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Original article

Analysis of peripheral blood chemiluminescence in horses

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Abstract

The aim of this study was to analyze the chemiluminescence (CL) of peripheral blood in clinically healthy horses of different sexes and ages. The tests were carried out on 119 half-breed horses, representing various forms of use (66 recreational horses and 53 sport horses). The test material was peripheral blood, which was collected under resting conditions, i.e. before physical activity related to the use of these animals. In the blood samples, spontaneous and stimulated CL with zymosan and phorbol myristate acetate were determined. It has been found that regular training effort increases the blood's pro-oxidative potential, which was demonstrated by significantly higher ($p<0.05$) CL values in sport horses compared to recreational animals. Analysis of the results did not show any statistically significant correlation between sex or age of the horses with chemiluminescence values in peripheral blood. The result of the research suggests the need to optimize the results of blood CL measurements, taking into account the number of neutrophils and the concentration of hemoglobin in the blood of tested animals. Analysis of non-optimized blood CL results may lead to premature conclusions.

Key words: horses, blood, neutrophils, chemiluminescence

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Introduction

The results of previous studies indicate that the diversity of the intensity of free radical processes accompanies many physiological and pathological conditions of humans and animals. In addition to commercial tests e.g. Bursttest (Phagoburst), which allow quantatative analysis of leukocyte oxadative burst using flow cytometry, chemiluminescence is also of value in research. Chemiluminescence enables the measurement of light emitted by activated phagocytes, and thus the assessment of the oxygen-dependent bactericidal potential of these cells (Bedouhène et al. 2017). It is characterized by high sensitivity and repeatability of the results, requires a small volume of research material, is relatively cheap, and allows a great deal of information to be obtained in a short time frame.

It should be emphasized that the vast majority of previous studies using CL measurements have been conducted in humans. These showed clear changes in the aerobic metabolism of blood cells, including acute bacterial and viral infections, cancer, metabolic disorders and inflammation, regardless of their etiology (Iranifam 2014, Hughes et al. 2018). The method also enables the study of congenital pathomechanisms or acquired defects in surface receptors and intracellular enzymes, such as deficiencies in glucose-6-phosphate dehydrogenase (G-6-P), myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase defects, and assessment of the effects of drugs and preparations that support the oxygen metabolism of neutrophils (Jimenez and Navas 2002). The usefulness of CL measurements in clinical practice has been confirmed in the World Health Organization report recommending their use to assess the state of innate immunity (Primary immunodeficiency diseases. Report of WHO Scientific Group 1997).

The use of the CL method is much less common in farm animals, including horses. It was limited, among others, to assessing the effect of exercise induced stress (Korhonen et al. 2000, Raidal et al. 2000, Krumrych et al. 2018) and local inflammation (Koenig et al. 2009, Krumrych and Danek 2012, Martin et al. 2017) on the aerobic metabolism of neutrophils. Most studies in horses were limited to spontaneous testing or used only one pathway of phagocyte activation, ignoring the possible impact of endo- and exogenous factors. Observations were usually made on isolated cells. However, it is believed that CL measurements in whole blood better reflect homeostasis than studies on isolated leukocytes, and therefore are more useful in clinical trials. They do not require any laboratory procedures to isolate neutrophils, which can affect the survival, activity and expression of these cell receptors (Papp and Smits 2007).

The aim of this study was to analyze the spontaneous and stimulated chemiluminescence activity of peripheral blood of clinically healthy horses, taking into account their use, sex and age.

Materials and Methods

All animal procedures in this study were approved by the Local Ethical Committee for Experiments on Animals at University of Life Sciences in Lublin, Poland (approval No. 9/2005). Laboratory procedures were carried out in accordance with the standards of good laboratory practice developed in the National Veterinary Research Institute in Pulawy (Poland).

Animals

The study was conducted on 119 half-breed horses of different sex (37 mares - M, 32 stallions - St and 50 geldings - G), aged 4 to 20 years. The horses were divided into four age groups (I – 4-6 years, II – 7-9 years, III – 10-12 years, and IV – ≥ 13 years). The animals were used in recreational (66 horses) as well as sports (53 horses) activities. The use of recreational horses in the spring and summer season was quite irregular. The average time of their daily work was 2-3 hours, during which they were ridden mainly by young people learning to ride. In turn, the routine load of the sport horses, representing the discipline of show jumping, included the initial phase (walk, trot and gallop alternating with trot, after about 10 minutes) and an intensive work phase (30-40 jumps over obstacles of 120-140 cm in height in 20-30 min).

