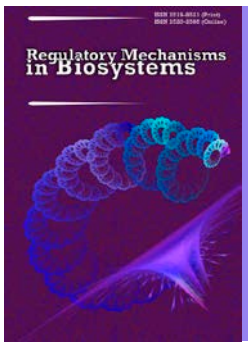

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Acid resistance and population structure of erythrocytes in trotter horses during and after exercise

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Regular physical exercise improves the cardiovascular system function, increases energy metabolism and antioxidant protection, provides greater muscle strength and physical endurance. However, exhaustive physical exercise can cause oxidative stress. During intense physical exercise, erythrocytes become more vulnerable to oxidative damage due to action of active oxygen forms, high concentration of polyunsaturated fatty acids. Physical loading of horses is accompanied by the development of tissue hypoxia, so one needs to study in detail changes of hematopoiesis indices, responsible for providing oxygen to tissues. When monitoring animal health, blood tests will detect a disease in early stages, because blood is a sensitive indicator of metabolic disorders, both of physiological and pathological body conditions. The purpose of present study was to examine changes of some hematopoiesis indicators (RBC, Hb, HCT, MCV, MCH), acid resistance of erythrocytesand population structure in trotting horses due to workloads of 1,600 m trot races (at rest, after race, after 90 min). In total, 20 clinically healthy horses of Orlovska and Russian trotting breeds, aged 2–3 years, were used in the study. The experimental animals included 5 mares, 15 stallions. All horses had active training. The horses were divided into two groups. The first animal group included animals that showed high results (2 min 04 s – 2 min 21 s); the second had trotters that covered the distance with worse time (2 min 28 s – 2 min 41 s). The erythrocyte couint in the leader horses after the loading increased, in the outsider horses it fell. After rest the leader horses' erythrocytes returned to their original values, in the outsider horses they did not change. The outsider horses' hemoglobin level after loading was lower than in the leader horses. The leader horses had higher hemoglobin content after rest (by 11.0% as compared with the values before the loading). The outsider horses had lower hemoglobin content. The leader horses hematocrit value increased, in the outsider horses – decreased. The leader horses' MCV after the loading was reduced, however after rest it recovered. The outsider horses showed MSV increase during the whole study period. The MCH in the leader horses did not change over the entire research period, in the outsider horses – it increased. The animals that showed better results, had an increasing number of "mature" and "old" erythrocytes due to their elimination from the depot. However, the outsider animals had an increasing number of "young" erythrocytes, resistant to hemolysis, but they were not able to provide tissues with needed oxygen.

Keywords: hematological parameters, performance, hemoglobin, hemolysis, mares, stallions

Introduction

Pedigree horse breeding has become prestigious in recent years, they are mainly used in sports. Horse riding is an active recreation for people, but also a challenge for these "noble" animals. Their loading must relate to a rigid training system. Racetrack training should include gradual loading, exercising endurance and consideration for the physical abilities of the horrse (Bulgakov, 2002). Trotting horses are prominent among the breeds, used in sports. They are characterized by the sports features of agility and endurance. All these elements depend on the course of metabolic processes, regulated by the hematopoietic system, because the health and performance of sport horses depend on its functional state. Operating performance of sport horses is determined by many biological and physiological factors. Understanding the interrelation of these factors is required to ensure effective training and achieving sound sport results. In equine sport medicine studies have been conducted to determine some hematological parameters and reliable criteria to evaluate horse performance during exercise and physical load (Hinchcliff et al., 2004). Knowledge of the functional and metabolic processes occurring in a particular sport discipline is extremely important for understanding what metabolic paths are involved, and which physiological processes are induced during various physical activities (Bergero et al., 2005). It has been proved that horses undergo changes between the balance of oxidant and antioxidant systems, depending on the exercise type, its intensity and duration, training condition, environmental conditions, and presence of diseases (Kirschvink et al., 2006; Williams and Bur, 2012).

