CHANGES IN SOME ACUTE PHASE RESPONSE PARAMETERS AFTER PHYSICAL EXERCISE IN HORSES WITH BOOSTER VACCINATION AGAINST EQUINE HERPES VIRUS 4/1 AND EQUINE INFLUENZA VIRUS

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Abstract. The purpose of the present study was to investigate the effect of physical exercise on acute phase response in horses, vaccinated against equine herpes virus 4/1 and equine influenza virus through easily available and efficient equine pathology biomarkers such as haptoglobin, fibrinogen and erythrocyte sedimentation rate (ESR).

Fifteen healthy Hanoverian stallions, 4 to 9 years of age were divided into three groups – group A – control, including 3 non-vaccinated horses; group B – consisting of 6 revaccinated horses and group C – 6 revaccinated horses submitted to physical exercise (barrier jumping for 4 consecutive days, beginning from post revaccination day 14).

Blood plasma haptoglobin concentrations were assayed by the patented method of ReactivLab (Glasgow, Scotland). Plasma fibrinogen was assayed with a commercial Hemostat Fibrinogen coagulation kit. ESR was determined by the method of Westergren.

The results demonstrated variable effects of physical exercise on the studied blood parameters. It lowered ESR on hour 0 and did not influence blood plasma haptoglobin throughout the experimental period. After one day, physical exercise produced a state accompanied by increased blood fibrinogen (within the reference range) and accelerated ESR.

The combination of ESR and blood fibrinogen levels could be useful for evaluation of physical exercise severity in horses during training and parcourt competitions indicating an occurring disease activity in revaccinated animals.

Keywords: acute phase response, equine vaccination, exercise influence

Introduction. The immune system response to physical exercise consists of numerous changes in non-specific and specific immune defence; however, most of their underlying mechanisms are still unclear. Immunological changes related to natural resistance include signs of inflammation such as release of inflammation mediators – interleukin 1, interferons (Weight et al.1991, Snaders 1995, Ueda et al. 2009) and interleukin 6 in the blood circulation (Meyer et al. 2001, Fäldt J. et al. 2004) and muscles (Liburt et al. 2010), TNFα, $PGF_2\alpha$ in blood (Donovan et al. 2007), activation of different white blood cell types (Scharhag et al. 2005, Buttner et al. 2007), complement system (Scoppetta et al. 2012) and induction of some acute-phase proteins (Northoff et al. 1991, Kazeem et al. 2012, Gondin et al. 2013).

It is currently believed that acute phase response (APR) is a dynamic process involving systemic metabolic changes, part of systemic non-specific defence before the triggering of the specific immune response (Gray 2009, Georgieva 2012). According to some researchers (Moon et al. 2012), 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. After physical exercise of horses, Donovan et al. (2007) established a systemic inflammatory response, mild transient endotoxaemia, leukocytosis, enhanced leukocyte expression of mRNA TNF-α, IL-1β, and IL-6 and increased circulating TNF-α and $PGF_2\alpha$ concentrations with most pronounced changes at failure and 2 hours post exercise. Cappeli et al. (2009) reported that moderate physical exercise could be beneficial for health while strenuous load induced an inflammation-like state. The literature data about the effects of exercise at the background of vaccination on acute phase response in horses are limited. Furthermore, the mechanisms of exercise-induced APR are still unclear. Therefore, the purpose of the present study was to determine whether physically trained horses vaccinated against equine herpes virus 4/1 (EHV4/1) and equine influenza virus (EIV) would exhibit any changes in systemic APR including circulating acute phase proteins haptoglobin and fibrinogen levels and erythrocyte sedimentation rate (ESR).

Material and methods

Horses

Fifteen healthy Hanoverian horses, 4 to 9 years of age, weighing 400–600 kg, were included in the study. Horses were housed in the stalls of the Trakia University Experimental Equine Base in Stara Zagora. They were reared under suitable living conditions (hygienic, safe and comfortable boxes with proper ventilation, under optimum light and temperature regimen) by experienced horse carers. Animals were fed commercial pelleted ration and alfalfa-grass hay. Salt and fresh water were offered *ad libitum*. Until the beginning of the experiment, the horses did not undergo active training and strenuous physical exercise.