The animals came from 5 horse recreation centers and 6 training facilities. Data from the interview conducted immediately before the implementation of the test procedure, clinical examination of the horses (measurement of rectal temperature, heart rate and respiratory rate, assessment of mucosal status and capillary filling time), as well as *ex post* results of laboratory tests indicated that the animals were healthy. Prophylaxis including deworming, and vaccination against influenza and tetanus were regularly used. Horse nutrition was balanced in terms of energy, protein, minerals and vitamins in accordance with the feeding standards of these animals (Frape 2010). The horses had permanent access to mineral licks and water. In the centers examined, the conditions of animal keeping were similar and took into account their welfare requirements.

The research was carried out in the spring (46 horses) and summer (73 horses) season (from April to September) in places where the horses live permanently.

Whole blood samples

The analyzed material was blood collected from the external jugular vein using a Vacuette closed vacuum system. Each time it was collected into 2 plastic tubes: 4 ml in a dipotassium ethylenediaminetetraacetate (K_2EDTA) tube and 9 ml in a lithium heparin tube. In order to limit the influence of circadian rhythm on the neurohormonal activity of the body, and thus, among other factors the number of blood cells and their function, samples were obtained each time between 9:00 and 11:00, and then stored at +4°C. Laboratory tests were carried out no later than 6 hours after collection.

Laboratory analysis

The number of red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), white blood cell (WBC) and the number of neutrophils (N) and lymphocytes (L) were determined in venous blood collected in K_2EDTA tubes. RBC, Hb, Ht and WBC determinations were performed using an ABCVet hematological analyzer (Horiba ABX, France), while the number of N and L was measured by microscopic analysis of May-Grünwald-Giemsa-stained blood smears.

Chemiluminescence (CL) tests of whole blood (collected in lithium heparin tubes) were performed with luminol (5-amino-2,3-dihydrophthalazine-1,4-dione; Fluka Chemie GmbH, Buchs, Switzerland) dissolved in 0.4% sodium hydroxide (NaOH) solution to a concentration of 28 µmol/ml. The measurements were carried out using a BioOrbit 1251 Luminometer apparatus (Pharmacia LKB, Turku, Finland) using the kinetic method for 40 minutes at 38.0 ºC, taking CL readings at 5-minute intervals. The results are presented as CL integration values, i.e. the area under the emission curve as a function of time (in mV/min).

Spontaneous CL (without stimulation - CL-WS) as well as CL stimulated with receptor involvement for the Fc fragment of the antibody and complement (CL-Z) and the non-receptor (CL-PMA) route were determined in blood samples. The following CL stimulators were used:

- zymosan A (Z): 100 mg zymosan (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was suspended in 10 ml PBS solution and diluted 10 times,
- phorbol myristate acetate (PMA): 5 mg PMA (Sigma Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in 5 ml of ethanol 95% and then diluted in PBS to a concentration of 0.32 µmol/l.

The evaluated sample contained 200 µl phosphate-buffered saline (PBS) solution or 100 µl PBS solution + 100 µl stimulator solution, 100 µl luminol solution and 150 µl whole blood collected in a heparin tube. Tests were started immediately after adding blood to previously prepared reagents. All measurements were repeated three times during simultaneous testing and the arithmetic mean was calculated. Due to the fact that the CL value is inversely proportional to the concentration of hemoglobin, whose light absorption spectrum weakens chemiluminescence, and directly proportional to the number of neutrophils in the sample, the recorded results were corrected, referring to the CL value to 1000 cells. The results were optimized according to the formula taking into account the volume of the blood sample tested (Krumrych and Wiśniewski 2006):

$$CL\ calculated = \frac{CL\ measured \times Hb}{(WBC \times N \times 150)\ /100}$$

Explanation: Hb – hemoglobin content in % (in relation to the mean value calculated for the particular group of horses), WBC ($\times 10^3/\mu l$) – absolute value of white blood cell count, N – percentage of neutrophils in the white blood cell picture, 150 – volume of blood in µl.