During intensive exercising, erythrocytes become more vulnerable to oxidative damage due to performance of active oxygen forms, high concentration of polyunsaturated fatty acids and haemoferrum (Petibois and Deleris, 2005; Çimen, 2008). Physical loading of horses is accompanied by the development of tissue hypoxia, so one needs to study in detail changes of hematopoiesis indices, responsible for providing oxygen to tissues. When monitoring animal health, blood tests will detect a

disease in the early stages, because blood is a sensitive indicator of metabolic disorders, both physiological and pathological body conditions (Andriichuk et al., 2014). Some hematological adaptation mechanisms are necessary to ensure oxygen and substrates supply, coming from the blood to the working muscles and releasing metabolites during training. Low efficiency of these mechanisms limits the physical performance of horses (Muñoz et al., 1997; Piccione et al., 2007). During physical loading some physiological changes happen in the horse's body, such as increased cardiac output, increased pulmonary arterial pressure, and blood flow is optimized by erythrocytes and hemoglobin (Terskov and Hitelzon, 1967).

The aim of this study was to examine the changes of erythrocytes' acid resistance and erythrocytes' population in trotting horses due to workloads of 1,600 m trot races (at rest, after race, after 90 min).

Materials and methods

There were used in total 20 clinically healthy horses of Orlovska and Russian trotter breeds, aged 2–3 years for this study. The experimental animals included 5 mares, 15 stallions. The horses' body weight averaged 509.8 ± 10.45 kg (450–581 kg). All horses had active training. All horses were studied for blood count and basic biochemical parameters, characterizing the functional state of organs and systems that were within the reference fluctuation. The mares were not pregnant. All horses were vaccinated and dehelmintizated, kept in the same conditions. All horses at the time of study were clinically healthy.

The horses' daily diet included: meadow hay (6 kg), oats (6 kg), wheat bran (2 kg), carrots (1 kg), and was divided into three portions. Salt and water were available without restriction. The study was conducted during trot races (with maximum trotter load of 1,600 m distance) The horses were divided into two groups. The first animal group (group A – 2 mares, 8 stallions; successfully completed, SC) included animals that showed high results (2 min 04 s – 2 min 21 s); the second (group B – 3 mares, 7 stallions; which had not successfully completed, nSC) had trotters that covered the distance with worse time (2 min 28 s – 2 min 41 s). The study was conducted at rest, after races and after 90 min.

Blood samples were taken from the jugular vein using a 16-gauge needle in to the vacuum blood tubes, 10 ml (Vacutest, Italy). Tubes of ethylenediaminetetraacetic acid (EDTA) were used to study the blood hematology. The blood hematology was analyzed by an automated hematology analyzer Mythic 18 (Orphee S. A., Switzerland) and the PZ Cormay S. A. (Poland) reagents. The red blood cell count (RBC), hemoglobin concentration (Hb) and mean cell volume (MCV) were measured directly; packet cell volume (PCV) and mean cell hemoglobin (MCH) were calculated automatically.

Acid resistance of erythrocytes was determined by the method of A. I. Terskyi and I. I. Hitelzon. Blood samples were placed into centrifuge tubes, with previously added heparin at 10 IU per 10 ml of blood. Plasma was separated by centrifugation (1500 g/min for 20 min). The erythrocytes' suspension was washed three times by cooled to 4 ºC (to prevent lipoproteins oxidation) 0.85% solution of sodium chloride followed by centrifugation under the same conditions. The suspension of erythrocytes was taken by capillary (0.02) and transferred to test tubes, which already contained 10 ml of isotonic sodium chloride solution. The capillary was washed in the upper layer of the solution and the tube contents were thoroughly mixed. Thus 0.2% suspension of erythrocytes was received.

Measurement of solutions optical density was carried by a photometer at 540 nm wavelength (cuvettes with 10 mm process solution thickness). Before the study, both cuvettes were filled for 5–10 min with hemolytic solution with the abovementioned concentration. The control cuvette was filled with 4 ml of 0.85% sodium chloride, and the experimental one was filled with 2 ml of hemolytic solution and added 2 ml of 0.2% erythrocytes suspension. The hemolytic with erythrocytes suspension was stirred by the tip of the pipette . The sample extinction was determined immediately after mixing. Extinction changes were recorded every 30 seconds until a constant indicator is reached (during 30 s).

