Praca oryginalna *Original paper*

From stable to stable: how does stress influence the first line of immune defense in sport horses

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Received 06.11.2023 *Accepted 20.11.2023*

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Summary

Stressful factors, including physical exercise, can influence the body's defense mechanisms and can be involved in the etiology and evolution of systemic diseases. The aim of this study was to investigate the influence of various stressors to which sport horses are commonly subjected on the innate immune system. For this, four experimental protocols were designed including different animals, namely: (a) resting horses subjected to everyday medium level exercise (n = 18, average age = 7 years, range: 6 to 11 years), (b) medium course transportation, where the distance of one-way trips represented 270 km (n = 8, average age = 7 years, range: 6 to 9 years), (c) a long course, with a 561 km one-way distance (n = 5, average age = 8.2, range: 7 to 11 years) and (d) pre- and post-competition investigations (n = 9, average age = 7 years, range: 6 to 11). Blood was sampled on heparine (50 UI/ml) before and after the workout or transportation. The phagocytic activity of leukocytes was measured by a carbon particle inclusion test, along with total immunoglobulin/opsonin levels (Ig) (24% Zn sulphate precipitation test) and circulating immune complexes (4.2% polyethylene glycol precipitation test). The results were statistically interpreted by use of Micrososft Excel program (Student's t test). The various types of stress differently influenced the equine athletes. While a moderate daily exercise did not substantially influence the humoral and cellular effectors of innate immunity, transportation exerted stronger beneficial effects based on the distance to the competition site. As a fast acting antimicrobial mechanism, the phagocytosis underwent the most pronounced changes, positively influenced mainly by the short course transportation. While the transportation stress enhanced phagocytosis, the competition decreased the potential of the cells to react, without significantly influencing their numbers. The experiment suggested that management measures, such as age-tailored exercise and maternal care and diet could help in alleviating stress from stable to stable, during the competition season, thus improving animal welfare and allowing a better health forecast.

Keywords: horses, exercise, transportation, phagocytosis, total immunoglobulins, circulating immune complexes

Sports horses, used as career competition animals, are frequently subjected to exogenous and/or endogenous stresses throughout their life, which over time could lead to the onset of severe health matters. Thus, at a systemic level, hyperkalemia, lactacidemia and hyperthermia resulting from competitive effort represent just as many problems. Oxidative stress, studied especially in recent years, can cause either respiratory or systemic alterations. The inadequate quality of inhaled air, transportation, high temperatures and/or humidity, microbial agents can also cause disease (1, 2).

Stressors, including physical exercise, can influence the body's defense mechanisms and can be involved in the etiology and evolution of systemic diseases. Given the complex interrelation between the nervous, endocrine and immune systems, all competitions are accompanied by emotional stress and could have a negative impact on both humans and animal competitors, in terms of their resistance to microbial disease. In equines, moderate intensity physical effort has beneficial effects, but either sustained effort during extended training periods or short-term but intense (competition) exercise, might negatively impact the

horse's health (13); sometimes it may be followed by dramatic consequences in the functioning of the immune system, which lead to immunosuppression (4, 6). This immunosuppression is defined by a decrease in the number of circulating lymphocytes, a decrease in the CD4+/CD8+ ratio, leukocytosis, a decrease in the proliferative response of circulating lymphocytes, changes in the function of NK cells, a decrease in the activity of neutrophils and macrophages and alterations in the concentrations of secretory IgA and IgM, of plasma cytokines and hormones (7, 16, 18).

The equine immune system represents a very sensitive indicator of health and welfare, as in other vertebrates (5). Its innate branch, represented mostly by phagocytes and molecules such as lysozyme, complement, opsonins or properdine, plays a critical role in the first line of defense by eliminating bacteria, viruses or other aggressors at mucosae or skin level, once mechanical barriers are overcome. Phagocytosis represents one of the first line protection mechanisms, with particular significance especially in preventing the penetration into the host of bacteria and viruses (11, 14).

Of phagocytes, the macrophages smoothen the way for launching the mechanisms and effectors of the adaptive immune response (T and B lymphocytes).

Transportation represents one of the major events amidst the life of competition of equine athletes and it could be either short or long duration, depending on the competition venue. Production loss and transport-related diseases were studied mainly in horses raised for meat, where the presence of numerous coexisting factors was recognised (15). Nevertheless, little is known about the impact of the duration of transports, long or short (14), on the innate immune system, its effectors and intervention mechanisms.

The aim of the study was to investigate the changes in phagocytic activity, immunoglobulins' levels and the complexation activity, indicated by the level of circulating immune complexes, used as a measure of the host's potential to clear microbes during the resting period, short and long term transportation to competition venue and during Eventing in continuously trained sport horses, as a measure of their susceptibility/resistance variations due to the competition cycle.

