ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS IN STRENUOUSLY TRAINED HORSES REVACCINATED AGAINST EQUINE HERPES VIRUS 4/1 AND EQUINE INFLUENZA VIRUS

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Abstract. The objective of the current paper was to study the impact of physical exercise on electrophoretic migrational profile of serum protein fractions in horses revaccinated against equine herpes virus 4/1 (EHV 4/1) and equine influenza virus (EIV).

Total protein concentration in serum was determined by the biuretic reaction using a commercial kit. Determination of protein fractions was performed through microelectrophoresis of the serum on a buffered agarose gel with pH 8.6.

Fifteen healthy Hanoverian stallions were used and separated into three groups – group A (3 non-vaccinated horses), group B (6 revaccinated horses) and group C (6 revaccinated and submitted to physical exercise horses). Group C horses performed barrier jumping for 4 consecutive days, beginning from day 14 following revaccination.

In horses of Group A there were no statistically significant changes in the studied serum protein fractions. In Group B, the concentration of total globulins and γ-globulins was above the reference range on day 1 (corresponding to day 18 after revaccination), resulting in the stimulation of the immune response.

Compared to horses from Group B, the combined effect of the revaccination and the physical exercise in Group C led to statistically significantly higher concentrations of β_1 – globulins on hour 2 and day 4, and increased albumin and total protein on hour 0 as well and to lower β_2 – globulins 2 hours after physical exercise, but all parameters were within reference intervals. Total globulin concentrations throughout the study period (except on day 4) and of γ-globulins on day 1 after physical exercise rose above the reference intervals.

The results obtained from this research show that the physical exercise of horses revaccinated against EHV 4/1 and EIV has a modifying effect on serum proteins, without suppressing the protective function of γ-globulins. This is important when assessing the health condition of revaccinated horses during training and parcourt competitions.

Keywords: electrophoresis, horses, physical exercise, revaccination

Introduction. Protein electrophoresis is a reliable technique for identifying protein components of plasma or serum in human or veterinary medicine and laboratory animal medicine (Matthews, 1982; Vavricka, 2009; Zaias, 2009; Cavalcante et al., 2012). Changes in the levels of protein fractions provide early and useful diagnostic and prognostic information for the defence systemic response to acute inflammation, malignancy, trauma, necrosis, infarction, burns and chemical injury (O'Connell et al. 2005; Atherton et al., 2013). However, serum electrophoresis is rarely used in equine medicine.

The study on the changes and individual protein fraction ratios allows for differentiation of diseases even when total protein concentration, an important clinical sign by itself, remains unchanged (Kamyshnikov, 2000).

A complex assessment of changes in all expressed protein fractions in a proteinogram has a particular importance in diagnosing diseases of the internal organs. Proteinogram can be used as a nonspecific parameter of animal health at the norm-pathology level (Yerokhina, 2009; Gerou-Ferriani et al., 2011). Measuring the serum protein fractions can be useful not only in identifying sick animals (Ahmadmahmudi et al., 2012; Tothova et al., 2012), but also in studying the severity and dynamics of the pathological process (Cavalcante et al., 2012).

Estimating total protein, albumin and globulin concentrations can provide information on horses' hydration status, state of infection, inflammation, increased protein loss, or decreased protein production (Rose et al., 1994). Moreover, these parameters are useful in assessing the fitness state during exercise and exhaustion during and after competition, so that horse athletes can be protected from diseases (Piccione et al., 2008). In most cases it is commonly believed that during continuous physical exercise, a dysfunction of the whole protein content is observed as a final result. It is realized through a reduction in the rate of protein synthesis and increased protein breakdown in liver (Dohm, 1986).

There are few studies on the activation of protein metabolism during physical exercise in racing horses, as well as on the possible role of individual amino acids in the beginning of their fatigue. Some of them investigate changes in amino acids during different types of physical exercise (Assenza, 2004; Bergero et al., 2005), while others explain total protein changes (Ahkenazi et al., 1998; Piccione et al., 2007). There are few data on the combined impact of the physical exercise and vaccination on blood serum protein fractions of these animals.

