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HEMATOLOGICAL CHANGES AND RESISTANCE OF ERYTHROCYTES OF CRIMEAN HORSES IN RESPONSE TO 32 KM RACES

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Aim. The aim of the present study was to investigate the alterations of some hematological parameters (haematocrit (HCT), haemoglobin concentration (HGB), the count of red blood cells (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW), as well as resistance of erythrocytes to urea and hydrogen peroxide in horses after 32 km endurance race. **Methods**. Seven horses from Crimea region (Bilohirsk, Crimean region) were involved in this study. Haematological parameters (haematocrit (HCT), haemoglobin concentration (HGB), the count of red blood cells (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)) were determined with use of hematological and biochemical methods. Blood samples have been investigated by centrifugation at 3,000 g for 15 min. The peroxide and osmotic resistance of erythrocytes were determined spectrophotometrically at 540 nm by monitoring the rate of erythrocytes disintegration by hydrogen peroxide. Endurance horses used in this study are trained and conditioned to perform over long distances at moderate speeds. The prolonged exercises were used in endurance race. The walk about 3 km/h for 20 min, the trot about 7 km/h for 15 min, and the canter about 5 km/h for 15 min and the walk about 1 km were repeated for 1 h (phase I); rest in an outdoor paddock without access to water for 30 min. And phase II: the walk about 3 km/h for 20 min, the trot about 7 km/h for 15 min, and the canter about 5 km/h for 15 min and the walk about 1 km was repeated for 1 h. **Results.** The results of the present study showed that adequate endurance race of low intensity could improve oxygen-dependent respiratory function in horses from Crimean region. Furthermore, the non-significant increase of red blood cells indices in endurance horses indicates good athletic level after 32 km endurance ride. Statistically significant differences in the percentage of hemolyzed erythrocytes between pre- and post-ride period were observed and thereby signify an oxidative stress-dependent impairment of erythrocyte stability. **Conclusions.** The haematological changes caused by various physical efforts reflect changes in the functions of different systems and can be used for health control and diagnosis of diseases. It also allows evaluating the level of sport performance, the accuracy of training, and physiological condition of horses.

Keywords: endurance ride, endurance horses, hematological parameters, resistance of erythrocytes, exercise.

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INTRODUCTION

Equine endurance races have become an important field of competition in recent years but unfortunately have received little attention from scientists [1, 2]. The training of endurance horses and athletic longevity is comparable to that of a human marathoner [3].

Most breeds have been tested and used for endurance races; the most competitive are Arabian or Arabian crosses due to their muscle fibre composition, but other breeds, including Thoroughbred, Quarter Horses, Mustangs, Appaloosas, Morgans, Standardbred [4, 5]. For endurance race, it often use horses of local breeds, bred directly in recreational areas. For example, widespread in Carpathians Hucul horses – bred in Poland and Ukraine or horses in Crimean Mountains [6]. The basis of Crimean horses breed was formed from the horses of Bashkir breed imported to the Crimea in the 60s of the last century. As a result of crosses under the influence of harsh conditions of maintenance of horses herd, breeds showed genotypic and phenotypic heterogenecity. The horses in Crimea Mountains are small, about 145 cm at the withers. They are wide in the body and deep-chested, with a thoracic circumference (girth) averaging about 180 cm; they have a large head and a short neck, low withers and a flat back. The legs are short with heavy bone; cannon bone diameter may reach 20 cm. The most common coat colours are bay, red, brown, chestnut, mouse grey. The mane and tail are thick and the coat is also thick. Today, horses in Crimean Mountains are widely used in endurance race and recreational riding [6].

Fig. 1. Maps of Crimean region in Ukraine, marked are Bilohirsk (Crimean region, Ukraine)

Metabolic responses during endurance races result from a build-up of free radicals in the muscles leading to poor performance and serious repercussions on health status, significant changes in internal homeostasis, blood volume, mean arterial pressure and plasma tonicity. There are several neuroendocrine mechanisms involved in the acute and chronic defense of internal homeostasis, which act to ensure an adequate blood flow to the working muscles and other vital tissues, together with the provision of a proper fluid volume for sweating and thermoregulation [1, 5, 7]. The working muscles of endurance horses depend on aerobic metabolism of glycogen stores, blood fatty acids and volatile fatty acids from hindgut fermentation, heart size and capacity to deliver large volumes of blood to the tissues [2, 5]. Certain cardiovascular and haematological adaptations are necessary to guarantee the correct supply of oxygen and blood substrates to active muscles during exercises and the release of metabolites. These systems could act as limiting factors to the aerobic potential and, thereby, could limit the physical performance [8].

