changes 2 hours after exercise [18]. Although it has been reported that moderate physical exercise could be beneficial for health while strenuous load induced an inflammation-like state [18–20], the literature data about the effects of exercise on APR in horses are limited, and the mechanisms of exercise-induced APR are still unclear.

The current survey aimed to determine whether horses trained with different training programs would exhibit any changes in systemic APR including circulating acute phase proteins SSA, haptoglobin, albumin, iron, and fibrinogen levels after a simulated exercise event. The obtained evidence-based data would improve the current knowledge on APR during exercise in horses and the possible usefulness of APP measurements for evaluation of physical exercise severity in horses during training and competitions.

2. Materials and Methods

2.1. Animals and Study Design

Protocols of animal husbandry and experimentation were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments.

The study was carried out on 20 clinically healthy horses (Italian Saddle) from the same training center Located in Sicily (Italy). Before starting the study, horses were subjected to clinical examination and routine hematology and biochemical analysis at rest conditions, to insure of their healthy status. All animals were housed in individual boxes (3.50 × 3.50 m) under natural spring photoperiod (sunrise at 6 AM, sunset at 6 PM) and 18°C–22°C indoor temperature. The horses were fed three times a day (7:00 AM, 1:00 PM, and 7:00 PM) with standard ratios constituted of hay (first cut meadow hay, sun cured, late cut, 8 kg/horse/day, 6.9% crude protein on average) and a mixture of cereals (oats and barley, 50% each about 3.5 kg/horse/day): dry matter 86.36% (9.11% horse's digestible protein, 13.05% crude protein, 20.7% crude fiber and 3.42% crude protein) and moisture 13.63%; water was available *ad libitum*.

All horses (11 geldings and 9 females, 9 ± 1 year old, average body weight of 425 ± 35 kg) were sedentary animals at enrollment time and were randomly divided into two equal groups according to the intensity of training programs they were subjected (Table 1). Briefly, the horses of group A (n = 10) were subjected to an intense training program, whereas horses of group B (n = 10) were subjected to a moderate training program. At the end of the training period, horses of both groups were subjected to the same simulated exercise event: animals performed a show jumping course of 400 m length with 12 obstacles (7 verticals and 5 large of 1.30 ± 0.10 m in height).

2.2. Blood Sampling and Laboratory Analysis

From each horse, blood samples were collected by jugular puncture at rest conditions, before exercise text (T_{REST}), and after 12 and 24 hours from the end of exercise ($T_{12\ h}$ and $T_{24\ h}$). Blood samples were collected into two different vacutainer test tubes: a test tube containing clot activators (Terumo Corporation, Tokyo, Japan) and a test tube containing 3.8% sodium citrate (Terumo Corporation, Tokyo, Japan).

After storage at room temperature for 20 minutes, the vacuum tubes containing clot activators were centrifuged at 1,308 × *g* for 10 minutes using the Thermo Scientific CL10 centrifuge (Thermo Fisher Scientific Inc, Waltham, MA) to obtain serum samples. The concentration of total proteins (TPs), albumin, iron, haptoglobin, and SAA was determined from the obtained sera using commercially available kits. An automated UV spectrophotometer (Slim, SEAC, Florence, Italy) was used to determine the concentration of albumin and TPs, whereas iron concentration was determined with colorimetric method (Wako Pure Chemical Industries) with an automated analyzer (Model 7070, Hitachi LTD, Tokyo, Japan). The concentration of SAA and haptoglobin was determined using enzyme-linked immunosorbent assay (ELISA) kits previously validated in equine species [21,22] (Multispecies SAA ELISA kit, and Phase Haptoglobin kit, Tridelta PhaseTM range, Maynooth, Ireland)

Table 1
Training program performed by group A (n = 10 horses subjected to intense training program) and by group B (n = 10 horses subjected to moderate training program).