The aim of the present study is to investigate the electrophoretic migration profile of serum protein fractions in horses revaccinated against influenza and herpes virus and submitted to physical exercise.

Material and Methods

Horses

For research, we used fifteen healthy Hanoverian stallions aged 4–9 years. Their body mass was between 400–600 kg. Horses were kept in suitable living

conditions inside the stalls of Trakia University Experimental Equine Base in Stara Zagora, and fed on a diet of commercially available pellets and alfalfa-grass hay. Salt and water were offered ad libitum. Until the experiment, the horses had not been subjected to a regular training routine or strenuous physical exercise. Horses were walked free on a paddock near the stalls for several hours a day.

Horses were divided into three groups: group A (3 non-vaccinated horses, controls), group B (6 horses with revaccination), and group C (6 horses with revaccination and physical exercise).

Revaccination

Horses from groups B and C were revaccinated by administering 1 ml oily adjuvated vaccine against influenza and herpes virus infection intramuscularly in the neck (Fluvac EHV 4/1 Plus, Fort Dodge, Iowa, USA). The same animals had received a prior vaccination against the above mentioned viruses a year before that. Controls (group A) were treated with an equivalent volume of sterile physiological saline as a placebo in syringes identical to those containing vaccine.

All procedures were performed in accordance with the Bulgarian animal welfare and protection legislation.

Physical exercise

The following physical exercise program was applied: preliminary warm-up 15 min walk at 100–120 m/min, 15 min trot at 250 m/min and 5 min canter at 350 m/min. Horses jumped over barriers 7 times initially, then 15 more in partcourt as the barrier height was gradually raised from 90 to 110 cm. The height was increased to eliminate the hypothalamic-pituitary-adrenal axis habituation resulting in attenuated responses. Horses were subjected to a peak exercise similar to a competition for four consecutive days, between 8 a.m. and 11 a.m. beginning on day 14 after revaccination at 19–21°C and relative humidity of 55–65%. Subsequently, they were not subjected to strenuous physical exercise.

Blood collection

Blood samples were taken from *v. jugularis externa* in tubes without anticoagulant before feeding. Samples were kept at a room temperature for 2 hours in order to coagulate. After that, they were centrifuged at 1200 x g for 10 min, and the serum was obtained. Serum samples were neither lypemic, nor hemolysed. For study purposes we have preferred serum to plasma, to avoid fibrinogen interference. The serum was used to determine total protein concentration and protein fractions before, immediately (0 hour) and on 2nd hour, relevant to 14th and 17th day after revaccination; also, on 1st, 2nd, 4th and 11th day after physical exercise, which corresponded to 18th, 19th, 21st and 28th day after revaccination.

Biochemical analysis

Total protein concentration was determined in serum by the biuret reaction: formation of a purple complex of proteins and cuproions in alkaline medium. A photometric colorimetric test of Human GmbH (Wiesbaden, Germany) was used.

Protein fraction determination was performed through microelectrophoresis of the serum from tested animals on a buffered agarose gel (Merck, Germany) with pH 8.6. Electrophoretic migration was performed for 35 min at 20°C and electric current of 10–12 mA for each sample and voltage of 150V. After migration, the gel was stained with Amido Black staining solution (Sigma-Aldrich, Germany); afterwards, it was destained in a solution of methanol, ice cold acetic acid and water (40:10:50) and dried completely. Protein fractions were quantified by densitometer (Carl Zeiss, Jena, Germany). The main protein fractions migrated towards the anode at a different speed in the following order: albumin, α_1-, α_2-, β_1-, β_2- and γ- globulins.