Statistical analysis

The results are presented as arithmetic mean and standard deviation (sd). Before performing the statistical analysis, we tested the residuals for normality of data distribution using the Shapiro–Wilk test. For each dependent variable, Levene's test was used to determine homogeneity of variance. To compare the values of the studied parameters, the multivariate analysis of variance (MANOVA) was used. In the case normally distributed variables, the significance of differences between groups was verified using the post-hoc Tukey's test. When the distribution was abnormal, the Kruskal-Wallis test was used. Statistical significance was set at $p<0.05$ in all cases. All statistical analyses were performed using Statistica for Windows, v. 6.0 software (StatSoft Inc., Tulsa, USA).

Results

The results of hematological measurements (RBC, Hb, Ht, WBC, L, N) are presented in Table 1. Their analysis showed significantly higher average values of red blood cell indices (RBC, Hb, Ht) in sport horses (S) as compared to recreational horses (R). However, no statistically significant differences were found between these groups in terms of white blood cell parameters (WBC, L, N).

Sex analysis of the tested animals showed that the stallions (St) of both groups were characterized by the highest RBC, Hb and Ht values, although this phenomenon was confirmed statistically only in com-

Table 1. Mean values (±sd) of the hematological indices in examined horses.

Horses			Hematological parameters					
			RBC (T/l)	Hb (mmol/l)	Ht (l/l)	WBC (G/l)	L (G/l)	N (G/l)
R (total) (n=66)			[a] 7.58±1.23	[a] 5.74±0.57	[a] 0.38±0.06	6.53±1.29	2.10±0.64	4.22±1.04
S (total) (n=53)			[b] 8.33±1.64	[b] 6.31±0.74	[b] 0.41±0.08	6.78±1.16	2.19±0.55	4.35±0.98
R	Sex	M (n=24)	7.35±0.78	5.75±0.45	0.35±0.03	7.09±1.61	2.12±0.76	4.73±1.31
		St (n=10)	7.99±0.94	6.22±0.52	0.40±0.07	6.11±1.01	1.99±0.43	3.73±0.87
		G (n=32)	7.53±1.53	5.56±0.59	0.38±0.07	6.17±0.97	2.06±0.63	3.95±0.76
S	Sex	M (n=13)	8.08±1.03	[ab] 6.32±0.81	[ab] 0.39±0.03	7.09±1.61	2.12±0.63	4.41±0.86
		St (n=22)	9.10±2.07	[a] 6.63±0.74	[a] 0.45±0.10	6.75±1.41	2.19±0.54	4.34±1.24
		G (n=18)	7.60±0.89	[b] 5.84±0.40	[b] 0.38±0.04	6.79±0.93	2.13±0.73	4.32±0.72
R	Age	I (n=20)	7.40±1.05	5.81±0.67	0.36±0.04	6.52±1.18	2.34±0.54	3.97±0.73
		II (n=18)	7.59±0.94	5.74±0.47	0.38±0.05	6.42±1.07	1.83±0.38	4.33±0.95
		III (n=16)	7.79±1.84	5.62±0.47	0.38±0.08	6.42±1.19	2.11±0.75	4.21±0.94
		IV (n=12)	7.29±0.90	5.74±0.69	0.39±0.07	6.63±1.97	2.02±0.86	4.42±1.63
S	Age	I (n=14)	8.66±1.08	6.17±0.51	0.41±0.05	6.46±1.04	2.13±0.68	3.93±1.03
		II (n=18)	8.80±2.38	6.60±0.88	0.44±0.11	6.74±1.34	2.14±0.66	4.33±1.05
		III (n=12)	7.79±0.86	6.23±0.71	0.39±0.05	7.35±1.11	2.22±0.36	4.81±0.85
		IV (n=9)	7.63±0.94	5.95±0.60	0.39±0.05	6.62±0.85	1.98±0.68	4.41±0.75

Explanation: sd – standard deviation; RBC – red blood cell count; Hb – hemoglobin concentration; Ht – hematocrit; WBC – white blood cell count; L – lymphocytes; N – neutrophils; R – recreational horses; S – sport horses; n – number of horses; M – mares; St – stallions; G – geldings; I – 4-6 years; II – 7-9 years; III – 10-12 years; IV – ≥ 13 years; [a,b] – mean values differ significantly for $p < 0.05$

parison with S geldings (G) in the Hb and Ht range. In contrast, no significant differences were observed in the mean values of other indicators (WBC, L, N) between mares (M), St and G. Despite the large diversity of the animals in terms of age (4-20 years), there was no significant effect of this factor on the mean values of the hematological parameters.