Endurance exercise in horses leads to variety of physiological changes, i.e. an increase of haematocrit (HCT), cardiac output, mean pulmonary arterial blood pressure, and the arterial hypoxemia [2, 5, 8]. In addition, muscle temperature increases significantly during an exercise; therefore, this also can affect on the reduction of erythrocyte resistance. Also acidosis, elevated level of catecholamines, dehydration, and compression of erythrocytes in capillaries within the contracting muscle are some important mechanisms that play a role in intravascular haemolysis during regular physical activity [9]. Intravascular hemolysis is one of the most emphasized mechanisms for destruction of erythrocytes during physical activity in horses [6, 10–12]. In endurance races, stress and fatigue are clearly expressed by changes in hematological and biochemical parameters of horses [2]. Additionally, blood haematological parameters can be good indicators of the response to treatment, the severity and the systemic effects of a disease, as well as horse welfare, health and fitness levels of horses [1, 2, 5, 7]. Therefore, the main goal of the present study was to investigate the alterations of some haematological parameters [haematocrit (HCT), haemoglobin concentration (HGB), the count of red blood cells (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)], as well as resistance of erythrocytes to hemolytic agents in Crimean horses after 32 km endurance race.

MATERIALS AND METHODS

Horses. Seven horses in Crimean region (Bilohirsk, Crimean region, Ukraine) were involved in our study (Fig. 1). The basis of local horses in Crimea was formed from the Bashkir Curly Horse which were imported to the Crimea in the 60s of the last century. Equestrian tourism in Crimea was established mainly in the mid-90s, when the horses ended up in private ownership, and each owner at his discretion led the subsequent reproduction of livestock. Thus, a lot of horses in Crimea were subjected to chaotic cross-breeding with stallions of various breeds: Akhal-Teke, Trakehner, Arab, Thoroughbreds, trotters, and other cold blood horses, including Bashkir Curly Horse. As a result of crosses under the influence of harsh conditions of herd maintenance, breeds showed genotypic and phenotypic heterogenecity. These horses come in a wide range of sizes from miniature to draft, and in different color. Their hoofs are almost perfectly round in shape. They also have stout round-bone; straight legs; flat knees; strong hocks; short back; round rump; powerful rounded shoulders; round chest, all of which contribute to their strength and endurance.

All horses participated in endurance race. Horses were subjected to herd maintenance with feeding (hay and oat) provided twice a day and water available *ad libitum*.

All horses were thoroughly examined clinically and screened for hematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant. Owners allowed to provide supplemental feed and salts to their horses. Information about supplementations in horse diets with antioxidant compounds, such as vitamin E or selenium, was not available. Information about previous physical activities was not available. A comprehensive physical examination was performed on all horses. The physical examination included monitoring horses' vital clinical signs (heart rhythm, respiratory rhythm and gut sounds). In addition, the hydration status, gait of the animal, and presence of any injuries, especially in the legs, girth, withers, and back, were recorded. Only horses that had normal clinical parameters were allowed to participate in the endurance race.

Endurance race. The prolonged exercises were used in endurance race. The walk about 3 km per h for 20 min, the trot about 7 km per h for 15 min, the canter about 5 km per h for 15 min, and the walk about 1 km was repeated for 1 h (phase I); the rest in an outdoor paddock without access to water for 30 min. Phase II consisted of the walk about 3 km per h for 20 min, the trot about 7 km per h for 15 min, the canter about 5 km per h for 15 min, and the walk about 1 km was repeated for 1 h.

Blood samples. Blood was drawn from jugular veins of the animals in the morning, 90 min after feeding, while the horses were in the stables (between 8:30 and 10 AM), and immediately after endurance race (between 11:00 AM and 2:00 PM). Blood was stored into tubes with K-EDTA and held on ice until centrifugation at 3,000g for 15 minutes. The plasma was removed. The erythrocytes' suspension (one volume) was washed with five volumes of saline solution three times and centrifuged at 3,000 g for 15 minutes. Plasma aliquots were frozen and stored at –25 °C until analyzed.

Hematological assays. Routine haematological parameters (haematocrit (HCT), haemoglobin concentration (HGB), the count of red blood cells (RBC), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red cell distribution width (RDW)) – were measured and counted with an automated hematology analyzer (Abakus Junior Vet, Austria).