The animals were divided into three groups: group A

– control (3 non-vaccinated horses), group B – 6 horses with revaccination, and group C – 6 horses with revaccination submitted to physical exercise. One year prior to the experiment, the horses from groups B and C were vaccinated against EIV and EHV 4/1. Horses were walked free on a paddock near the stalls for several hours a day.

Revaccination

The horses from groups B and C were revaccinated intramuscularly in the neck with 1 ml oil adjuvant vaccine against influenza and herpes virus infection (Fluvac EHV 4/1 Plus, Fort Dodge, Iowa, USA).

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 related to the rearing, vaccination and physical exercise of horses were developed and approved in compliance with Regulation 44/2006 from 20 April 2006 for veterinary medical requirements of animal rearing facilities; the Code of Conduct for the Welfare of Horses of The International Federation for Equestrian Sports (FEI) and Directive 86/609/EEC.

Physical exercise

The following physical exercise programme was applied: preliminary warm-up of 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 parcourt 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 response. Horses were subjected to a peak exercise similar to a competition for four consecutive days, always between 8 and 11 a.m. beginning on day 14 after revaccination at 19–21°C and relative humidity of 55–65%. The physical training of horses did not threat their welfare. All obstacles were designed in a way ensuring the safety of animals. Parcourt was performed by professional jockeys. Subsequently, horses were not subjected to strenuous physical exercise.

Blood samples and analyses

Blood samples were collected from *v. jugularis externa* in heparinized tubes for haptoglobin assay and in tubes with 3.8 % sodium citrate for fibrinogen determination. The two analytes were analysed before exercise (baseline, i.e. the 14th post revaccination day), on hour 2 (17th post revaccination day) and on the 1st, 2nd, and 4th day after physical exercise (18th, 19th, and 21st post revaccination days).

Citrate-anticoagulated blood (3.8 % sodium citrate) was also used for erythrocyte sedimentation rate (ESR) determination. ESR was evaluated from the height of settled red blood cells (in mm) at min 15 and 30, hours 1, 2 and 24 before exercise (baseline), immediately after exercise (hour 0), and post exercise hour 2 and days 1, 2 and 4 corresponding to post revaccination days 14, 17, 18, 19 and 21.

Blood plasma haptoglobin concentrations were assayed by a patented method of ReactivLab (Glasgow, Scotland). The results were obtained with a blood biochemical analyzer (Pentra 400, Horiba ABX). Plasma fibrinogen was assayed with a commercial Hemostat Fibrinogen coagulation kit (Human Diagnostica, Wiesbaden, Germany).

ESR was determined by the method of Westergren recommended by the International Council for Standardization in Hematology as a method of reference. Westergren glass tubes, filled with citrated blood, were placed upright on a special holder. During the analysis, the room temperature did not exceed 25°C.

Statistical analysis

The results were presented as means and standard error (SE). The level of significance was $p<0.05$. The differences between the groups were analysed by unpaired t-test. Correlation coefficients were obtained by the Pearson Correlation test (Statmost for Windows, DataMost Corporation, USA).

Results

The data from haptoglobin, fibrinogen and erythrocyte sedimentation rate assays are presented in Tables 1 and 2.

Control horses (group A) did not demonstrate significant changes in blood haptoglobin, fibrinogen and ESR.

Table 1. **Changes in blood haptoglobin and fibrinogen in horses from group A** (controls, n=3), **group B** (revaccination, n=6) **and group C** (revaccination+exercise, n=6). **Data are presented as mean values** (SE). **Level of significance: $^{b}p<0.01$ versus baseline; $^{8}p<0.01$ between groups B and C**

