

RESEARCH ARTICLE

The Effect of Different Types of Musculoskeletal Injuries on Blood Concentration of Serum Amyloid A in Thoroughbred Racehorses

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Abstract

Background

Training-induced muscle, skeletal and joint trauma may result in acute phase response reflected by the changes in the blood concentration of serum amyloid A (SAA) in racehorses. It remains yet unclear if such systemic reaction could be triggered by sport injuries and what is the impact of different types of musculoskeletal trauma on SAA concentrations in racehorses. This study aimed to determine changes in the SAA blood concentration in racehorses with different types of injuries of musculoskeletal system.

Materials and Methods

The study involved 28 racehorses diagnosed after the race with bone fractures (n = 7), dorsal metacarpal disease (n = 11), joint trauma (n = 4) or tendon and muscle trauma (n = 6) and 28 healthy control racehorses. Serum samples were collected twice, between 1 and 4 days of the injury or successful completion of the race. SAA concentration was measured using the commercial ELISA kit. Differences between mean SAA concentration in respective groups were analyzed using ANOVA and Tukey post-hoc test.

Results

Mean SAA concentration within the first 4 days of the injury of muscle and tendon was significantly higher than in bone fractures, dorsal metacarpal disease, joint trauma or in the healthy horses (p<0,001). There were no significant differences between the other groups.

Conclusions

Strain injuries of muscle and tendons can cause a moderate increase in SAA blood concentration in racehorses, reflecting the occurrence of the acute phase response. Similar reaction is not observed in the stress-related bone injuries.

Introduction

Serum amyloid A (SAA) is the main acute phase protein (APP) in horses, massively released into the bloodstream in the initial phase of inflammation, known as the acute phase response (APR). Elevated blood concentration of the positive APPs is one of the main effects of the APR. Their production in the liver is mediated by the proinflammatory cytokines—interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor α (TNF α), secreted by the phagocytic cells in response to various types of tissue damage [1]. Due to the fast and intensive reaction to cytokine stimulation, the main APPs are considered sensitive biomarkers of the diseases accompanied by systemic inflammation [2,3]. In horses affected by inflammatory disorders of gastrointestinal, respiratory and reproductive system or developing complications after surgical procedures, SAA level can exceed the physiological range from 10 to 1000 times and may be useful in disease diagnostics and prognostication [3–8]. Much less is known about the relation between the APPs and disorders of the musculoskeletal system. Injuries of bone, muscle and tendon resulting from repetitive mechanical overload are the main health issues in performance horses. In racehorses they are responsible for the greatest number of the lost training days [9]. Early recognition of horses on risk of suffering the injury is crucial and the range of blood biomarkers have been examined in that context [10–12].

We previously reported that racehorses with stress-related injuries of musculoskeletal system showed higher SAA levels than the non-injured horses in the 3rd-4th day after the race [13]. The examined group included horses with injuries of different location and pathogenesis and large individual variations in SAA concentration were observed. Our hypothesis is that the ability of the stress-related musculoskeletal injuries to induce the APR, and thus increase the SAA level, could vary depending on the type of the injury and the tissues involved. Therefore, we aimed to compare changes in SAA blood concentration among racehorses diagnosed with different types of stress-related injuries of musculoskeletal system and healthy racehorses subjected to exercise. Unlike most of the reports on naturally occurring injuries, this study analyzed SAA level with regard to the time of acquisition of the injury. The group of injuries of musculoskeletal system associated with the SAA reaction was identified.

Materials and Methods

Horses and injuries

This case-control study involved 56 Thoroughbred racehorses aged 2 to 7 years (median age of 2 years, interquartile range from 2 to 3 years), stabled and regularly trained in different horse racing facilities in Poland. This study was approved by the III Local Ethical Committee for Animal Experiments at Warsaw University of Life Sciences—SGGW (permit number: 68/2013), the trainers and the owners of the horses.