Assays of osmotic resistance of erythrocytes. The osmotic resistance of erythrocytes was measured spectrophotometrically at the wavelength of 540 nm as described by Kamyshnikov (2004) [13]. The method is based on the determination of differences between osmotic resistance of erythrocytes to a mixture containing various concentration of sodium chloride and urea. Absorbance of mixture contained erythrocytes and 0.3 M urea solution was determined as 100 % hemolysis (standard). The level of hemolysis in every test tube (%) was calculated in respect to the absorbance of standard. Hemolysis of erythrocytes (%) in every test tube with different urea concentration was expressed as curve [13].

Assay of resistance of erythrocytes to hydrogen peroxide. The peroxide resistance of erythrocytes was determined spectrophotometrically at 540 nm by monitoring the rate of erythrocytes disintegration by hydrogen peroxide as described by Gzhegotskyi *et al.* [14]. The mixture contained 0.25 mL of washed erythrocytes, 0.08 mL of 4 mM phosphate buffer (pH 7.4) with 4 mM sodium azide for inhibition of catalase activity, and 0.17 mL of 30 μM hydrogen peroxide dissolved in phosphate buffer (pH 7.4). In the blank, hydrogen peroxide was substituted by phosphate buffer. Absorbance of mixture containing erythrocytes, distilled water and hydrogen peroxide was determined as 100 %. The peroxide resistance of erythrocytes (hemolysed erythrocytes fraction) was expressed in % [14].

Statistical analysis. Results are expressed as mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between states before and after riding, Wilcoxon signed-rank test was applied to the data [15].

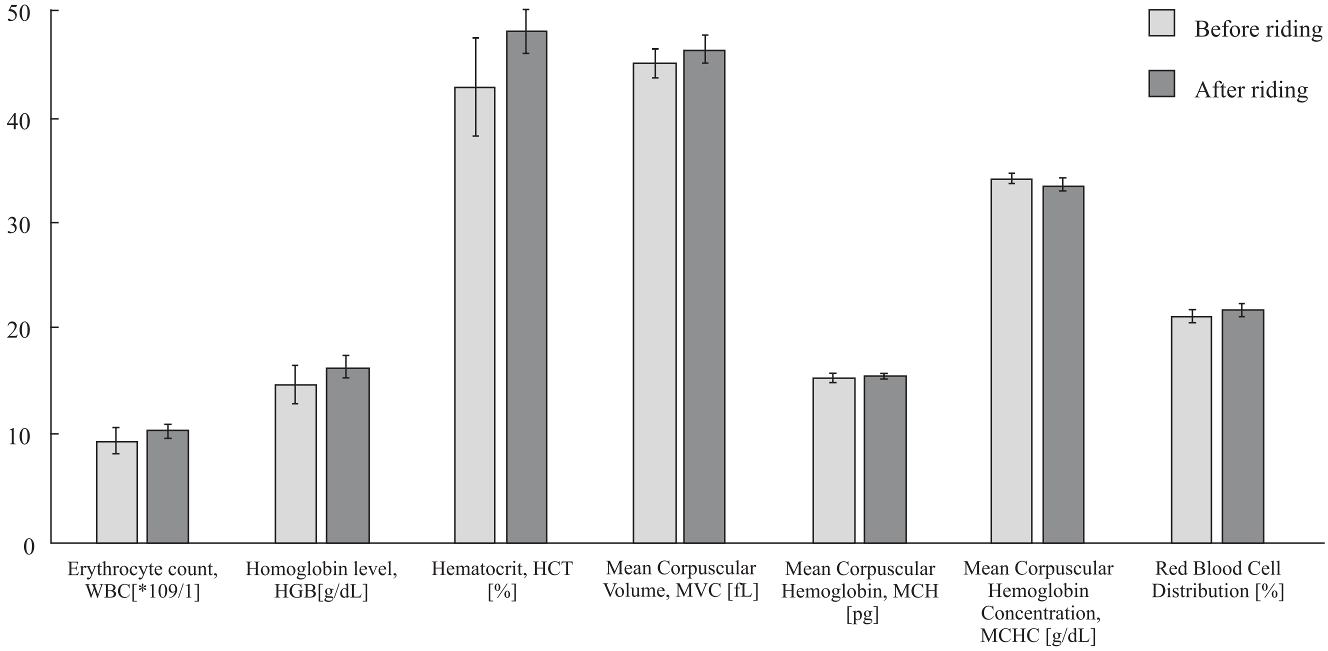

Fig. 2. Values of red blood cell indices of Crimean horses during 32 km riding

All statistical analyses were performed using STATISTICA 8.0 software (StatSoft, Poland). In addition, the relationships between values of hematological indices of all individuals were evaluated using Spearman's correlation analysis [15].