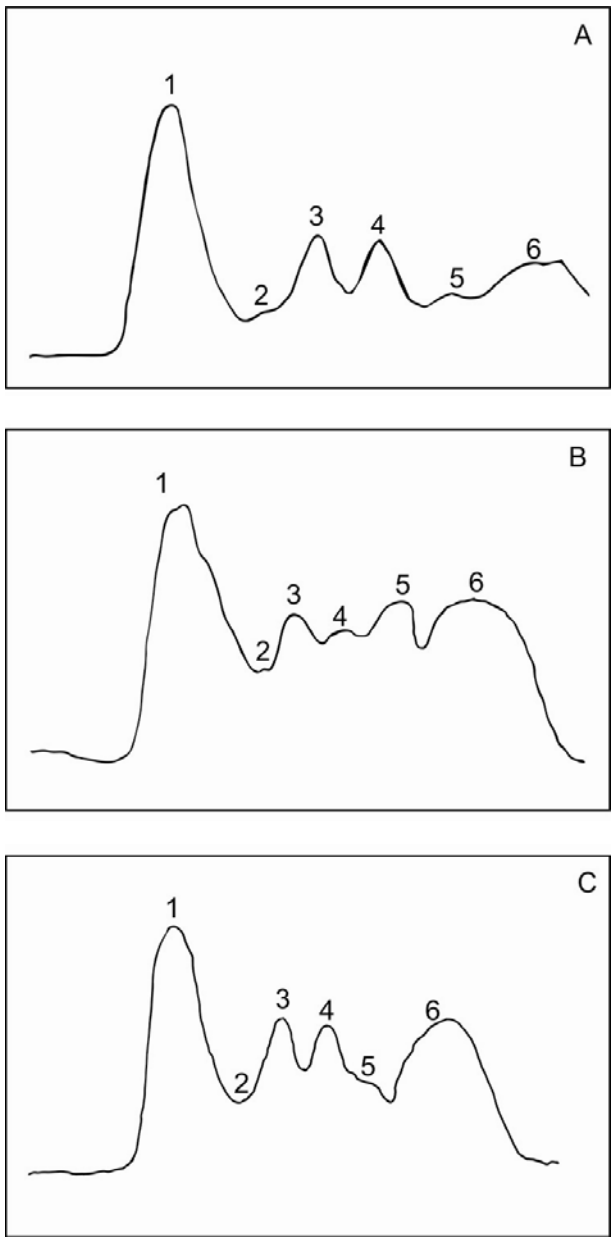

Fig 1. **Electrophoretic patters of serum proteins**: A. healthy horse from group A; B. horse from group B and C. horse from group C 1 day after physical exercise demonstrating increase in γ-glubulins. 1: albumin; 2: α_1-globulin; 3: α_2-globulin; 4: β_1-globulin; 5: β_2-globulin; 6: γ-glubulins.

The absolute concentration of individual fractions presented in g/L was calculated from the percentage obtained by densitometering each protein fraction and the amount of total protein in the sample. The total globulin concentration in serum and albumin/globulin (A:G) ratio were calculated from the total protein and serum albumin concentration.

Statistical analysis

Results were statistically processed by one-way analysis of variance (ANOVA) and presented as a mean (SE) at statistical significance of $P<0.05$. An unpaired t-test was used to analyze the differences between the groups.

Results

The control horses (Group A) exhibited the normal equine serum electrophoretic pattern consisting of albumin, α_1-, α_2-, β_1-, β_2- and γ-globulins (Fig. 1A). Statistical analysis results obtained for different protein fractions of serum electrophoresis in horses were presented in Tables 1, 2 and 3. No statistically significant differences vs initial level for α_1-globulins in horses of Group A were detected. In Group B, which included revaccinated horses, there was a slight increase of α_1-globulins up to day 1. Their concentration decreased afterwards, resulting in a statistically significant value vs initial level on day 4 ($P<0.05$). Horses from Group C showed a slight decrease of this parameter between 0 hour and day 11 vs initial level without statistical significance.