Parameters	Days after revaccination				
	14	17	18	19	21
	Period after physical exercise				
	Baseline	2 hours	1 day	2 days	4 days
Group A (control)					
Haptoglobin (g/L)	1.44 (0.06)	1.59 (0.15)	1.35 (0.17)	1.43 (0.04)	1.49 (0.14)
Fibrinogen (g/L)	1.43 (0.09)	1.42 (0.06)	1.53 (0.12)	1.52 (0.13)	1.48 (0.10)
Group B (revaccination)					
Haptoglobin (g/L)	1.53 (0.05)	1.70 (0.18)	1.68 (0.12)	1.60 (0.14)	1.55 (0.09)
Fibrinogen (g/L)	1.52 (0.04)	1.52 (0.03)	1.47 (0.08)	1.51 (0.05)	1.51 (0.04)
Group C (revaccination + exercise)					
Haptoglobin (g/L)	1.75 (0.26)	1.42 (0.13)	1.56 (0.10)	1.53 (0.11)	1.49 (0.08)
Fibrinogen (g/L)	1.48 (0.09)	1.57 (0.04)	1.73 (0.02)^{8b}	1.61 (0.04)	1.57 (0.05)

In group B, blood haptoglobin and fibrinogen were neither considerably altered. ESR decreased statistically significantly vs baseline from hour 0 to day 4 as followed: at min 15, 30 and 45 (post exercise days 2 and 4), at hour 1 and 2 (post exercise hours 0 and 2 and days 2 and 4) and at hour 24 (post exercise day 4). The ESR values were also significantly different vs control group at min 15 (post exercise days 2 and 4) and at min 30 on the day after the exercise.

Blood plasma haptoglobin was insignificantly reduced throughout the experiment in group C. On the day following the exercise, fibrinogen increased both vs baseline ($p<0.01$) and vs group B ($p<0.01$), although remaining within the reference range. By post exercise hour 0, ESR decreased statistically significantly vs baseline at all measured intervals except for the 15th min. When compared to group A, substantial differences were established for ESR at min 30 (baseline) and at min 45 (post exercise days 1 and 4). Compared to the group of revaccinated non-exercised horses, ESR values were significantly higher on post exercise days 1 and 4 at all measurement intervals. ESR values at hour 1 for the three studied groups are depicted on Fig. 1.

Table 2. **Changes in erythrocyte sedimentation rate in horses from group A** (controls, n=3), **group B** (revaccination, n=6) **and group C** (revaccination+exercise, n=6). **Data are presented as mean values** (SE). **Level of significance: [a]p<0.05; [b]p<0.01 versus baseline; [1]p<0.05; [2]p<0.01 between groups A and B; [4]p<0.05; [5]p<0.01; [6]p<0.001 between groups A and C; [7]p<0.05; [8]p<0.01 between groups B and C**

Interval	Days after revaccination					
	14	17		18	19	21
	Period after physical exercise					
	Baseline	0 hour	2 hours	1 day	2 days	4 days
Group A (control)						
15 min	17.0 (1.15)	18.0 (1.1)	19.0 (1.2)	20.0 (2.6)	19.0 (2.6)	22.3 (2.6)
30 min	32.3 (5.2)	31.3 (5.2)	33.3 (4.3)	33.7 (4.4)	32.0 (5.0)	34.6 (7.3)
45 min	64.3 (17.4)	67.0 (17.9)	68.3 (13.5)	51.0 (9.6)	47.0 (11.5)	47.6 (3.2)
1 hour	75.0 (17.0)	71.0 (16.5)	74.3 (13.7)	67.6 (11.1)	72.0 (13.5)	73.7 (7.0)
2 hours	92.3 (17.2)	89.0 (18.5)	96.7 (16.5)	85.0 (14.2)	90.3 (11.6)	95.3 (7.4)
24 hours	102.0 (16.9)	103.0 (18.5)	101.0 (9.7)	103.6 (16.7)	104.6 (15.7)	104.3 (7.0)
Group B (revaccination)						
15 min	29.2 (6.5)	15.0 (3.2)	18.7 (2.3)	25.2 (4.2)	9.7 (1.7) [a1]	11.2 (2.5) [a1]
30 min	63.3 (10.5) [1]	37.7 (6.0)	47.3 (4.3)	57.8 (6.0) [2]	25.8 (3.7) [b]	30.0 (6.9) [a]
45 min	81.7 (10.9)	55.7 (7.6)	57.2 (2.9)	65.7 (6.1)	45.2 (5.2) [a]	46.3 (8.2) [a]
1 hour	94.3 (8.0)	67.7 (8.6) [a]	72.5 (5.2) [a]	80.7 (4.7)	61.3 (6.6) [b]	58.7 (9.1) [a]
2 hours	118.2 (7.7)	90.3 (8.5) [a]	91.8 (6.0) [a]	110.5 (4.1)	83.0 (8.6) [a]	84.5 (9.0) [a]
24 hours	133.3 (5.7)	119.3 (5.9)	120.0 (8.8)	124.2 (2.1)	118.3 (4.3)	112.8 (5.0) [a]
Group C (revaccination + exercise)						
15 min	31.3 (7.9)	16.3 (2.1)	14.2 (3.1)	42.0 (5.3) [5,7]	23.2 (7.1)	26.8 (4.9) [7]
30 min	65.5 (12.7) [4]	34.8 (3.3) [a]	49.2 (7.3)	75.2 (6.7) [6]	50.0 (13.3)	52.2 (7.3) [7]
45 min	85.7 (12.6)	51.7 (4.1) [a]	63.3 (8.0)	86.3 (6.5) [4,7]	67.5 (13.4)	71.0 (7.7) [4,7]
1 hour	97.5 (11.7)	64.2 (4.7) [a]	80.5 (8.6)	102.3 (5.6) [8]	84.3 (13.5)	85.3 (7.6) [7]
2 hours	116.3 (8.3)	90.0 (4.8) [a]	108.6 (6.4)	121.8 (3.0) [7]	101.3 (12.1)	112.3 (5.5) [7]
24 hours	135.3 (4.3)	114.0 (4.7) [b]	123.0 (3.6)	128.7 (3.0)	131.5 (5.6)	130.2 (2.3) [8]