The difference between the initial and final (after hemolysis) optical density is seen as 100% and the ΔE percentage was calculated (the next extinction index was subtracted from the previous one and seen as "X"), which reflects the relative percentage of non-hemolyzed erythrocytes every 30 s. This calculation excludes dependence of the results on the number of erythrocytes and hemoglobin concentration. The obtained data was depicted graphically. The horizontal axis reflects time from 0 and every 30 s, the ordinate axis – the erythrocytes hemolysis percentage. The left side of the diagram indicates hemolysis of "old" erythrocytes populations, the central part with peak is formed by the destruction of "mature" and partially "young" cells, the right side is the hemolysis result of only "young" erythrocytes (Terskov and Hitelzon, 1967).

Erythrocytes population structure was determined by fractionation of sucrose density gradient by I. Sizova method.

Blood samples were put into centrifuge tubes, with previously added heparin at 10 IU per 10 ml of blood. Plasma was separated by centrifugation (1500 g/20 min). The erythrocytes suspension was washed three times by cooled to 4 ºC (to prevent oxidation of lipoproteins) 0.85% sodium chloride solution followed by centrifugation under the same conditions.

The erythrocytes suspension was taken by a 0.1 ml pipette and moved to Florinski test tubes, previously filled with 0.9 ml of isotonic sodium chloride solution. The pipette was washed in the upper layer of the solution and the tube contents was thoroughly mixed (thus obtaining 10% erythrocytes suspension). 0.5 ml of erythrocytes suspension was introduced into the 45o tilted column, and sucrose solution was put layer over layer on the column wall (the first layer – with the highest concentration, the last – with the lowest). This provides the distribution of erythrocytes in different concentrations of sucrose, depending on the erythrocytes' age.

The column was carefully leveled at 90o to avoid mixing of the sucrose layers, and every last layer was carefully separated into a separate graded tube (7 in total). Isotonic sodium chloride solution was added into each tube, totaling volume to 10 ml. All 7 tubes were thoroughly mixed and checked for the optical density.

Measurement of the solutions' optical density was carried by a photometer at 520 nm wavelength (cuvettes with 10 mm working layer thickness). A 0.85% sodium chloride solution was a control one. The received extinction amount was seen as 100%, and each tube index was seen as "X" and thus calculating the percentage of a particular faction. The indicators of the first three tests (in sucrose solutions of 30%, 26% and 22% concentrations) were added. The obtained result was a percentage indicator of the "old" erythrocytes number. The same was done with the fourth and fifth indicators (18% and 14% solutions), by calculating the concentration of "mature" cells, while adding percentage of the sixth and the seventh results (10% and 6% sucrose solutions) and we obtained a relative indicator of the "young" populations content (Sizova et al., 1980). After the releas of erythrocytes from the red bone marrow to peripheral blood, they undergo three age stages: "young", "mature" and "old". It depends on cell membranes' oxidation intensity and their antioxidant capacity (Wijk and Solinge, 2005).

Mathematical analysis of the study results was conducted in Statistica 6.0 (StatSoft Inc., USA). Differences between average values were considered statistically significant at $P < 0.05$ (ANOVA).

Results

Number of erythrocytes of the first and the second horse groups before the exercise was on the same level. After races , the leader horses showed significant ($P < 0.01$) increase in erythrocyte count. The outsiders, on the contrary, tended to show a decrease in the number of erythrocytes as compared to the period before exercise. After races, the erythrocyte count in the outsider horses was 16.5% less as compared to the leaders. 90 min after the trot race finished the erythrocytes number in the leader horses declined, but did not return to the original values. At the same time, the erythrocyte count in outsiders decreased during the recovery period, and was 15.8% lower ($P < 0.05$) as compared with indicators before exercise, and by 9.9% after the end of the races apparently due to exhaustion of the blood depot reserve capacity. The outsider horses in the recovery period had a 22.0% ($P < 0.01$) lower erythrocyte count compared to the leaders.

The hemoglobin content in outsider horses at rest was 10.5% lower as compared to the leaders. In the next period (after trot races) the value of hemoglobin in animals of both groups tended to increase, but the leaders showed higher figures. The leaders in the recovery period had further hemoglobin increase and it obtained probability ($P < 0.05$) and was 11.0% higher as compared to the period before exercise, which obviously points to increased performance of bone marrow and intensive formation of hemoglobin under physiological hypoxia. The outsiders had hemoglobin decreases compared with the after race period and this was almost similar to the value before exercise.