α_2-globulins in group A did not change significantly during the whole period of the study. In Group B, this parameter exhibited a change similar to that of α_1-globulins from this group – a slight increase up to day 1, and from day 2 to day 11 – a statistically significant decrease vs initial level on day 4 ($P<0.05$). A statistically significant difference between groups B and A for this parameter was present on day 4. In Group C, α_2-globulins decreased on day 2 ($P<0.05$), day 4 ($P<0.001$) and on day 11 ($P<0.05$) vs initial level. A statistically significant difference ($P<0.05$) was detected at initial level between groups B and C for this parameter and on day 4 between groups A and C.

β_1-globulins in horses from Group A did not exhibit any significant changes versus the initial level. In Group B, they decreased until the end of study, statistically significantly vs initial level on days 4 and 11. The dynamics of this parameter was similar in horses from group C, with a statistically significant decrease on days 4 and 11. Initial β_1-globulins values were statistically significantly higher than those of group A ($P<0.05$). In this group, the parameter was considerably higher versus group B on hour 2 and on day 4 ($P<0.05$).

No statistically significant changes were noted in the concentrations of β_2-globulins in the three studied groups versus initial level. However on hour 2, in comparison with the control group, a statistically significant increase ($P<0.05$) was observed in revaccinated horses, while in Group C, the decrease ($P<0.05$) in the concentration of this parameter versus group B was marked.

Table 1. **Changes in the concentration of protein fractions, globulins, total protein and A:G ratio in Group A horses** (n=3). Results are presented as mean (SE). Level of significance: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$ versus initial level

Analytes	Days after revaccination						
	14		17	18	19	21	28
	Time intervals after the end of physical exercise						
	Initial level	0 hour	2 hour	Day 1	Day 2	Day 4	Day 11
	Group A (controls)						
α_1-globulins (g/L)	5.09 (0.24)	4.86 (0.37)	5.0 (0.06)	5.46 (0.44)	4.98 (0.16)	4.95 (0.43)	5.61 (0.28)
α_2 – globulins (g/L)	9.81 (1.27)	9.95 (1.03)	9.38 (0.94)	10.34 (0.86)	9.44 (0.96)	9.12 (0.18)	9.21 (0.86)
β_1-globulins (g/L)	6.89 (0.83)	7.89 (1.04)	7.17 (0.92)	7.64 (0.51)	8.26 (1.15)	6.83 (0.94)	8.48 (1.44)
β_2 –globulins (g/L)	6.31 (0.75)	6.43 (0.67)	5.99 (0.5)	6.15 (1.05)	6.38 (0.78)	7.06 (1.93)	7.59 (1.0)
γ-globulins (g/L)	16.43 (0.68)	17.31 (0.98)	16.32 (0.96)	17.87 (0.95)	16.69 (0.91)	16.58 (0.42)	16.88 (1.37)
Albumins (g/L)	27.49 (1.84)	28.07 (0.98)	28.66 (0.88)	26.73 (1.13)	27.13 (1.94)	27.4 (0.36)	27.58 (1.05)
Globulins (g/L)	44.85 (1.71)	44.27 (0.62)	42 (2.0)	41.93 (2.01)	43.8 (2.22)	42.53 ±3.73	43.75 (3.42)
Total protein (g/L)	72.33 (1.2)	71 (1.53)	70.67 (1.45)	68.67 (0.88)	70.67 (1.45)	69.67 (4.18)	71.33 (3.84)
A:G ratio	0.62 (0.06)	0.64 (0.02)	0.69 (0.05)	0.64 (0.06)	0.62 (0.06)	0.66 (0.05)	0.64 (0.05)

A:G – albumin:globulin ratio

Table 2. **Changes in the concentrations of protein fractions, globulins, total protein and A:G ratio in group B horses** (n=6). Results are presented as mean (SE). Level of significance: [a]P<0.05; [b]P<0.01; [c]P<0.001 versus initial level; [1]P<0.05; [2]P<0.01; [3]P<0.001 between groups A and B in each interval