Days of week	Gait	Duration (min)	Obstacle Height (m)
Group A			
I and III	Walk	5	
	Trot	30	
	Canter (400 m/min)	20	
	Obstacle	1	0.90 (n = 8)
	Walk	5	
II, IV, and VI	Walk	5	
	Trot	25	
	Canter (400 m/min)	25	
	Obstacle	1	0.80 (n = 6)
	Walk	5	
V	Walk	5	
	Trot	30	
	Canter (400 m/min)	20	
	Obstacle	1	1.20 (n = 13)
	Walk	5	
VII	Rest	—	
Group B			
I and III	Walk	5	
	Trot	30	
	Canter (350 m/min)	20	
	Obstacle	1	0.90 (n = 4)
	Walk	5	
V and VII	Walk	5	
	Trot	30	
	Canter (350 m/min)	20	
	Obstacle	1	1.20 (n = 6)
	Walk	5	
II, IV, and VI	Rest	—	

Each training section per day lasted 1 hr.

by means of a microtiter plate reader (EZ Read 400 ELISA, Biochrom, Cambridge, UK). All calibrators and samples were run in duplicate. Samples exhibited parallel displacement to the standard curve. The intra-assay coefficient of variation for haptoglobin and SSA was <7% and <5%, respectively.

The vacuum tubes containing sodium citrate were centrifuged, within 15 minutes from blood collection, at 908 g for 15 minutes (Thermo Scientific CL10 centrifuge; Thermo Fisher Scientific Inc). From the obtained citrated plasma samples, fibrinogen concentration was measured using a commercial standard kit made especially for Clot 2 automatic coagulometer (SEAC, Florence, Italy). The assay procedure consisted of placing 200 μL of diluted plasma (diluted 1:10 by the combination of 100 μL of plasma + 900 μL of buffer) in a test tube preheated at 37°C, incubating for 2 minutes at 37°C, and then adding 100 μL of the fibrinogen reagent. Immediately after the addition of fibrinogen reagent, a stopwatch was started, and the clotting time was measured. The time (seconds) until clot formation was automatically converted into mg/dL by the automated mechanical endpoint coagulation instrument.

2.3. Statistical Analysis

Obtained values were tested for normality using the Kolmogorov-Smirnov test. Two-way analysis of variance for repeated-measures procedure was used to determine the statistically significant effects of exercise text and training program on studied parameters. Bonferroni's multiple comparison tests were applied for post hoc comparison. Values of $P < .05$ were considered statistically significant. Data were analyzed using statistical software Prism v. 4.00 (GraphPad Software Ltd, 2003).

3. Results

Obtained data passed the Kolmogorov-Smirnov normality test ($P > .05$). All results are expressed as mean values ± standard deviation (Table 2). Statistical analysis showed a significant effect of exercise test on SAA ($P < .001$), fibrinogen ($P < .01$), and iron ($P < .001$) both in group A and in group B (Fig. 1), whereas the serum levels of TPs, albumin, and haptoglobin remained unchanged between groups throughout monitoring period ($P > .05$).

Briefly, an increase of SAA and fibrinogen has been observed after 12 and 24 hours from the end of exercise test ($T_{12\ h}$ and $T_{24\ h}$) with respect to the baseline values (T_{REST}), whereas iron showed lower values at $T_{12\ h}$ than T_{REST} and $T_{24\ h}$. A statistically significant effect of training intensity was found on SAA concentration (Fig. 1).

Table 2
Mean values ± standard deviation (M±SD) of serum total proteins (TPs), albumin, haptoglobin, serum amyloid A (SAA), iron, and plasma fibrinogen measured at rest, before exercise text (T_{REST}), after 12 and 24 hr from the end of exercise ($T_{12\ h}$ and $T_{24\ h}$) in group A (n = 10, horses subjected to intense training program) and by group B (n = 10, horses subjected to moderate training program).

Parameters	Groups	T_{REST} M ± SD	$T_{12\ h}$ M ± SD	$T_{24\ h}$ M ± SD
TPs (g/dL)	Group A	5.9 ± 0.5	6.0 ± 0.9	6.2 ± 0.4
	Group B	5.7 ± 0.5	5.8 ± 0.3	6.0 ± 0.2
Albumin (g/dL)	Group A	2.8 ± 0.3	2.8 ± 0.4	2.8 ± 0.5
	Group B	2.9 ± 0.4	2.9 ± 0.3	2.8 ± 0.2
Haptoglobin (mg/dL)	Group A	1.9 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
	Group B	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.2
SAA (μg/mL)	Group A	24.0 ± 1.0	41.8 ± 2.3	49.2 ± 1.1
	Group B	23.7 ± 0.9	39.2 ± 1.3	45.5 ± 1.5
Iron (μg/mL)	Group A	172.0 ± 2.1	140.8 ± 6.8	163.1 ± 5.2
	Group B	170.6 ± 2.2	146.9 ± 10.5	166.2 ± 6.0
Fibrinogen (mg/dL)	Group A	156.4 ± 5.6	167.7 ± 9.8	164.1 ± 4.0
	Group B	153.1 ± 4.4	162.6 ± 3.3	161.5 ± 5.6