RESULTS AND DISCUSSION

In our study, all hematological parameters of horses in Crimean region were within the reference values. In the present study, post-ride values of red blood cell indices did not significantly change compared to pre-ride period (Fig. 2).

Exercises have variable effects on the erythrocyte indices depending on work intensity, fitness and training levels, environmental conditions and breed of horses [6, 8, 16, 17]. Predominantly, the increase in the value of RBC indices in horses is caused by a release of erythrocytes from the spleen, where about 50−60 % of the general number of these blood cells are located [16]. Exercise increases sympathetic activity in horses and thus increases hematocrit. The number of cells released from the spleen in response to exercise is not "all-or-none," but rather it is related to the extent of the increase in sympathetic activity that is related to exercise intensity. However, the increase of hematocrit could also be attributable to changes in plasma volume in relation to thermoregulatory processes, mainly by sweating and evaporation from the respiratory mucosa and to fluid shift derived from physical activity [18]. Adamu *et al.* (2012) reported significant increases in RBC, HGB and HCT ($p < 0.0001$) which could indicate metabolic crisis and poor performance in endurance horses [4]. However, numerous studies have shown that horses exposed to high altitude have significantly higher RBC, HGB and platelet corpuscular volume (PCV) values, compared to animals that live at less altitude [16, 19]. It is considered a compensatory mechanism for the lower content of oxygen in the atmospheric air, which is proportionally reduced to the altitude [19].

Given fact that horses from Crimean region were housed under altitude of the Crimean Mountains, our results are consistent with previous studies [19]. Moreover, one of reasons of the slight increase of red blood cells indexes in the blood of Crimean horses can be their inhabitation on the altitude. Long term hypoxic exposure and/or stress to altitude can lead to an increment of red blood cells, hemoglobin, density of capillary blood vessels, and myoglobin density in skeletal muscle [20, 21], resulting in enhancement of oxygen delivery capacity. In the cellular level, hypoxic exposure can accelerate the proliferation of mitochondria in the muscles [22], increase the buffering capacity for lactic acid [23], and subsequently enhance endurance capacity in the high altitude environments. In spite of this theoretical rationale, the majority of studies investigating athletes who returned to the sea level from high altitude training reported no changes or

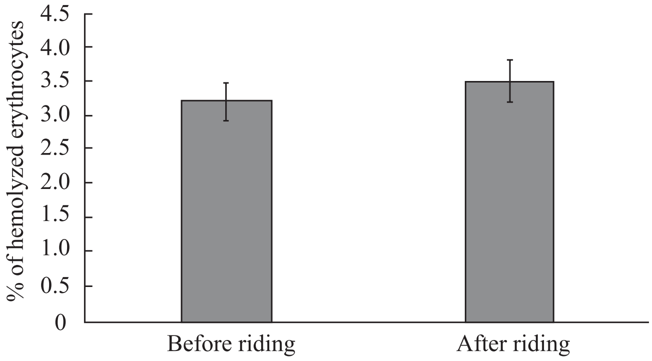

Fig. 3. Resistance of erythrocytes to H_2O_2 exposure in Crimean horses during 32 km riding

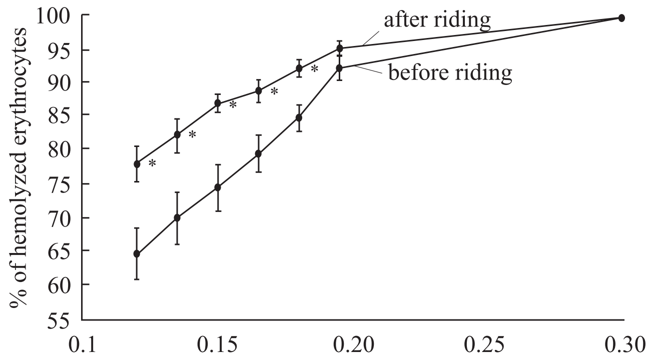

Fig. 4. Osmotic resistance of erythrocytes (% of hemolyzed erythrocytes in solutions with different urea concentration) of Crimean horses during 32 km riding. *Statistical significance ($p < 0.05$) between means before and after 32 km riding

even reduction of the level of physical performance. And few studies demonstrated an improvement of physical performance after the altitude training [24]. This statement can be meaningful in connection with evidence of Adamu and coauthors which have shown that there are no significant changes of red blood cells indices in post-ride period in endurance horses with good performance level [2, 4].