Material and methods

Horses were included in the experiment based on their experience in competitions and training program, most of them being involved in Eventing (Dressage, Cross Country, and Show jumping). Four experimental protocols were designed, namely: (a) for resting horses subjected to everyday medium level exercise (n = 18, average age = 7 years, range: 6 to 11 years), (b) medium course transportation, where the distance of a one-way trip represented 270 km (n = 8, average age = 7 years, range: 6 to 9 years), c) a long course, with a 561 km one-way distance (n = 5, average age = 8.2, range: 7 to 11 years) and d) pre- and post-competition investigations (n = 9, average age = 7 years, range: 6 to 11). Blood samples were obtained by jugular vein puncture from all animals, whenever possible within the legal frame of pre competition testing performed by the Veterinary authority. For the traveling animals, samples were taken before departure and upon arrival to the home stable. For the immediate pre- and post-competition samplings, the official veterinarian of the competition supervised the sampling. All maneuvers were carried out with regard to animal welfare and protection (EU Regulation 2016/429). Blood was sampled on heparine (50 UI/ml) at the specified moments of the experimental protocols.

Leukocyte subpopulations, N/L index. Blood was smeared and stained according to the usual technique and stained with Fast Panoptic (ITW Reagents International). Subpopulations of the leukocytes were counted and the N:L ratios were calculated as an indicator of the stress level caused by the effort of the athlete.

Carbon particle inclusion test (phagocytic activity) (9, adapted). For that, 0.450 ml portions of heparinized blood were mixed with 2 µl of India ink supernatant, obtained by centrifugation at 1308 g for 60 min (Hettich, Germany). Subsequently, 0.150 ml of the mixture were transferred immediately to 2 ml of saline and the rest was incubated for a total of 60 min at 37°C, with 0.150 ml being extracted at 45 and 60 min of incubation. All tubes containing saline plus the blood+India ink mixture were centrifuged at 419 g and the supernatants were read spectrophotometrically (λ = 535 nm, d = 0.5 cm). The decrease in absorbance over time, as the carbon was phagocytized was expressed as the phagocytic activity index and was calculated as the difference between the natural logarithms of the optical densities of the phagocytosis 45 and 60 min divided by time.

Circulating immune complex measurements (9, adapted). The measurement of the levels of circulating immune complexes (CIC) stands for the molecular clearance capacity of the host. The blood sera, after clotting of the blood for 30 min at 37°C were removed and kept at –20°C until tested. The precipitating agent for CIC most frequently used is a 4.2% polyethylene glycol (PEG) solution in borate buffer. The precipitation test was was completed in a 96-well-plate, while 196.7 µl of borate buffer and PEG solution were mixed each with 3.3 µl of each of the sera, in duplicate. After 60 min allowed for precipitation, the results were read spectrophotometrically (λ = 450 nm, d = 0.5 cm) (multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). The CIC values expressed as optical density units (ODU) were obtained by substracting from the optical densities of PEG treated samples those of the control buffer treated pair sera.

Immunoglobulin measurements (9, adapted). To quantify the total Ig levels in equine sera, a volume of 6.6 µl of each serum was mixed with 193.4 µl of a 0.024% barbital buffer zinc sulphate solution for 30 minutes at 22-23°C, in 96-well plates, in duplicate. Optical densities (ODU) were then read by spectrophotomety (λ = 475 nm, d = 0.5 cm).

Statistical analyses. Average values and standard error were calculated by use of Excel program. Student's t test was applied to evaluate the statistical significance of the differences, $p < 0.05$ was considered significant.

Results and discussion

Horses represent unique creatures, whose development based on "flight-fight" reaction to the environmental threats created the utmost efficient athlete. Nevertheless, this proficiency came at a high cost, sometimes with deleterious effects on both the animal's physiology and psychology (2). Coping daily with environmental factors which interfere with survival can lead to adaptation or permanent prejudice to horses, pushing them beyond their physiological limits, such as extended high difficulty exercise (4).

The complexity of the mammalian immune system relies on its double sided, innate and adaptive, yet intricate structure and functionality. The direction adaptive immunity takes, that is the cooperation of macrophages and T and B lymphocytes, depends on the initial stages of the phagocytic and pro-inflamatory responses. Antimicrobial protection in mammals is mainly based on adaptive immunity, with the mechanisms subject to external influence of various stresses (8).

Recent studies indicated that changes resulting from one demanding exercise involve the decrease in IgA concentrations, chemotaxis and chemiluminescence of phagocytes but not the mitogen-induced blastogenesis of lymphocytes (20). Training of high intensity but short duration has similar effects to long duration, prolonged and ample exercise or over exercising in suppressing the immune response (7). The longer the exercise period or its intensity is, the deeper the immune suppression of immunity becomes (7, 16, 17).