The experimental group consisted of 28 racehorses (median age—2 years, interquartile range from 2 to 3 years) enrolled on the basis of the clinical signs of acute musculoskeletal injury (lameness, heat, swelling, sensitivity to palpation) observed after the race or intensive

training session (breezing), which led to suspension of training for the minimum of 7 consecutive days. Diagnosis of the injuries was based on clinical examination and diagnostic imaging (radiographs and ultrasound) performed by the equine veterinary practitioner. In the days following the injury horses were subjected to stall rest or light exercise (30 min in horse walker) depending on the veterinary recommendations. According to the diagnosis, horses were divided into four groups: bone fractures (n = 7), dorsal metacarpal disease (n = 11), joint trauma (aseptic arthritis) (n = 4) and muscle and tendon trauma (n = 6).

Fractures

All fracture cases included in the study were closed, of nontraumatic aetiology and manifested after intensive exercise. Accordingly, they were classified as stress fractures. The fractures were confirmed by radiography or ultrasound and located in the distal limb (the carpus and lower bones).

Dorsal Metacarpal Disease (DMD)

The cases of DMD were primarily identified by trainers, who recognized acute lameness, heat, sensitivity to palpation and swelling at the dorsomedial surface of the metacarpus in one or both front legs. Diagnostic imaging was requested by trainers in single cases only—therefore, the inclusion criteria in this group involved, besides the clinical manifestation of DMD, missing at least 7 days of regular training due to the condition.

Aseptic arthritis (joint trauma)

Horses allocated in the aseptic arthritis group were identified with unilateral or bilateral joint effusion associated with pain, lameness or reluctance to train, and did not show any bone or tendon abnormalities in radiographical and ultrasound examination. The trainers of the horses refused to perform the analysis of the synovial fluid, however, the acute synovitis emerging after intensive training, lack of radiological changes and remission of clinical signs within several days of rest substantiated the diagnosis of stress-related aseptic arthritis.

Muscle and tendon injury

Cases in this group included tendonitis of the front SDFT, confirmed in the ultrasound examination by the enlargement of the cross-sectional area of the tendon compared with the contralateral leg. Muscle injury was represented by exertional rhabdomyolysis (ER), manifested by muscle pain and firmness developing short after the beginning of exercise and confirmed by the high creatinine kinase and aspartate aminotransferase blood levels.

The control group included 28 healthy Thoroughbred horses (median age— 2 years, interquartile range from 2 to 3 years) that did not have any hematological abnormalities and lacked clinical signs of the injury after completion of the race and in the following days. Control horses were all subjected to the light exercise in horse walker in the sampling period due to the end of the racing season. Hematology control was performed in all horses in order to minimize the possible effect of concomitant health issues other than musculoskeletal injury and as a general health control in the non-injured horses. Racing and training distances for all horses varied from 1200 to 1600 m, accordingly to their age and experience and depended on the horse's trainer.

Blood samples

Blood was collected from injured horses on the 1st (16 horses) or 3rd (12 horses) day after the injury depending when the injury was notified by an owner. Exact time of blood collection is

presented in [Table 1](#). Control horses were blood-sampled on both 1st and 3rd day after the race. All blood samples were acquired by a jugular venipuncture into 20 ml syringes and transferred immediately into K2-EDTA tubes for hematological tests and plain tubes for SAA analysis. EDTA blood samples were kept in +4°C and examined within 5 h for routine hematological parameters in an automated analyzer (ABC Vet, Horiba ABX). Plain tubes were centrifuged at 4380g for 5 minutes, collected serum was frozen and stored at -20°C for SAA analysis. SAA concentrations were measured using an enzyme linked immunosorbent assay (PHASE SAA Assay, Tridelata Ltd) previously validated for use in equine studies [[13–17](#)]. Basic dilution of the samples was 1:1000. Samples with SAA values above the detection limit were further analyzed at the dilution of 1:2000.