Table 2 presents mean CL values in whole blood of tested horses. They indicate a relatively low emission of blood CL of these animals in the spontaneous test (CL-WS). Blood CL analysis after prior activation of the phagocytic cells by the receptor (CL-Z) and non-receptor (CL-PMA) pathway showed high efficiency of zymosan (Z), which induced about 8/9 times more light emission than in the spontaneous test. A clearly weaker effect was obtained with PMA, which caused about a 2-fold increase in CL in blood.

Comparison of mean blood CL values of recreational and sport horses showed clearly higher CL in regularly trained and intensively trained (S) animals than in horses with relatively less physical activity (R). Statistical analysis confirmed the significance of these differences for the spontaneous test (CL-WS), as well as for determinations using stimulators (Z and PMA).

Analysis of the results shows the highest CL-WS, CL-Z and CL-PMA values in mares (with the exception of CL-PMA in S horses), but in comparison with stallions and geldings these differences were not signifi-

Table 2. Mean values (±sd) of spontaneous and stimulated chemiluminescence (CL) of whole blood in examined horses.

Horses			Chemiluminescence of whole blood		
			CL-WS (mV/min)	CL-Z (mV/min)	CL-PMA (mV/min)
R (total) (n=66)			[a] 2663 ± 900	[a] 24772 ± 9971	[a] 4754 ± 1665
S (total) (n=53)			[b] 3853 ± 1383	[b] 29658 ± 6294	[b] 6547 ± 1453
R	Sex	M (n=24)	2887 ± 851	25141 ± 8353	5168 ± 1758
		St (n=10)	2516 ± 867	23163 ± 11171	4792 ± 1785
		G (n=32)	2545 ± 952	24942 ± 9892	4656 ± 1614
S	Sex	M (n=13)	4033 ± 915	31578 ± 5912	6351 ± 1609
		St (n=22)	3707 ± 1659	28747 ± 6279	6556 ± 1429
		G (n=18)	3902 ± 1345	29386 ± 6621	6679 ± 1434
R	Age	I (n=20)	2784 ± 963	24065 ± 10283	4941 ± 1767
		II (n=18)	2935 ± 940	25940 ± 9721	4623 ± 1791
		III (n=16)	2479 ± 938	25405 ± 9922	5089 ± 1707
		IV (n=12)	2373 ± 689	23075 ± 7661	4725 ± 1435
S	Age	I (n=14)	4078 ± 1857	32270 ± 7230	6937 ± 1469
		II (n=18)	3942 ± 921	29847 ± 6037	6718 ± 1553
		III (n=12)	3974 ± 1585	29042 ± 6036	6031 ± 1295
		IV (n=9)	3155 ± 959	26040 ± 4209	6288 ± 1404

Explanation: WS - without stimulation; Z – zymosan; PMA – phorbol myristate acetate; sd, R, S, n, M, St, G, I, II, III, IV, [a,b] – as in Table 1

cant. There were no significant differences between the age groups studied, although there is a tendency to slightly decrease their mean values as the age of horses increases.

The results of CL determinations in the blood of tested horses with reference to 1000 neutrophils are presented in Table 3. They indicate a significantly higher chemiluminescence activity of these cells in sport horses compared to recreational animals. This phenomenon has been reported both for spontaneous testing as well as after previous receptor and non-receptor stimulation.

Considering the sex of the tested horses, in contrast to the non-optimized results, showed that the highest spontaneous and stimulated CL values were found in stallions. The statistical analysis carried out did not show, however, a statistical significance between the mean values of these parameters in comparison with mares and geldings.

No significant differences in the analyzed indicators were found also between the examined age groups, although there is a decreasing trend of the activity of CL neutrophils as the horses' age increases. This phenomenon was most clearly manifested in the case of CL-WS and CL-Z in both groups of horses and CL-PMA in S horses.

Statistical analysis of mean CL values (optimized and non-optimized) in R and S horses did not show any significant difference between results obtained during spring compared to results obtained during summer.