Analytes	Days after revaccination						
	14		17	18	19	21	28
	Time intervals after the end of physical exercise						
	Initial level	0 hour	2 hour	Day 1	Day 2	Day 4	Day 11
	Group B (revaccination)						
α_1-globulins (g/L)	5.84 (0.53)	6.06 (0.65)	6.12 (0.54)	6.08 (0.43)	4.81 (0.35)	4.47 (0.27)[a]	4.8 (0.33)
α_2 – globulins (g/L)	9.17 (0.32)	10.72 (0.99)	9.58 (0.54)	10.86 (0.69)	8.15 (0.52)	6.69 (0.79)[a1]	8.21 (0.5)
β_1-globulins (g/L)	8.21 (0.76)	8.19 (1.04)	8.11 (0.5)	7.55 (0.49)	6.91 (0.73)	5.51 (0.31)[b]	5.98 (0.32)[a]
β_2 –globulins (g/L)	7.66 (0.49)	8.65 (1.04)	8.53 (0.72)[1]	8.67 (0.95)	7.0 (0.71)	5.91 (0.65)	7.72 (0.86)
γ-globulins (g/L)	16.91 (0.81)	17.26 (1.31)	17.62 (0.87)	21.21 (1.24)[b]	14.43 (0.98)	15.58 (1.53)	15.87 (0.75)
Albumins (g/L)	26.1 (1.6)	24.31 (0.82)[1]	24.2 (0.67)[2]	24.96 (0.9)	23.2 (1.24)	23 (1.35)[2]	20.42 (1.17)[c1]
Globulins (g/L)	45.24 (1.15)	48.86 (2.21)	48.8 (2.23)	50.54 (1.99)[a1]	41.3 (1.69)	38.16 (1.71)[b]	42.57 (1.15)
Total protein (g/L)	71.33 (1.67)	73.17 (1.7)	73.0 (1.63)	75.5 (1.4)[2]	64.5 (1.82)[b1]	61.17 (1.24)[c]	63.0 (2.0)[b]
A:G ratio	0.58 (0.04)	0.51 (0.04)[2]	0.50 (0.04)[1]	0.50 (0.04)	0.57 (0.04)	0.62 (0.06)	0.48 (0.02)

A:G – albumin:globulin ratio

Table 3. Changes **in the concentrations of protein fractions, globulins, total protein and A:G ratio in group C horses (with revaccination and physical exercise)** (n=6). Results are presented as mean (SE). Level of significance: [a]P<0.05; [b]P<0.01; [c]P<0.001 versus initial level; [4]P<0.05; [5]P<0.01; [6]P<0.001 between groups A and C in each interval; [7]P<0.05; [8]P<0.01; [9]P<0.001 between groups B and C in each interval

Analytes	Days after revaccination						
	14		17	18	19	21	28
	Time intervals after the end of physical exercise						
	Initial level	0 hour	2 hour	Day 1	Day 2	Day 4	Day 11
	Group C (revaccination and physical exercise)						
α_1-globulins (g/L)	5.62 (1.03)	5.05 (0.32)	5.08 (0.58)	4.92 (0.42)	4.44 (0.22)	4.63 (0.14)	5.11 (0.63)
α_2 – globulins (g/L)	10.63 (0.46)[7]	10.52 (0.69)	9.72 (0.69)	9.82 (0.93)	8.07 (1.05)[a]	6.78 (0.57)[c5]	8.25 (0.56)[a]
β_1-globulins (g/L)	9.67 (0.56)[4]	9.22 (0.7)	9.94 (0.49)[7]	8.76 (1.24)	7.59 (0.66)	7.44 (0.64)[a7]	6.83 (0.58)[b]
β_2 –globulins (g/L)	6.56 (0.88)	6.87 (0.34)	6.58 (0.22)[7]	7.77 (0.66)	6.94 (0.86)	5.09 (0.69)	6.59 (0.70)
γ-globulins (g/L)	15.88 (1.19)	18.69 (0.88)[a]	18.38 (0.56)[a]	20.61 (1.18)[c]	16.75 (0.76)	13.91 (0.73)[5]	15.83 (0.61)
Albumins (g/L)	24.30 (0.63)	27.49 (0.56)[a8]	24.8 (0.59)[4]	22.03 (1.25)[4]	21.38 (0.70)[a]	23.99 (0.99)	20.23 (0.86)[b5]
Globulins (g/L)	48.36 (1.75)	50.34 (0.82)[6]	49.7 (1.25)[4]	54.3 (1.19)[b5]	43.79 (1.16)[a]	37.85 (1.38)[c]	42.61 (0.79)[b]
Total protein (g/L)	72.67 (2.20)	77.83 (0.48)[b4,7]	74.5 (0.92)	76.33 (1.26)[a6]	65.17 (1.01)[c4]	61.83 (1.19)[c]	62.83 (0.95)[c]
A:G ratio	0.51 (0.02)	0.55 (0.02)[4]	0.50 (0.02)[4]	0.41 (0.03)[a4]	0.49 (0.02)	0.64 (0.05)[b]	0.48 (0.02)