Assessing the hematocrit value, it should be noted that in the leaders after exercise it tended to increase, while in the outsiders it did not change, and after 90 min, this figure decreased by 5.0% as compared to the period before the exercise ($P < 0.05$). The MCV in the leaders after exercise decreased by 11.0% ($P < 0.05$), and after 90 min of rest it returned to original values. In the outsiders the reverse pattern occurred, after exercise the MCV tended to increase (by 8.7%), and in the recovery period it was higher by 15.4% as compared with the values before exercise ($P < 0.05$). When determining the MCH, it was found that in the leaders this ratio did not change during the entire study period, but in the outsiders after exercise it increased and obtained probability 90 min after exercise ($P < 0.01$; 25.0%). Such changes of red blood indexes in second group animals indicate slowed adaptation processes of "red" blood cells to physiological hypoxia during exercise and appearance of oversaturated macrocytic erythrocytes forms in the bloodstream.

The population structure of erythrocytes in both animal groups did not differ at the beginning of the experiment (Table. 1). At the beginning of the experiment there was no significant difference in erythrocyte's acid resistance between leaders and outsiders. However, if the left part of the chart (hemolysis of "old" and "mature" population) almost coincided, still the main peak in horses that showed high results was at 7.2 min, which was by 1.1 min longer as compared to the outsiders (Fig. 1).

Table 1
Erythrocytes population structure indexes in trotting horses (%, n = 10)

Experimental periods	"old"		"mature"		"young"	
	leaders	outsiders	leaders	outsiders	leaders	outsiders
Rest	8.8 ± 1.06	7.6 ± 0.92	33.1 ± 2.05	34.8 ± 4.26	58.0 ± 1.57	57.3 ± 5.10
After race	6.9 ± 1.44	6.0 ± 0.35	29.8 ± 2.44	30.5 ± 1.31	63.2 ± 3.80	63.4 ± 1.31
After 90 min	4.9 ± 0.32	4.5 ± 0.20**	25.8 ± 2.43	22.3 ± 1.07**	69.2 ± 2.28	73.1 ± 1.16***

Note: ** – $P < 0.01$, *** – $P < 0.001$, compared with the previous period.

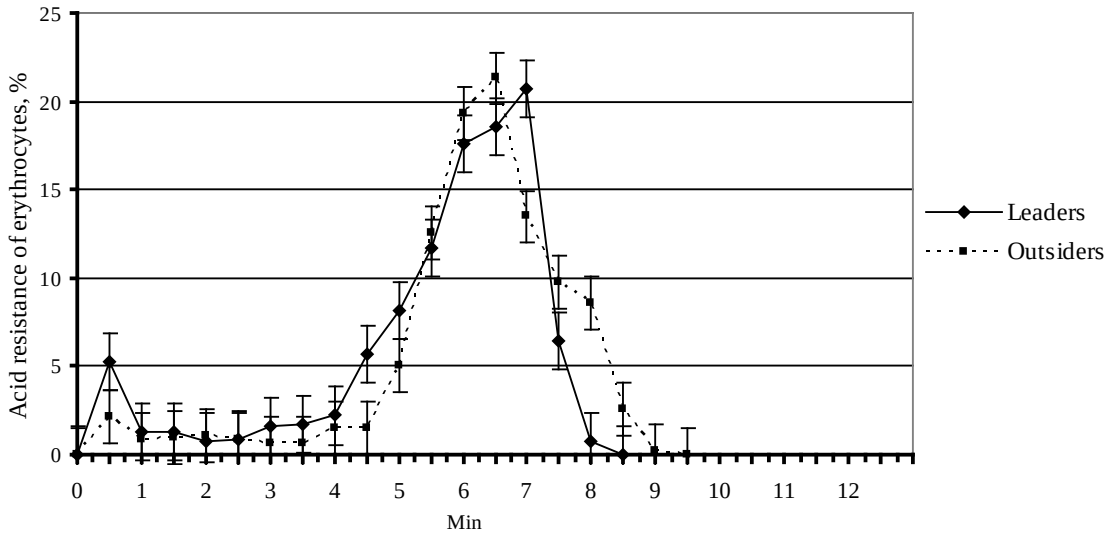

Fig. 1. Acid resistance of erythrocytes (% of hemolyzed erythrocytes) of trotter horses at rest: data are the mean ± standard error of the mean (n = 10)