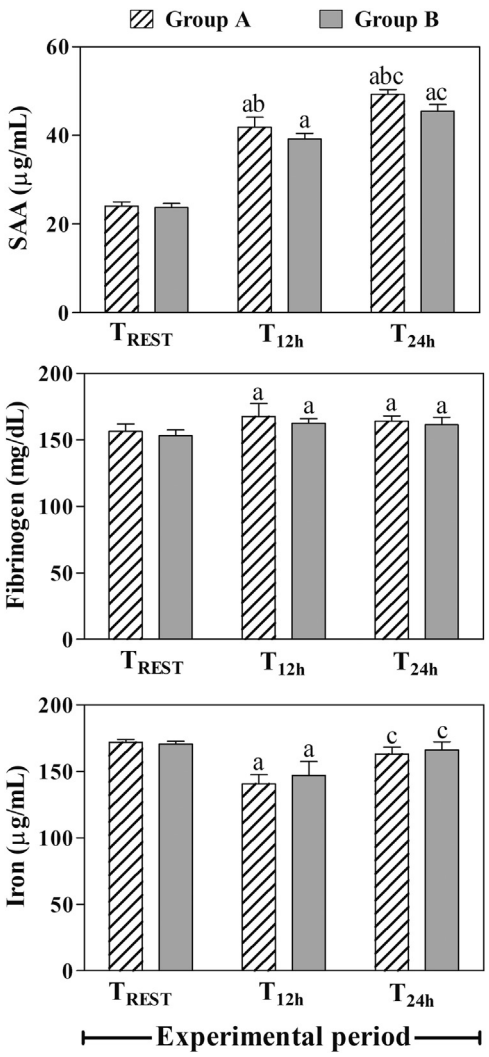

Fig. 1. Trend (mean values ± standard deviation) with related statistical significances of serum amyloid A (SAA), iron, and plasma fibrinogen measured at rest, before exercise test (T_{REST}), after 12 and 24 hr from the end of exercise ($T_{12\ h}$ and $T_{24\ h}$) in group A (n = 10, horses subjected to intense training program) and by group B (n = 10, horses subjected to moderate training program).

In particular, the horses subjected to an intense training program (group A) showed higher SAA levels with respect to the group subjected to a moderate training program (group B) at $T_{12\ h}$ ($P < .01$) and $T_{24\ h}$ ($P < .001$). No significant effect of training was found on the other parameters ($P > .05$).

4. Discussion

The scientific community is currently interested in studying the hematological and biochemical changes resulting from physical exercise to better understand the adaptations that arise during the stressful event such as exercise and to adopt the best measures to arise the upmost of equine athletes' physical performance [20,21]. A deep knowledge of the adaptive responses of the athletes during physical exercise and even better understanding the time of re-establishment of their homeostasis would allow the design the more appropriate training programs to improve athlete's capabilities. It is widely accepted that physical and psychological stress elevates circulating APPs levels both in experimental animals and horses [22–24].

The results obtained in the present study showed that the circulating levels of SAA, fibrinogen, and iron were influenced by simulated exercise event suggesting the onset of an APR and, it would seem that training program intensity the horses underwent also affected the degree of response, although only SAA values were significantly different between groups.