Table 3. Mean values (±sd) of spontaneous and stimulated chemiluminescence (CL) after optimalisation (for 1000 neutrophils) of whole blood in examined horses.

Horses			Chemiluminescence of 1000 neutrophils		
			CL-WS (mV/min)	CL-Z (mV/min)	CL-PMA (mV/min)
R (total) (n=66)			[a] 438 ± 182	[a] 4039 ± 1835	[a] 774 ± 306
S (total) (n=53)			[b] 617 ± 281	[b] 4861 ± 1931	[b] 1082 ± 481
R	Sex	M (n=24)	439 ± 200	3740 ± 1649	750 ± 268
		St (n=10)	505 ± 270	4397 ± 2135	964 ± 501
		G (n=32)	418 ± 135	4179 ± 1810	775 ± 277
S	Sex	M (n=13)	616 ± 227	4771 ± 1264	958 ± 332
		St (n=22)	649 ± 377	5172 ± 2466	1195 ± 622
		G (n=18)	580 ± 167	4547 ± 1591	1034 ± 347
R	Age	I (n=20)	479 ± 195	4028 ± 1706	832 ± 263
		II (n=18)	458 ± 163	4056 ± 1709	711 ± 271
		III (n=16)	401 ± 154	4269 ± 2203	830 ± 321
		IV (n=12)	406 ± 229	3813 ± 1621	796 ± 428
S	Age	I (n=14)	680 ± 259	5608 ± 1638	1211 ± 362
		II (n=18)	678 ± 368	5112 ± 2577	1174 ± 672
		III (n=12)	562 ± 209	4219 ± 1176	885 ± 309
		IV (n=9)	472 ± 120	4055 ± 1180	963 ± 281

Explanation: sd, WS, Z, PMA, R, S, n, M, St, G, I, II, III, IV, [a,b] – as in Table 2

Discussion

The hematological tests allowed for a more complete characterization of the health status of the studied animals, and in addition some of them (WBC, N, Hb) were used to optimize the results of chemiluminescence measurements. It was found that the mean values of these parameters were within the ranges of physiological norms assumed for clinically healthy horses (Walton and Lawson 2021). Significantly higher mean values of red blood cell parameters (RBC, Hb, Ht) in sport horses compared to recreational animals overlap with other observations (Burlikowska et al. 2015). They indicate that the type of regularly used exercise loads (training) affects the resting values of these indicators, which in turn translates into greater aerobic capacity of sport horses.

Implementation of the research, based on the CL measurement of peripheral blood of horses, allowed objective results to be obtained, assuming that the total CL blood corresponds to the size of the generation of reactive oxygen species (ROS) by neutrophils, which are the dominant population of phagocytes in peripheral blood. This is justified because the share of monocytes in the emission of light from blood samples is relatively small due to their low number and low, compared to neutrophils, ROS generation (Okunnu and Berg 2019). Not only was spontaneous CL blood determined,

but also stimulated, after earlier activation of phagocytes by the receptor (Z) and non-receptor (PMA) pathways. This arrangement of tests allows for indirect assessment of the ability of neutrophils to produce ROS, as well as allowing for more complete insight into their functional efficiency by assessing the expression and distribution of surface receptors and enzymatic proteins necessary for the proper and effective course of the phagocytosis process.

The results of our own research, carried out on a relatively large number of recreational and sport horses, showed a low resting CL activity in the blood of the tested animals, comparable to the results of previous observations (Krumrych and Danek 2012, Krumrych et al. 2013). This fact, which is evidence for the resting metabolic activity of cells, along with a clear reaction after stimulation with Z and PMA, indicates, in conjunction with clinical and hematological tests, the good health of horses. It has been shown that in unhealthy individuals phagocytic cells may be in a pre-activated, activated or depleted state, which means that despite stimulation with various stimulators they do not respond with a significant increase in ROS production (Li et al. 2000).

The use of stimulating compounds resulted in an increase in free radical activity of neutrophils in the studied horses. Binding of receptors for the Fc-antibody fragment and complement components (Z) caused the greatest 'efficiency' of this phenomenon, while stimulation by the non-receptor (PMA) pathway induced a 'respiratory burst' of neutrophils to a much smaller extent. This result is consistent with our own previous research, carried out in clinically healthy breeding horses of noble half-breed, showing the generation of much larger amounts of superoxide anion radical by horse neutrophils after Z stimulation than after using PMA (Krumrych and Wiśniewski 2006).