Correlations between ESR-1 hour with either haptoglobin or fibrinogen in groups A, B and C are shown in Table 3. In controls, a strong negative correlation was established between ESR and haptoglobin at post exercise hour 2 and day 1, and an even stronger relationship on post exercise day 2. A very strong positive correlation between ESR and fibrinogen was observed on day 1 and a less strong on day 4.

In group B, ESR exhibited a strong positive correlation with haptoglobin on post exercise hour 2 ($r=0.897$, $p=0.015$) and day 4 ($r=0.827$, $p=0.042$). The correlation between ESR and fibrinogen in the same group was negative on day 1 ($r= -0.590$, $p=0.217$).

In physically trained horses from group C, ESR was positively related to haptoglobin from baseline to the 4th day except for day 2 when it was moderate ($r= 0.498$, $p=0.314$). A strong negative correlation between ESR and fibrinogen was observed 2 hours after exercise ($r= -0.631$, $p=0.179$) and the association of both parameters was stronger and negative on day 4 ($r= -0.843$, $p=0.035$), and moderate and positive – on day 1 ($r= 0.383$, $p=0.454$).

Discussion and conclusions

According to our results, blood haptoglobin and fibrinogen in vaccinated horses did not change during the experimental period. Eckersall et al. (2008) demonstrated an immediate post vaccination acute phase response, stimulated via proinflammatory cytokines, which play an important role in the relationship between innate and adaptive immune response. Stimulation of acute phase response after vaccination was also reported by Stokka et

al. (1994). In our study, blood haptoglobin in physically exercised horses declined slightly, but within the reference range described by Eckersall (2008a) (1–2 g/L). The data reported by Weight et al. (1991) and Inoue et al. (2005) showed a statistically significant reduction of blood serum haptoglobin after exercise. Pelegrini Masini et al. (2003) have also established substantially decreased haptoglobin concentrations immediately and 10 min after exercise of horses. Kazeem et al. (2012) demonstrated reduced haptoglobin levels following prolonged exercise and suggested that they could indicate intravascular haemolysis during exercise. However, the research data of Hanzawa et al. (2002) indicated that physical exercise increased total haptoglobin concentrations. According to Gondin et al. (2013), polo exercise was also accompanied by acute phase response of a relatively short duration, manifested via increased haptoglobin, ceruloplasmin and α1-antitrypsin concentrations, and was characterised as non-inflammatory and related to the physical exercise stress.