However, the leaders full time of hemolysis ended within 8.5 min, and in outsiders 1 min later (9.5 min), indicating the circulation of a larger number ofr "young" cells in peripheral blood. After trot races both animal groups trended to decrease "old" (by 1.9% – in leaders; by 1.6% – in outsiders) and "mature" (by 3.3% and 4.3%, respectively) erythrocyte forms. However, the proportion of "young" populations increased by 5.2% and 6.1% respectively, probably due to bone marrow reflex stimulation as a result of excessive physical activity (Table 1).

A confirmation of these changes was depicted in the erythrocytes acid resistance diagram. The erythrocytes diagrams in leaders and outsiders coincided and almost overlapped. The left and right parts of the chart were the same, hemolysis time completed within 9 min. Only the main peak for the leaders was somewhat lower and smoother (Fig. 2), indicating the gradual destruction of "mature" and "young" cells under the influence of hemolytic and stable saturation with lipid components of erythrocytes' membranes in the first group of horses.

After 90 min bone marrow hyper regenerative response occurred in both animal groups, due to the deepening process of issue hypoxia after exercise. It should be noted that in the third period of study (after 90 min) the leaders showed only a tendency to reduce "old" and "mature" erythrocyte forms (in 10% of animals) while increasing the "young" (in 20% of horses) ones. However, the outsiders showed credible changes of these indicators, namely the number of "young" populations increased by 9.7% ($P < 0.001$; Table 1), while "mature" and "old" ones were less by 8.2% and 1.45% respectively. Obviously, this animal group even after rest manifests increased production of cells by bone marrow erythroid, leading to release of "young" cells into peripheral blood which are more resistant to hemolysis. Evidence of this is the erythrocyte diagram that reflects the age structure of red cells and the activity of biochemical connections between the membrane components. It was established that in outsiders the main peak – happened at 6.75 min (by 1.5% more as compared to leaders) and amounted to 22.5% of populations (Fig. 3). The full time of hemolysis ended in 10 min, while in horses that showed better results it was in 8 min, indicating the circulation in the outsiders of greater proportion of immature "young" cells in the bloodstream (due to bone marrow reflex stimulation), which are unable to fully perform the function of oxygenation, which in turn negatively affects the muscular activity of this group of animals.

Discussion

Exercise has variable effects on hematological parameters. These differences may depend on the intensity and duration of physical exercise. The erythrocyte count increase along with of hemoglobin concen-

tration increase after exercise is probably the result of splenic contraction and subsequent release of erythrocytes, accompanied by change in erythrocyte indices. Increase of the erythrocyte count, hemoglobin concentration and hematocrit value allows increased oxygen transportation to body tissues. It may also be associated with physical exercise and redistribution of fluids in the body, but these changes are secondary in relation to catecholamine release and splenic contraction (Robert et al., 2010; Gerber et al., 2014; Scantlebury et al., 2014; Padalino et al., 2017). The study results showed that physical exercise causes increase of red blood cells, hemoglobin and hematocrit concentration, immediately after exercise, returning to initial values within 1 hour after exercise (Vazzana et al., 2014).