Analysis of the results of blood chemiluminescence measurements showed that recreational animals had significantly lower CL (spontaneous and stimulated) values compared to horses undergoing regular competitive training. It should be noted that the significance of these differences was also confirmed in relation to the results optimized for 1000 neutrophils. This result may suggest a relationship between resting neutrophil pro-oxidative activity and the regular physical activity of horses. It seems that both the type, intensity and time of effort could have been factors determining the resting pro-oxidative potential of neutrophils, although the sustained effect of exercise loads of the tested horses from the day preceding the blood collection should also be taken into account. The results correspond to the results of our own previous research (Krumrych et al. 2018) and Escribano et al. (2005), who showed a significantly higher resting oxygen metabolism of neutrophils and their greater phagocytic activity in horses undergoing moderate and intensive training programs than in untrained horses. A similar impact, beneficial for the protection of the body, of regular and moderate training on indices of non-specific immunity was found in humans (Nieman and Wentz 2019). However, many studies have found a transient, post-exertional impairment of peripheral blood phagocyte function (Raidal et al. 2000, Simpson et al. 2015). This phenomenon was usually a consequence of extensive and long-lasting training programs, indicating the importance of the intensity of exercise loads for the functional activity of these cells. The precise mechanisms determining the activity of phagocyte oxidative burst are still unclear and probably multifactorial (Terra et al. 2012, Kraemer et al. 2020). It is thought that the post-exertional increase in neutrophil oxidative activity could be the result of a number of mediators among them, most frequently, TNF-α, INF-γ, IL-6, IL-8 and IL-1β. The increase of these cytokines in peripheral blood was shown to be consequent to inflammation resulting from microtrauma of skeletal muscle, especially after intensive exercise (Liburt et al. 2010, Cywińska et al. 2014). On the other hand, post-exertional supression of neutrophil function is related to, among other factors, an increase in blood cortisol concentration (Kraemer et al. 2020). In vitro experiments demonstrated that high doses of hydrocortisol decrease the mobilization of complement receptor expression (CR1 and CR3) resulting in reduced phagocytosis and oxidative burst activity (Forslid and Hed 1982). As a consequence, increased blood cortisol concentration, post-excertional neutrophilia is connected to a release of immature cells with limited phagocytic functions from the bone marrow (Robson et al. 2003). The immunosuppressive influence on the described cells can also be attributed to a post-exertional increase of hormonal factors such as: growth hormone, catecholamines and changes in the concentration of metabolic factors (glutamine, glucose, lipids) in blood. (Ortega, 2003, Hyyppä 2005).

Numerous clinical and experimental studies, performed on human and animal models, suggest that endogenous factors may also affect the non-specific immune response. One of the more frequently analyzed factors in physiological and pathological conditions is sex. Underlying this factor is the identification of sex hormone receptors on various immune cells, suggesting a direct effect of steroids on immune functions (Bereshchenko et al. 2018). Most experimental studies have shown that progesterone, estradiol, and testosterone induce suppression of neutrophil oxidative burst, as reflected by reduced production of superoxide anion

($O_2^{\bullet -}$) (Marin et al. 2010, Bartoskova et al. 2014). These observations seem to confirm the research of Doucet et al. (2010), who showed a higher activity of neutrophil oxidative burst in rats after ovariectomy than in animals with preserved gonads. The immunostimulatory effect of sex hormones should also be mentioned. Indeed, in vitro studies have shown that low testosterone levels promote neutrophil phagocytosis as opposed to higher concentrations that inhibited the phagocytic activity of these cells (Marin et al. 2010). The interaction of sex hormones and the innate immune system appears to be complex, and precise knowledge of the underlying mechanisms remains unknown. The consequence of this is the diversity of results of previous observations of phagocytic activity in individuals of different sexes. Studies on isolated cells have shown that male neutrophils, regardless of age, produce greater amounts of $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) compared to women (Szuster-Ciesielska and Kandefer-Szerszeń 2001). However, this phenomenon was not confirmed statistically due to large inter-individual differences. Different results were presented by Siddiqi et al. (2001), indicating higher oxidative burst activity of neutrophils in healthy women than in men.