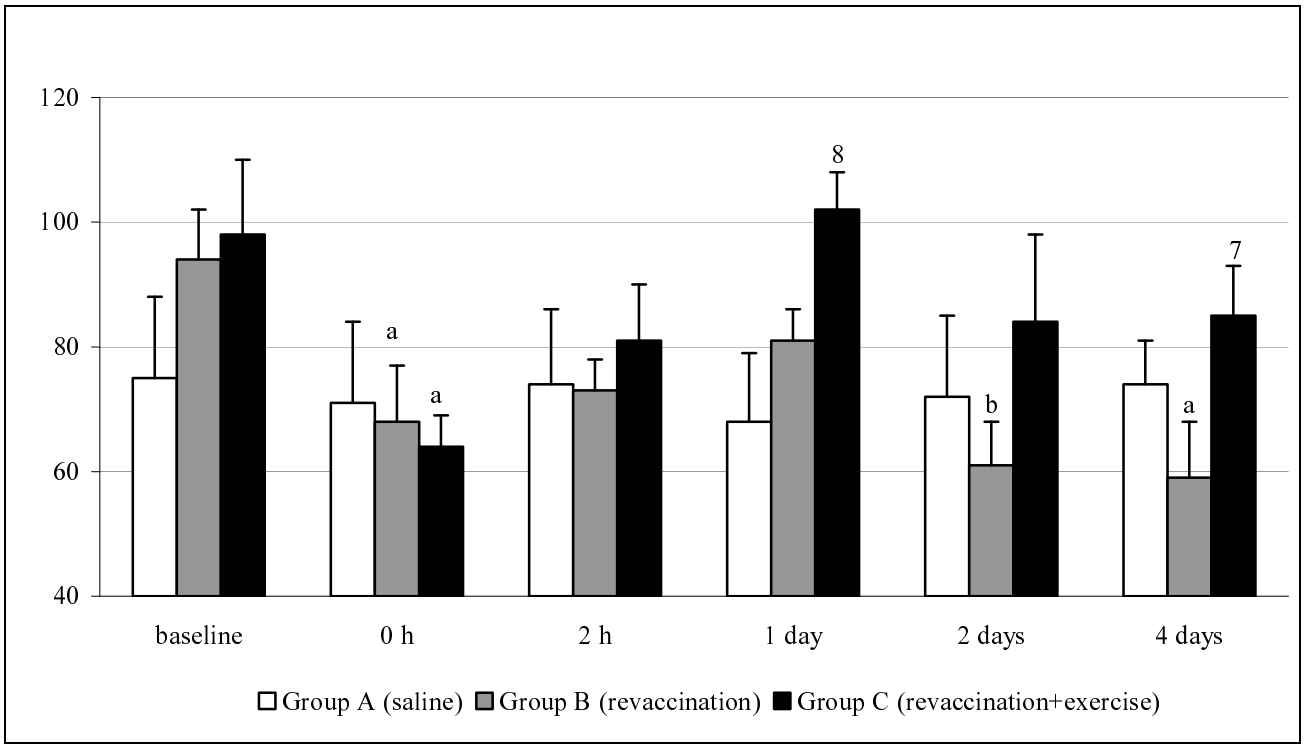

Fig. 1. **Changes in erythrocyte sedimentation rate on the 1st hour** (mm) **in horses from control group A, group B** (revaccination) **and group C** (revaccination+exercise). **Data are presented as mean values and SE. Level of significance: [a]p<0.05; [b]p<0.01 versus baseline; [7]p<0.05; [8]p<0.01; between groups B and C.**

Table 3. **Pearson correlation coefficients** (r) **between erythrocyte sedimentation rate** (ESR 1 h/mm), **blood haptoglobin** (Hp) **and fibrinogen** (Fib) **in horses from group A** (control), **group B** (revaccination) **and group C** (revaccination+exercise)

Parameters and group		Days after revaccination				
		14	17	18	19	21
		Period after physical exercise				
		Baseline	2 h	1 day	2 days	4 days
ESR *vs* Hp Group A	r	0.582	-0.815	-0.758	-0.938	-0.220
	p	0.604	0.394	0.453	0.226	0.859
ESR *vs* Fib Group A	r	0.255	0.390	0.920	0.134	0.646
	p	0.921	0.745	0.226	0.914	0.553
ESR *vs* Hp Group B	r	-0.441	0.897	0.385	-0.200	0.827
	p	0.381	0.015	0.450	0.703	0.042
ESR *vs* Fib Group B	r	-0.026	0.282	-0.590	0.110	0.059
	p	0.961	0.587	0.217	0.836	0.911
ESR *vs* Hp Group C	r	-0.632	0.662	0.509	0.498	0.703
	p	0.178	0.152	0.303	0.314	0.119
ESR *vs* Fib Group C	r	-0.189	-0.631	0.382	0.282	-0.843
	p	0.721	0.179	0.454	0.589	0.035