A:G – albumin:globulin ratio

No significant changes in γ –globulin subfraction in control horses were observed during the whole study period. In groups B and C, this parameter showed an increase, which was statistically significant for group B on day 1 (corresponding to day 18 after revaccination) and for group C – from 0 hour to day 1. Serum electrophoretic patterns on day 1, demonstrating increase in γ–globulin fraction in groups B and C, are presented in Figs 1B and 1C respectively. A statistical significance between groups C and A was detected on day 4 ($P<0.01$).

There were no changes in albumin concentration in Group A. In Group B, the concentration of this analyte marked a gradual significant decrease on the 11th day ($P<0.001$) versus initial level and on hour 0 ($P<0.05$), 2nd hour ($P<0.01$), day 4 ($P<0.01$) and day 11 ($P<0.05$) versus Group A. In Group C, albumin concentration demonstrated a statistically significant increase on 0 hour ($P<0.05$), followed by a decrease on day 2 ($P<0.05$), and day 11 ($P<0.01$) versus initial level. There was a marked decrease on hour 2, day 1 ($P<0.05$), and day 11 ($P<0.01$), as well as increase on hour 0 ($P<0.01$) versus Group B.

Total globulin concentration calculated through subtracting the albumin concentration from the total protein concentration did not demonstrate significant changes in the dynamics of control horses. In revaccinated horses, a gradual increase with a peak on day 1 ($P<0.05$) was observed, followed by decrease on day 4 ($P<0.05$ versus initial level). Their concentration on day 11 remained lower vs initial level without statistical significance. In the group submitted to physical exercise, this concentration augmented, and reached its highest point on day 1 versus initial level. From day 2 to day 11, globulin concentration was statistically lower than the initial one. Compared to the controls, the total globulin concentration in revaccinated horses was higher on day 1 ($P<0.05$). Statistically significant differences for this parameter in Group C versus Group A were established on 0 hour ($P<0.01$), 2 hour ($P<0.05$), and on day 1 ($P<0.01$).

Total protein concentration in horses from Group A did not alter throughout the studied period. Revaccinated horses showed a statistically significant decrease on day 2 ($P<0.01$), day 4 ($P<0.001$) and day 11 ($P<0.01$) versus initial level. In Group C an increase in the concentration for this indicator was noticed versus initial level on 0 hour ($P<0.01$) and on day 1 ($P<0.05$) while a steady decrease from day 2 to day 11 ($P<0.001$) coincided with the above trend. A significant difference between groups C and A on hour 0 ($P<0.05$), on day 1 ($P<0.001$) and on day 2 ($P<0.05$), was registered, and a similar difference between groups C and B on hour 0 ($P<0.05$) was marked as well.

The A:G ratios in groups A and B were not statistically significantly changed versus initial level. In horses submitted to physical exercise there was a marked reduction of this parameter on day 1 ($P<0.05$) and an increase on day 4 ($P<0.01$) vs initial level. The comparison between groups A and B showed statistically significant differences on hours 0 ($P<0.01$) and 2 ($P<0.05$). Such differences were observed between controls and physically trained horses on hour 0, hour 2 and day 1 ($P<0.05$).