Researchers have indicated that the N : L ratio could be a more reliable indicator of transportation stress when compared to blood cortisol levels, due to its increase, caused by the reduction in lymphocyte numbers (12). Nevertheless, no significant differences between costisol levels and N : L ratios were found between two groups of horses subjected to differentiated transportation treatments: 24 h continuous transportation or 12 hour transport – 12 hour break – 12 hour transport.

It has been indicated that the N : L ratio is variable on the workout type in different horse breeds (1.143 – draft, 1.836 – leisure and 1.288 – endurance), exercise increases these values (1.256 – draft, 2.389 – leisure and 1.473 – endurance, respectively), supporting the influence of the stressor on this parameter (19).

The values of the N : L ratio in resting horses in this experiment reached 1.54 ± 0.45, when compared to that calculated for competition (before: 1.46 ± 0.05 and after: 1.11 ± 0.034) and showed no statistically significant differences either versus each other, or versus values cited in literature, except the one for leisure. No values were obtained from transported horses in this experiment. It is possible that the individual differences or the small group size led to lower post exercise values in this experiment.

Phagocytosis has been described as one of the first lines of defence in mammalian immune response, leading to desintegration of microbes as well as subsequent antigen presentation. Opsonins, also known as total immunoglobulins (Ig), play a crucial role in microbial clearance by enhancing the complement activity and phagocytosis. The phagocytic cells, but also the lymphocytes, respond very promptly to stresses, excercise included, with the intensity of the replica depending on the length of the stresfull action (7). They are activated by surface pathogen structures (pathogen-associated molecular patterns, PAMPs) but also internal stimuli released by cell damage or inflamation such as (damage-associated molecular patterns, DAMPs) therefore they are present very fast at the injury site (3, 10).

Research carried out on racing horses, after completion of an 80 km distance track, indicated an increase of serum cortisol and circulating monocytes' and neutrophiles' numbers; however, lymphocyte numbers, phagocytes' oxidative burst process and glucose levels significantly ($p < 0.05$) decreased till after three days subsequent to the competition (17).

The phagocytic activity per minute in this study encountered high variability by the investigated category. While in resting animals, the values did not change between samplings, the transportation induced an increased per minute phagocytosis, with higher values in equine exposed to shorter distances and significantly lower values in animals exposed to longer distances ($p < 0.01$). A decrease in phagocytosis was also observed due to the competition stress, where probably the number and variety of stress factors was higher (Fig. 1). It is visible that the short course transportation ranked first among the four situations differing by type of stressor and induced the highest per minute value of phagocytosis, followed by long course transportation.

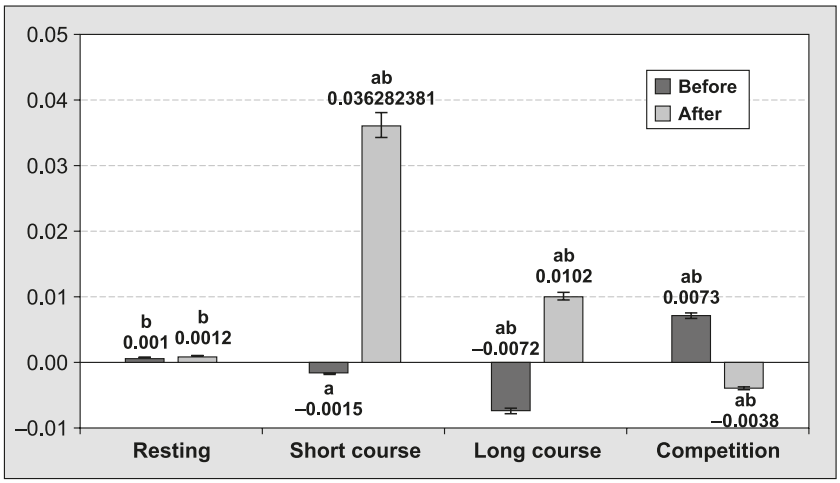

Fig. 1. Phagocytic activity by minute depending on the protocol
Explanations: a – indicates the statistical significance of the differences $p < 0.05$, 0.01, 0.001 within the category; b – $p < 0.05$, 0.01, 0.001 between initial and final values of different categories

Apparently, the phagocytosis decreased as per minute value during the competition effort, standing for a sharper decrease of functionality of phagocytes. The mechanism underlying these processes remain to be investigated.

Total immunoglobulin levels increased subsequent to exercise, although the differences between initial and final values within each category were not significant between transportation duration, but differed significantly between the competition and all other segments, where $p < 0.001$ (Fig. 2). Interestingly, both types of transportation enhanced phagocytosis, while the competition effort depressed it. This might be due to the intensity of the stressors' action, where the competition effort, although short, was probably more intense than the transportation one.