Our results showed that fibrinogen concentration was the highest 1 day after the physical exercise, although within the reference range reported by Thrall et al. (2005) (1–4 g/L). This elevation was statistically significantly different both vs baseline and vs the vaccinated horses. In the opinion of Northoff et al. (1991) one of possible explanations is physical exercise-induced tissue damage in muscles, causing an inflammation condition associated to local release of IL-6 by macrophages at concentrations, inducing acute phase response. An alternative hypothesis, according to Donovan et al. (2007), is endotoxaemia induced by short-time strenuous physical exercise; endotoxin could enhance the release of numerous pro-inflammatory cytokines, including high levels of IL-6 and TNFα triggering acute phase response. In their studies, Coyne et al. (1990) also showed increase in blood fibrinogen following exercise. On the contrary, Scoppetta et al. (2012) reported that after prolonged exertion of horses fibrinogen, haptoglobin and albumin concentrations were lower. 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 towards APR activation or towards reduction, when it acts as an anti-inflammatory agent on cytokine production by macrophages and monocytes (Jensen et al. 1998, Murata, 2007). In our previous studies, we also found out increased blood cortisol levels immediately after exercise, which could probably influence fibrinogen production (Goundasheva et al. 2005).

Erythrocyte sedimentation rate was detected at 15-min intervals until the 1st hour. Compared to other animal species, in the three studied groups of horses, erythrocyte sedimentation occurred more rapidly during the first hour after blood sampling due to rouleaux formation.

Baseline ESR values on the 1st hour in control horses were 5 mm/h. Our data were comparable to the data outlined by Nikolov et al. (2009) (55–75 mm/h). Other researchers affirm 88–110 mm/h as normal ESR values in horses (Gul et al. 2013). However, in this species, large variations in ESR have been reported (Ju et al. 1993, Binev et al. 2006).

In vaccinated horses (group B), ESR values between post exercise hour 0 to day 4 (corresponding to post vaccination days 17 to 21) remained lower than baseline ones. On the 2nd hour, ESR correlated strongly and positively with haptoglobin, indicating that this protein had an effect on ESR.

According to our results, ESR was slowed down statistically significantly immediately after physical exercise (hour 0). The established strong negative correlation between ESR and fibrinogen on hour 2 showed that it was not a result from changes in either fibrinogen concentration or haptoglobin (belonging to α_2 globulin fraction), which did not change significantly over the entire period of the study. Most probably, slowed down ESR could be due to higher red blood cell counts observed at that post exercise interval (unpublished data). As stated by Fallon et al. (2001), ESR was slowed after moderate and severe physical exercise.

One day after physical exercise, ESR values were statistically significantly higher compared to group B. During that period, both APP changed proportionally to ESR showing a strong positive correlation between ESR and haptoglobin (r=0.509; p=0.303) and a moderate positive one for ESR vs fibrinogen (r=0.382; p=0.454). Therefore, ESR depended at a large extent on acute phase proteins. The data reported by Husain et al. (2002), Cha et al. (2009), and Lee et al. (2009) also support our results. Allen (1988) observed a strong correlation between fibrinogen and ESR in thoroughbred racehorses suggesting that the changes in this acute phase protein influence the extent of aggregation and rouleaux formation by equine erythrocytes.

In conclusion, the results demonstrated variable effects of physical exercise on ESR (mm/h) and acute phase proteins haptoglobin and fibrinogen. It slowed down ESR on hour 0 and did not influence blood plasma haptoglobin throughout the experimental period. After one day, physical exercise produced a state accompanied by increased blood fibrinogen (within the reference range) and accelerated ESR. The combination of ESR and blood fibrinogen levels could be useful for evaluation of physical exercise severity in horses during training and parcourt competitions, indicating an occurring disease activity in revaccinated animals.

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