Discussion and conclusions

Serum electrophoresis in healthy horses is characterized by lack of prealbumin stripe and presence of 6 different stripes: albumin, α_1-, α_2-, β_1-, β_2- and γ-globulins (Carapeto et al., 2006). We have observed the same protein spectrum in the serum samples from the three groups studied by agarose gel electrophoresis (Fig. 1).

In the revaccinated group, we have registered a decrease on day 4 for α_1-globulins, α_2-globulins, β_1-globulins, whose values remained within the reference range, reported by Kaneko et al. (2008) and Cavalcante et al. (2012). β_2-globulins varied also within the reference range.

The subfraction of α_1-globulins is associated with acute phase proteins - α_1 antitrypsin, α_1 acid glycoprotein, α_1 fetoprotein, thyroid-binding globulin and transcortin. An increase in the levels of this subfraction has been observed in acute and chronic diseases such as hyperadrenocorticism, diabetes mellitus, and renal failure (Batamuzi, 1996).

Perhaps the administered vaccine did not provoke an inflammatory response during the studied period, which explains the lack of pathological changes in the concentrations of α_1-, α_2-, β_1-, and β_2- globulins in this group. Other authors (Wolf et al., 2008) have also found no statistically significant changes in β- and γ-globulin fractions after vaccination of rhinoceroses with two types of commercial equine vaccines – killed vaccine and recombinant viral-vectored vaccine against West Nile virus infection. Eckersall et al. (2008), however, refuted these data and reported an increase in haptoglobin concentration, which belongs to α_2-globulins with a peak on 24th or 48th hour after revaccination in lambs.

The γ- globulin fraction is composed by immunoglobulins (IgA, IgM, IgE and IgG) (Thrall, 2004; Kaneko 2008; Alberghina et al., 2010). Some authors call on the effect of C-reactive protein, which has often been localized between β- and γ-globulin fractions (O'Conell et al., 2005). Our results show that in revaccinated horses (Group B) there was an increase of total globulins on day 1, corresponding to day 18 after revaccination. This is probably is due to the γ-globulin fraction, which was also increased during this period. A decrease of total globulins within the reference range reported by Kaneko et al. (2008) (26.2-40.4 g/L) was detected on day 4 in Group B. This is probably not related to the production of immunoglobulins from class G, because we have found an increased antibody titre against EHV-1, EHV-4 and equine influenza virus type A_1 and A_2 in a previous study (Goundasheva et al., 2005). It is possible that this decrease might be the result of decreased production of other immunoglobulins, such as IgM, IgA, IgD, as well as of C-reactive protein, included in the γ- globulin fraction.

In Group C horses, there were no statistically significant changes in α_1-globulins; however, there was a marked downward tendency in the period immediately after physical exercise until the end of the study. A

decrease in the concentration of this subfraction has also been reported by Piccione et al. (2007, 2008). The passage of these proteins, which have relatively small molecular weight in the extravascular space, observed by other authors (Coyne, 1990) after acute physical exercise – horse racing – might have led to reduced levels of α_1-globulins. Another possible reason might be the enzyme breakdown of plasma proteins, described in horse racing (Ferguson, 1981).

The observed statistically significant difference between groups C and B for α_2-globulins in the initial level suggested that this parameter varies widely within the reference limits. Other authors (Ahmadmahmudi et al., 2012; Cavalcante et al., 2012; Niedzwiedz et al., 2013) support this view, reporting that the variations in this as well as other protein parameters are influenced by the effects of electrophoresis or genetic, age, geographical and nutritional differences. According to the presented sources, α_2-globulin fraction includes a number of acute-phase proteins such as α_2-macroglobin, haptoglobin, ceruloplasmin, serum amyloid A and α_2-antiplasmin, which increase during inflammation and under the influence of stress factors (Kaneko, 1997; O'Conell et al., 2005). We have found that the level of this parameter in Group C was reduced similarly to Group B (day 4), whilst this decrease had begun earlier – on day 2 and was more prolonged – until day 11. Despite the fact that we did not detect any statistical difference between groups B and C during the studied period, there was a marked impact of the above-mentioned acute-phase protein on α_2-globulin dynamics. Indeed, the pattern of haptoglobin decrease was similar to that of the α_2-globulin fraction (unpublished data).