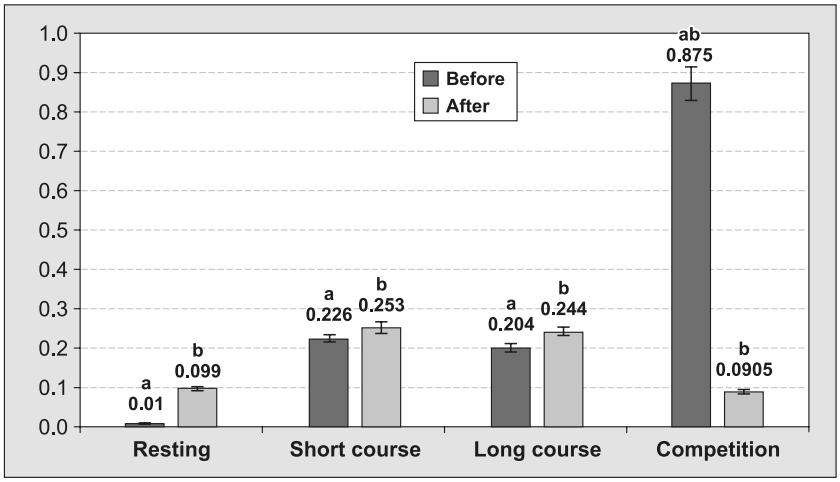

Fig. 2. Total immunoglobulin levels depending on the protocol
Explanations: a – indicates the statistical significance of the differences $p < 0.05$, 0.01, 0.001 before; b – $p < 0.05$, 0.01, 0.001 between initial and final values of different effort categories

The very high values of CIC in resting horses are connected with their low levels of total immunoglobulins. The clearance process is enhanced by transportation and also by competition (Fig. 3), suggesting an improved antimicrobial potential. The levels of CIC generally indicate the potential of the host to clear any kind of antigen, relying on the opsonine pool as binding immune globulin, rather than on specific antibodies. In the present study, this test was used as an indicator of the equine host to clear any potential microbial aggressor, and not to test the specific immunity against one or the other of potential microbes present. As such, the process seems to be impeded by any type of effort, although the differences were non-significant between the groups exposed to stress, standing for a reduced activity of opsonins, given their relatively high values (Fig. 2). A larger size of the study batches coupled with a dynamic study of the CIC levels, including several sampling moments during the stressful event could be of help in obtaining a more relevant picture of this parameter.

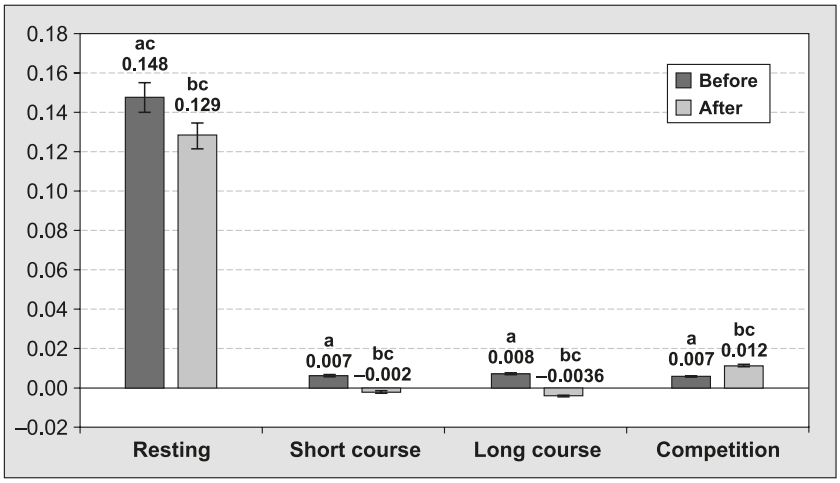

Fig. 3. Circulating immune complexes depending on the protocol
Explanations: a – indicates the statistical significance of the differences $p < 0.05$, 0.01, 0.001 before; b – $p < 0.05$, 0.01, 0.001 after; c – between initial and final values

Conclusion. The effects of various types of stress differently affected the innate immunity of equine athletes. While a moderate daily exercise in resting horses did not substantially influence the humoral and cellular effectors of immunity in their response from before the daily effort till after that, transportation exerted stronger beneficial effects based on the distance to the competition site, on both phagocytosis and total Ig levels. As a fast acting antimicrobial mechanism, the phagocytosis underwent the most pronounced changes. While the transportation stress enhanced phagocytosis, the competition effort, without significantly influencing the numbers of cells, decreased their potential to react. Nevertheless, the experiment suggested that personalized management measures, including the maternal care and diet along with exercise regime designed to be adequate to the age of the foal when envisaging a sport career for it, could help in alleviating stress from stable to stable, and mainly during the competition season, thus improving animal welfare and allowing a better health forecast.

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