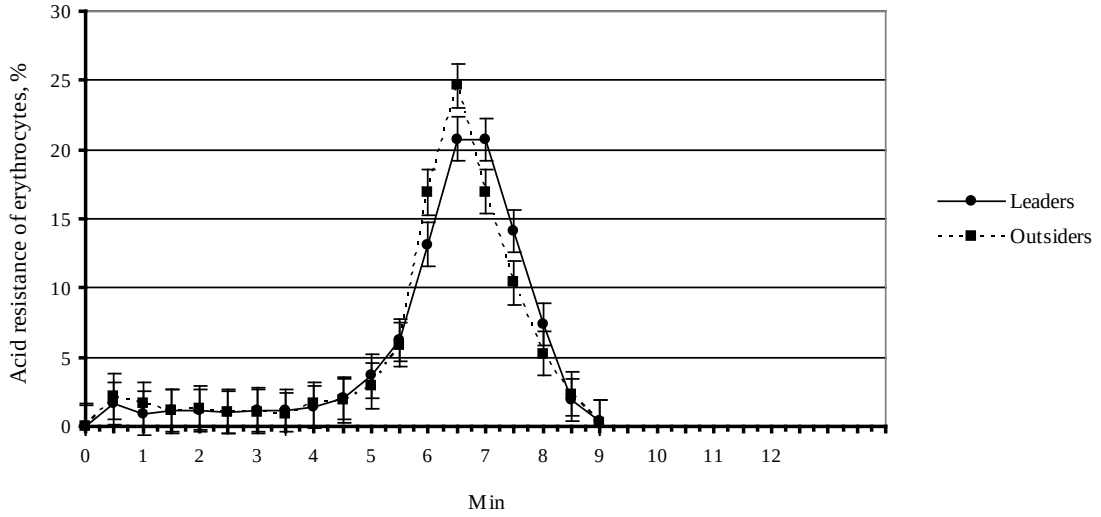

Fig. 2. Acid resistance of erythrocytes (% of hemolyzed erythrocytes) of trotter horses after race: the data are the mean ± standard error of the mean (n = 5)

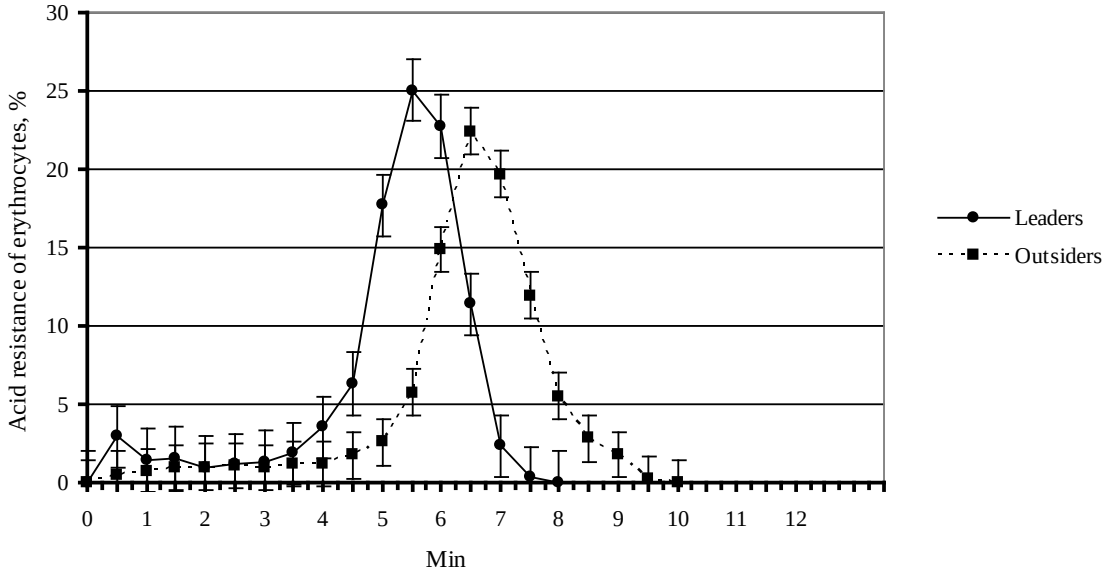

Fig. 3. Acid resistance of erythrocytes (% of hemolyzed erythrocytes) of trotter horses after 90 min: the data are the mean ± standard error of the mean (n = 5)

Cardiovascular and hematological adaptations are required for supplying tissues with oxygen and nutrients for active working muscles during physical exercise and release of metabolites (Piccione et al., 2007). This study showed changes in the erythrocyte number and hemoglobin concentration after exercise in horses of both groups, but more dramatic changes occurred in leader horses. Physical exercise has a different effect on the erythrocytic indices depending on exercise intensity, ability and professional training, environmental conditions, and horse breed (Krumrych, 2006). Our research results showed some differences in hematopoiesis indices in trotting horse of leaders and outsiders. Changes in the number of erythrocytes, Hb, Ht, MCV, MCN, acid resistance and population content of erythrocytes can be used to monitor the state of health and level of horse training.