The trend of APPs found in the present study seems to reflect a classical APR in the equine species. Indeed, it is well stated that intense exercise is a noninflammatory condition that is able to induce an APR through the induction of interleukin-6, which increases in blood 30 minutes to 1.5 hours after the beginning of exercise and peaks in a couple of hours [25]. In turn, this could be related to the increase of circulating levels of SAA, which may increase several 100- or 1,000-fold [26]. As a matter of fact, the SAA values measured in the current survey showed a rise after 12 and 24 hours from the end of exercise ($T_{12\ h}$ and $T_{24\ h}$). Among the APPs, the SAA seems particularly suited for real-time monitoring of inflammatory activity [26]. The low or undetectable SAA levels in healthy horses facilitate interpretation of mildly elevated concentrations, and the quick response time and short half-life of SAA cause its blood levels to increase quickly after an inflammatory stimulus has occurred [27] (start increase 6–12 hours, peak 48 hours after inflammatory stimulus) and to decrease in close parallel with successful treatment and/or resolution of disease [28]. This is in contrast to fibrinogen and haptoglobin, which are less suited for monitoring of the disease activity because these APPs are present in high levels in blood of healthy horses, and their amplitude of response is much lower than that of SAA. Moreover, it takes days for their levels to increase after an inflammatory stimulus (start increase 12–24 hours, peak 72–144 hours), and concentrations stay elevated for an extended period after the disease has been resolved [29]. It is established that, during an APR, blood concentrations of fibrinogen will increase modestly (up to 10 times healthy values) and this agrees with the trend found in the present study where an increase of this parameter was observed after 12 and 24 hours from the end of exercise. Coyne et al [30] also showed an increase in blood fibrinogen after exercise. On the contrary, Scoppetta et al [31] reported that after prolonged exertion of horses, fibrinogen concentration was lower. The increase of fibrinogen after exercise could be related to the physical exercise–induced tissue microdamage in muscles, which leads to an inflammation condition associated to local release of IL-6 by macrophages at concentrations, inducing acute phase response [32]. Fibrinogen changes could be also attributed to the effects of cortisol, released during exercise, on acute phase protein response. Cortisol could act synergically with cytokines toward APR activation or toward reduction, when it acts as an anti-inflammatory agent on cytokine production by macrophages and monocytes [33].

According to previous studies [34,35], also serum iron levels were influenced by physical effort in the current survey with decreased values at $T_{12\ h}$ followed by an increase at T24 hours until baseline values. The decrease in serum iron levels observed in both animal groups might reflect an exercise-induced inflammatory response. Indeed, inflammatory cytokines including interleukin-1b, interleukin-6, tumor necrosis factor α are released during inflammation [35] as well as during exercise [34] and lead to an hepatic upregulation of the hormone hepcidin that negatively influences iron transportation and absorption. Iron is a very sensitive marker of inflammation with a fast kinetic and, according to the results herein found, it decreases within hours after an inflammatory insult and return to baseline levels starting from 1 day after inflammation has waned [36,37]. Noteworthy, in the present study, both groups showed the same trend of the analyzed APPs; however, horses subjected to an intense training program showed a statistically significant higher SAA levels and, though not statistically significant, lower iron concentration compared to animals subjected to a moderate training program. Although training has been shown to cause a reduction in the postrun APR in humans [38], thus suggesting that an improved physical condition protects against the exercise-induced APR, the findings herein obtained seem to suggest that an intense training induces an inflammation-like state stronger than a lighter physical activity program [19]. It has been stated that the APR to exercise seems to be related to exercise duration and degree of muscle injury [39,40]. Studies in humans, dogs, and horses that have investigated the effect of acute exercise of a shorter duration at high intensity [41–43] showed that shorter running distances also caused an APR. By contrast, a recent study of horses participating in limited distance endurance rides (34 or 60 km) showed no postrace change in APPs [44]. The APR after acute, short-duration exercise has been investigated to a limited degree in horses. A study carried out on Thoroughbred horses reported no SAA response 1 hour after a 1,600 m race [45], whereas another survey carried out on the same athletic category demonstrating increased fibrinogen concentrations during the last weeks of an 80-d training program suggesting that this could be an effect of both the adjustments to training and subclinical disease becoming manifested over time [46].

Although the APR is recognized for its antibacterial and antiviral actions, it also promotes clearance of damaged tissue, and noteworthy, it sets the stage for repair and growth [14]. These latter activities may be a theological explanation for why an APR is initiated by exercise, recognized as a stressful event, that can result in tissue microdamage in muscles.

5. Conclusion

It is well recognized that APPs are not only useful for monitoring inflammatory processes for diagnostic and prognostic purposes but also for analyzing various noninflammatory conditions.

The results of the present study demonstrated variable effects of physical exercise on some APPs as SAA, fibrinogen, and iron. Noteworthy, the horses subjected to the intense training program showed a stronger increase of SAA levels after exercise event with respect to animals' group subjected to a moderate training program. Although the findings herein obtained suggest that jumping exercise induces an inflammation-like state, further studies are advocated to better evaluate the mechanisms by which exercise activates APR in the athletic horse. It is certain that the raised APPs have any animal health implications; therefore, APP measurements could be useful for evaluation of physical exercise severity in horses during training and competitions and might became a valuable tool for veterinarians and/or trainer for monitoring health status in race and performance horses.

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