Statistical analysis

Two-factor analysis of variance (ANOVA) including two categorical variables as fixed effects—type of injury (5 categories— 4 injuries and a control) and a day of blood collection (2 categories— 1st or 3rd day)—was performed to control for various days of blood collection. To cope with unbalanced study design (unequal replications of categories in 5x2 table) and achieve at least proportional replication of categories [[18](#)] 28 control horses were randomly split in two groups of 16 and 12 horses and blood samples collected from them on the 1st or 3rd day, respectively, were included in the analysis. As data were non-normally distributed according to a Shapiro-Wilk test ($p < 0.001$ for all horses included; strong right-hand skewness—Pearson’s coefficient of skewness = 2.43) as well as heterogeneity of variances was present among groups according to a Levene’s test ($p < 0.001$), natural logarithmic transformation was applied to satisfy assumptions of a two-factor ANOVA (after transformation: Shapiro-Wilk test $p = 0.065$ for dorsal metacarpal disease to 0.999 for joint trauma, Pearson’s coefficient of skewness = -0.02; Levene’s test $p = 0.073$). A Tukey test for unequal samples was used in post-hoc analysis. A p-value below 0.05 was considered to indicate statistical significance. Statistical analysis was carried out in Statistica 10 (StatSoft Inc.). For the needs of reporting results transformed units were converted back to the original ones and SAA concentration was given as median, inter-quartile range (IQR) and range. Morphological parameters, normally distributed according to a Shapiro-Wilk test were presented as the arithmetic mean \pm standard deviation.

Results

Analysis with two-factor ANOVA indicated a significant difference between types of injuries. Moreover, interaction between a type of injury and a day of blood collection was significant ([Table 2](#)). Having taken a day of blood collection into account the mean SAA concentration proved significantly higher in horses with tendon/muscle trauma compared to horses with other injuries ($p < 0.001$). No significant differences were observed between other types of injury ([Table 3](#), [Fig 1](#)). All hematological parameters in all horses remained within the reference ranges for Thoroughbred horses [[19](#)] ([Table 4](#)).

Table 1. Number of injured horses blood sampled on the 1st and 3rd day.

Injury type	1 st day	3 rd day
Bone fracture (n = 7)	3	4
Dorsal metacarpal disease (n = 11)	7	4
Joint trauma (n = 4)	3	1
Tendon/muscle trauma (n = 6)	3	3
Total (n = 28)	16	12

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Table 2. Results of two-factor ANOVA.

Source of variation	df	Mean square	F	p
Type of injury	4	13.49	16.35	<0.001
Day after the race	1	3.03	3.67	0.062
Interaction	4	2.43	2.94	0.030
Error	46	0.83	-	-

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Discussion

Distribution of the injury types within the examined group was typical for the stress-related musculoskeletal lesions in young Thoroughbred racehorses [20]. Our results have shown that SDFT tendonitis and muscle injury, represented in this study by the exertional rhabdomyolysis (ER), are the only injuries of the locomotor system that result in SAA elevation when compared with the control group [3]. Moreover, SAA levels in this group reached the values that are considered indicative for systemic reaction in horses with inflammatory diseases (50 mg/l) [3,21]. ER has been previously reported to cause the radical increase in SAA serum concentration in Arabian horses [17]. Our study included one case of ER that showed the highest SAA value among all of the examined horses (50 mg/l), although lower than the levels described by El-Deeb et al. [17]. This single result does not allow to hypothesize about the influence of breed or type of effort on the SAA level in horses with ER, nevertheless, it confirms the systemic effect of this muscle disorder. Another study by the same research team [22] indicated the significant increase in serum IL-6, TNF- α and PGF2- α in horses affected with ER. IL-6 is the main cytokine produced by the contracting skeletal muscle [23–25]. The release of IL-6 from muscle during exercise in human was suggested to reach sufficient intensity in order to account for its noticeable accumulation in blood [24]. Numerous studies have shown significant increase of IL-6 and other proinflammatory cytokines (IL-1 β , TNF- α , interferon γ - INF- γ) in blood in human and horses after intensive or prolonged exercise [25–31]. Several authors proposed the stress-induced damage of muscle fibers as the main cause of blood cytokine increase after exercise, supported by the positive correlation between cytokines and muscle enzyme concentrations or the delayed onset muscle soreness (DOMS) [22,29,32]. Whereas direct link between exercise-related muscle damage and the induction of the APR still needs to be elucidated, the evidence of local cytokine production in muscle that leads to the systemic response may facilitate search for similar mechanism in other tissues of the musculoskeletal system.