Interesting observations were provided by the analysis of blood chemiluminescence R and S horses. The highest, non-optimized CL values were found in mares although, in comparison with stallions and geldings, the differences were not statistically significant. In turn, after optimizing the results (with reference to 1000 neutrophils), it was noted that stallions were characterized by the highest mean CL values. It seems that this apparent inconsistency is the effect of taking into account the Hb concentration in the blood of tested horses in the optimization of results. It was demonstrated that stallions were characterized by higher Hb concentration in comparison with mares and geldings. This fact is important because, as shown earlier, heme compounds, due to their light absorption spectrum, weaken CL and cause light scattering (Lewkowicz et al. 1999). Therefore, the results of own research indicate a higher CL activity of neutrophil in stallions compared to mares and geldings, although the recorded results did not differ significantly from each other. The observations cited above indicate the validity of optimizing blood CL measurement results by referring them to a specific number of neutrophils and taking into account the Hb content. The assessment of 'raw' (not optimized) blood CL results may lead to unjustified conclusions.

A analysis of the age of the horses showed a tendency to a decrease in blood CL activity with age. This phenomenon was recorded in both groups of horses (R and S), both in the case of non-optimized and optimized results. Although the significance of differences between the studied age groups has not been statistically confirmed, this result corresponds to previous observations indicating a progressive decrease in phagocytic and bactericidal activity of phagocytes in humans and animals (Wenisch et al. 2000, Albright et al. 2016). It is believed that the age-related impairment of the functional efficiency of cells, which are the first line of defense against pathogens, is caused, among others, by damage to signal transduction pathways, including those with Toll-like receptors; inactivation of cytosolic enzymes (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase) involved in the synthesis of NADPH, increased intracellular Ca concentration and reduced hexose uptake (Wenisch et al. 2000, Shaw et al. 2010). Although there are many publications documenting phagocyte dysfunction during aging, the causes of this phenomenon are not fully understood. Meanwhile, these issues seem interesting in relation to, for example, susceptibility to infections, cancer and autoimmune disorders, and age-related diseases. It seems that the lack of significant differences between the studied age groups was due to large inter-individual differences in blood CL measurements, as well as the lack of older horses over the age of 20, among whom the greatest decrease in functional activity of phagocytes should be expected. Therefore, further studies on the functional activity of phagocytes are required, including foals and old individuals.

It is worth mentioning that the immune functional activity of neutrophils is influenced by seasonal variability. Studies in humans and in animals have shown among other things that the adhesive abilities of neutrophils, production of ROS and the expression of CD11b/CD18 were increased in samples collected during summer (Yellon et al. 1999, Klink et al. 2012). It is thought that this seasonal modulation of the immune system is caused by the annual light/dark cycle mediated by melatonin (Nelson 2004). Our own studies were conducted only during spring and summer and unfortunately do not take into account potential seasonal variability in blood CL in horses. The observations of other authors cited above also suggest the need to carry out such studies throughout the year. Knowledge of seasonal rhythms in neutrophil functional reactivity could be useful in long term studies, and in planning immunotherapeutic strategies against chronic infectious disease.

It should be emphasized that apart from measurements of the respiratory burst using CL cited in this study, other methods, albeit infrequently, are also used in horses such as commercial tests based on flow cytometry. (McTaggart et al. 2001, Robson et al. 2003, Cywińska et al. 2010). These allow measurement of the the percentage of cells producing reactive oxidants and their enzymatic activity reported as mean fluorescence

intensity. The results of the relatively infrequent studies using these tests correspond to those using the CL method. The usefulness of both methods in studies of oxidative activity in neutrophils seems to be confirmed by a correlation between luminol-amplified chemiluminescence and flow cytometry assays (Caldefie-Chézet et al. 2002).

Conclusion

In summary, this study showed that regular training effort increases the blood pro-oxidative potential, which was demonstrated by significantly higher CL values in sport horses compared to recreational animals. Analysis of the results did not show a statistically significant difference with regard to endogenous factors (age, sex) on the CL values in the peripheral blood of horses. The results of the study also suggest the need to optimize the results of blood CL measurements, taking into account the specific number of neutrophils and the concentration of Hb in the blood of tested animals. Analysis of non-optimized blood CL results may lead to unjustified conclusions.

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