The amount of β_1-globulins in Group C was higher than that of Group B ($P<0.05$) on hour 2 and on day 4 after physical exercise in horses, but within the reference range, reported by Kaneko et al. (2008) (4.0-15.8 g/L). The β_1-globulin subfraction, including mainly transferrin, hemopexin, plasminogen (Kaneko, 1997; Barrera et al., 2010) and β-lipoproteins (O'Conell et al., 2005), was apparently not affected significantly by the applied physical exercise.

Group C β_2-globulin concentration did not change statistically significantly vs initial level, but was lower ($P<0.05$) on hour 2 in comparison to Group B. Other authors indicate that the most important proteins in the composition of this protein fraction are fibrinogen, C3, C4, C1q complement components and C-reactive protein, which as acute-phase proteins are included in the stress response to external factors (Cavalcante, 2006; Bernabucci et al., 2009).

We found an increase in γ-globulin fraction concentration after physical exercise up to day 1. Similar to our data are the findings of other authors (Coyne et al., 1990) who report an increased concentration of γ-globulin fraction after physical exercise in horses. Furthermore, an increase in α_1-, α_2-, β_1-, β_2- levels has been also registered. An increase in the relative quantity of γ-globulin was noted in long distance horse racing by Piccione et al. (2008).

Group C showed an increase with a peak on day 1 ($P<0.01$), followed by a statistically significant decrease of total globulins vs initial level from day 2 until the end of the study. Except for day 4, total globulin concentration was above the reference values, reported by Kaneko et al., (2008). This is probably related to changes in the intensity of total globulin synthesis as an after-effect of physical exercise. An exception has been found for antibodies against EHV-1, EHV-4 and equine influenza virus type A_1 and A_2, which increase on day 4 and day 11 (corresponding to day 21 and day 28 after revaccination) in revaccinated horses submitted to physical exercise (Goundasheva et al., 2005). Piccione et al. (2009) describe changes in the serum concentration of total globulins after physical exercise, but they have not found any steady tendency in their dynamics.

In this study, we established increased total protein and albumin concentrations probably due to hemoconcentration, occurring after severe perspiration, leading to dehydration after physical exercise in horses. According to some authors (Lucke et al., 1979), the change in plasma albumin is not a correct parameter for dehydration. Stockham (1995) reported that glucocorticoids are known to increase albumin synthesis and cause hyperalbuminemia in different species, including horses. An increased glucocorticoid level after physical exercise has been observed by us as well (Goundasheva et al., 2005). A hyperproteinemia, associated with hyperalbuminemia has been found by other authors (Coyne et al., 1990; Hanzawa et al., 2000) in support of our results.

In Group C, we observed a decreased A:G ratio on day 1, in result of the increase in the serum total globulin concentration, respectively γ-globulin. Coyne et al. (1990) also established a decrease in this ratio after physical exercise in horses. We registered an increase of this ratio on day 4, probably due to a reduced amount of the above-mentioned total globulins, and the restoration of albumin to its initial level.

In conclusion, the present study showed that the administered EHV 4/1 and EIV vaccine to vaccinated horses (group B) did not induce an inflammatory response. The increase in total globulin and γ-globulin concentrations suggested stimulation of the immune response. In revaccinated and exercised horses (group C), physical exercise had an effect but did not suppress the immune response. Therefore, we can affirm that the applied physical exercise on revaccinated horses had a modifying effect on serum protein concentrations. This is an important factor in monitoring the health of physically trained revaccinated horses during training and parcourt competitions.

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