The endogenous production of proinflammatory cytokines in tenocytes stressed by application of cyclical strain, hypoxia and in tendon injuries is well documented [33–37]. Increased expression of IL-1 β , IL-6, TNF α , interleukin 8 (IL-8) and monocyte chemoattractant protein

Table 3. Serum amyloid A concentration in different types of injuries and in healthy horses.

Injury type	SAA concentration [mg/L]
Control (n = 28)	1.73 (0.87–3.25; 0.17–9.44)
Bone fracture (n = 7)	4.66 (1.23–12.45; 1.08–14.85)
Dorsal metacarpal disease (n = 11)	5.23 (2.30–10.06; 0.22–20.61)
Joint trauma (n = 4)	5.96 (4.51–7.95; 3.70–9.29)
Tendon/muscle trauma (n = 6)	40.22 (26.65–50.00; 24.11–50.00)*

Median (IQR; range)

* SAA concentration significantly higher than in the rest of groups (p<0.001)

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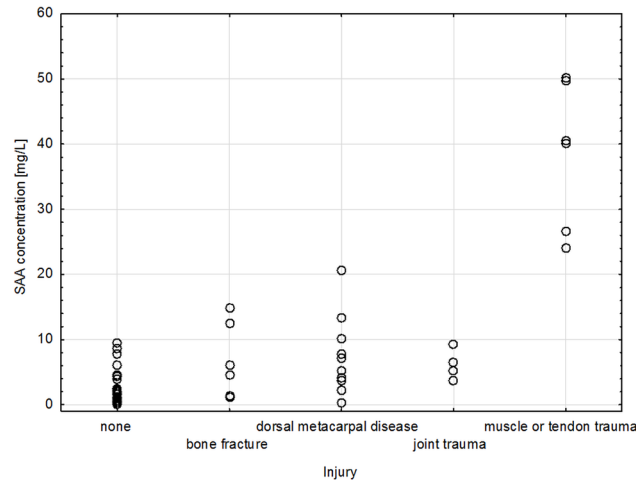


Fig 1. SAA concentration in five groups of horses. * SAA concentration significantly higher than in the rest of groups ($p < 0.001$).

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1 (MCP-1) was observed in rodent and rabbit models of tendon damage, human tenocyte culture and in the injured tendon samples. The only available equine study showed positive immunohistochemical staining for IL-1, TNF- α and INF- γ in the fragments of the inflamed SDFT, although it did not evaluate the IL-6 production [37]. While the auto- and paracrine effects of cytokines in tendon tissue and their role in injury healing is being investigated, it is still unknown to what extent the locally produced mediators of inflammation affect the general homeostasis. Langberg et al. compared the IL-6 concentrations measured simultaneously in plasma, muscle and peritendinous tissue in the human long-distance runners [38]. The results demonstrated that interstitial concentration of IL-6 in connective tissue surrounding the Achilles tendon was approximately 10-fold higher than collected from muscle and corresponded in time with the increase of IL-6 level in plasma. In our study tendonitis of the SDFT, the structure functionally and clinically equivalent to the human Achilles tendon, resulted in the elevated SAA blood level. This finding appears to corroborate the hypothesis that local inflammatory response within the connective tissue of the tendon, can reach the blood threshold necessary to induce the APR. Further investigation of this relationship is warranted.

There are several reports on the SAA response in serum and synovial fluid in horses with naturally acquired or experimental joint disease [39–41]. In the clinical cases of joint disease, SAA concentration tends to increase in horses with an infectious arthritis or suspected of the

Table 4. Hematological parameters in the injured and the control horses.