The process of hematopoiesis (erythrocytopoiesis) is represented by the first morphologically identified germ cell, in which the cytoplasm begins to synthesize hemoglobin – it is a perioperiblast. During 4 days of development, it loses the "blast" morphology of the nuclei, passing the prognosis and normocyte stages. After the last mitosis, the picnotically modified nucleus is crimped out of the cell, there is denucleation (by carioexpression and carriolysis) and the formation of reticulocyte.

A "young" cell after many processes and transformations becomes a "mature" red blood cell, which performs many physiological functions: transportation of oxygen to the tissues and carbon dioxide from them, the adsorption of amino acids, proteins, and their decay products, antigens, antibodies, enzymes, synthesis macroergic compounds and others. The aging processes are accompanied by chronic oxidative stress and inhibit the metabolism of red blood cells leading to lipid membranes and protein degradation and the reduction of ATP (Dice, 1993).

Drainage and the postpartum period cause the occurrence of hypoxia, which leads to irritation of the "red" bone marrow and changes in the ratio of red blood cell populations in the peripheral blood system. According to experimental studies, it was found that Russian trotting

breed horses within three months after birth increase the number of "young" red blood cells forms, in which there is no definite stabilization of the structural elements of erythrocyte membranes. As to stallions of this breed, a decrease in "mature" and increase in "young" red blood cells forms was found, acid resistance of erythrocytes to hemolytics was increased, which probably indicates a genetic predisposition to significant physical activity and adaptive cytogenesis of erythroid sprout bone marrow for oxygenation (Piddubnyak et al., 2014).

Concerning horses at the latent course of leptospirosis and herpesvirus infection, there are changes in the erythrocytopoesis system, in particular, an increased number of "old" forms of erythrocytes with the simultaneous gradual decrease in "young" have been found, indicating the inhibition of erythrocytopopse processes, inhibition of erythrocyte maturation processes and increased aging in the peripheral blood system. The reflection of the morpho-functional state of the erythrocytopoetic system in peripheral blood can be traced on a graph of hemolysis rate of erythrocyte populations under the influence of acid solution. Erythrograms of horses at the latent course of leptospirosis and herpesvirus infection had a right shift of their major peaks and the complete time of hemolysis in animals was completed more quickly, which may prove a blocking effect of infectious agents on the elements of the bone erythroid bone marrow, inhibition of erythrocyte maturation processes and increased aging.

According to scientists, concerning horses at the process a combined invasion – paraskarasis and strontiglyidoses, the qualitative composition of erythrocytes varies even in the case of weak intensity of the invasion. This is evidenced by the reduction of the "old" and "mature" forms of red blood cells and the increase in "young" ones, which are intensively destroyed and are unable to carry out fully the processes of oxygenation. The configuration of erythrograms among invasive horses indicates its shift to the right side, which is due to the appearance of "young" red blood cells in the peripheral channel, but the reduction of the complete time of hemolysis indicates that their membranes are depleted by protein-lipid components because of the activation of free radical processes with the help of the life products of helminths (Piddubnyak et al., 2010). Neuropathia among horses suspected of having an alkaloid mycotoxicosis and encephalitis is caused by changes in the erythrocytic pool and hypoxia. Destructive changes in erythrocytes are reflected in their acid resistance. It is reduced in patients, which is confirmed by the reduction of full hemolysis time and rapid destruction of populations of "red" blood cells.

In conclusion we can state that the results of this study show that exercise is a very important issue in life of a sports horse, and that hematological research is vital in evaluating sports horses. Changes of some hematological parameters are related to the degree of horse physical training.

Conclusions

Thus, according to the performed studies, the efficiency of sport horses depends on the trained level of animals. During its assessment, one should take into account not only conventional indicators (erythrocyte count, hemoglobin content, hematocrit value and "red" blood indexes), but especially important physical-chemical properties of erythrocytes (buoyancy, acid resistance) and regulation mechanisms of gas exchange. Thus, in animals that showed the best results, the number of "mature" and "old" erythrocytes at the expense of elimination them from the depot. However, the number of "young" erythrocytes, resistant to hemolysis increased in outsider animals, but they are unable to satisfy tissue demand for oxygen.

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