Injury type	WBC (G/L)	RBC (T/L)	HCT (%)	HGB (g/dL)
Control (n = 28)	6.83±1.54	10.19±1.02	43.90±4.23	14.50±1.37
Bone fracture (n = 7)	8.26±1.60	10.51±1.29	44.63±5.54	13.40±2.52
Dorsal metacarpal disease (n = 12)	9.35±1.79	9.54±1.37	40.19±6.28	11.58±3.10
Joint trauma (n = 4)	9.57±1.73	9.44±0.87	38.56±4.72	11.54±2.22
Tendon/muscle trauma (n = 6)	9.29±3.58	9.96±1.23	43.36±10.45	14.50±3.12

Mean \pm standard deviation

WBC—white blood cell count, RBC—red blood cell count, HCT—packed cell volume, HGB—hemoglobin concentration

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bacterial contamination of the joint but remains low in the non-infectious joint disorders [41]. Our study included four horses diagnosed with the aseptic, stress-related arthritis, typically encountered in young racehorses introduced to the high-intensity training. None of the affected horses showed SAA serum level higher than the control group, presumably due to the mild character of joint inflammation (limited to the synovial membrane of the joint capsule) and lack of the infectious agent.

Cases of bone injuries enrolled in this study showed different degree of severity, however, all of the fractures were categorised as closed, stress-related lesions of the distal limb accompanied with a limited soft tissue damage. Four of them were intraarticular fractures located in the carpus. Neither bone fractures nor periostitis (DMD) resulted in the marked APR, unlike the bone injuries in human and mice [42–44]. To authors' best knowledge, there are few studies on this subject referring to horses. Occurrence of the intraarticular fracture in horses caused the radical increase of IL-6 and TNF- α in the synovial fluid in contrast to the osteoarthritis not associated with fracture [45,46]. The early work on the serum concentration of the moderate APP, fibrinogen, did not show any abnormalities in horses with fractures of the distal leg—however, the authors did not report the time from the injury to the blood collection, so it may be suspected that it was not sufficiently long for fibrinogen to reach its peak concentration [3,47]. In the recent study we tested the SAA level in the 1st and 3rd day after injury. In this time SAA has been reported to reach its highest concentration in the course of the APR [3,8,13,16,39,40,48], therefore, it is unlikely that the SAA reaction in any of the groups in this study was overlooked. The lack of SAA release from the liver in horses with distal bone fracture may suggest that the release of IL-6 on the site of injury is limited compared to the injuries of the soft tissues and as a result, the concentration of this cytokine in blood is too low to stimulate the systemic reaction and SAA synthesis. In stress fractures of the distal limb the mass of the inflamed tissues is smaller than in the injuries of tendon and muscle. Together with the relatively poor vascularization of the bone, it could affect the secretion of locally produced cytokines into the blood, resulting in decreased attraction of the leucocytes to the site of injury and more restrained systemic reaction.

In conclusion, injuries of the soft tissue structures within musculoskeletal system may result in the increased serum SAA concentration in Thoroughbred racehorses, high enough to be interpreted as the APR, while damage to the bone and cartilage does not induce the systemic reaction. On the basis of this observation we cannot determine the cause of this difference, although lower mass of the affected hard tissues and their reduced vasculature may be a limiting factor for the release of locally produced proinflammatory mediators, mainly IL-6, to the blood stream. Injuries of muscle and tendon related to mechanical stress associated with race training can affect the serum level of SAA and should be considered in the interpretation of this biomarker in racehorses.

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Author Contributions

Conceived and designed the experiments: AC AT. Performed the experiments: AT AC AJ AN MS HB. Analyzed the data: MC. Contributed reagents/materials/analysis tools: LW MC AW AN. Wrote the paper: AT MC. Revised and corrected the manuscript: AC AW LW AN MS HB.

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