The resistance of erythrocytes to H_2O_2 exposure in Crimean horses before and after 32 km riding was presented in Fig. 3.

No significant changes in resistance of erythrocytes to H_2O_2 exposure in Crimean horses during 32 km riding were observed (Fig. 3).

Exercise-induced hemolysis has been confirmed under various conditions in stallions [12, 25], mares [12, 26] and mixed population [6, 27]. In our study, erythrocytes exposed to the different concentrations of urea after endurance race had a higher level of haemolysis compared to the pre-ride period (Fig. 4). Increase of hemolyzed erythrocytes percent by 20.58 % ($p = 0.013$), by 17.3 % ($p = 0.023$), by 16.75 % ($p = 0.006$), by 11.57 % ($p = 0.015$), and by 8.73 % ($p = 0.008$) in 0.12, 0.135, 0.15, 0.165, and 0.18 M urea solutions, respectively, was observed in horses after endurance race.

Erythrocytes appear much more vulnerable to oxidative damage during intense exercise because of their continuous exposure to high oxygen fluxes and their high concentrations of polyunsaturated fatty acids (PUFAs) and heme iron [9, 28, 29]. Our findings are consistent with data of Devi *et al*. (2009) which have shown that osmotic stress at 0.3 % and 0.4 % NaCl imposed hemolysis in animals exposed to altitude and thereby signifies an oxidative stress-dependent impairment of erythrocyte stability [30]. It has been shown that lipid peroxidation and oxidation of proteins by free radicals play a major role in many oxidative erythrocytes damage and cause profound alterations in the structural organization and functions of the cell membrane including decreased membrane fluidity, increased membrane permeability, inactivation of membrane-bound enzymes and loss of essential fatty acids [28, 29]. Based on our results, it is possible to affirm that enhanced percent of hemolyzed erythrocytes in horses after endurance ride indicates to an impairment of erythrocyte stability induced by oxidative stress.

CONCLUSIONS

Adequate endurance race of low intensity could improve oxygen-induced hematological function in Crimean horses. Furthermore, the non-significant increase of red blood cells indices in endurance horses indicates good athletic level after 32 km endurance ride. Statistically significant differences in the percentage of hemolyzed erythrocytes between pre- and post-ride period were observed. It signifies an impairment of erythrocyte stability induced by oxidative stress. The haematological changes caused by various physical efforts reflect changes in the functions of different systems and can be used for health control and diagnosis of diseases. It also allows evaluating the level of sport performance, the accuracy of training, and physiological condition of horses.

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Показники червоної крові та резистентність еритроцитів у кримських коней, які беруть участь у дистанційних пробігах на 32 км

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Однією з прикладних галузей конярства, які наразі активно розвиваються, є дистанційні пробіги та кінний туризм. В Україні, враховуючи регіональні ландшафтні особливості, дистанційні пробіги є найпоширенішими в Криму та на заході країни. До коней, що використовують у дистанційних пробігах, існують певні вимоги, але, на жаль, у наукових роботах небагато досліджень, пов'язаних з аналізом складу залучених коней, а також гематологічних та біохімічних показників крові. **Мета.** Проаналізувати кількісні зміни показників червоної крові та резистентності еритроцитів у коней, які брали участь у дистанційному пробігу на 32 км. **Методи.** Об'єктом досліджень слугували сім коней кримського типу. Гематологічні показники (гематокрит (HCT), концентрація гемоглобіну (HGB), кількість еритроцитів (RBC), середня концентрація гемоглобіну в еритроцитах (MCHC), середній об'єм еритроцитів (MCV), середній вміст гемоглобіну в еритроциті (MCH), індекс анізоцитозу (RDW)) визначали з використанням гематологічних та біохімічних методів. Зразки крові досліджували центрифугуванням при 3000 g протягом 15 хв. Пероксидну та осмотичну стійкість еритроцитів встановлювали спектрофотометрично при довжині хвилі 540 нм за швидкістю розпаду еритроцитів під дією пероксиду водню. **Результати.** Не виявлено достовірних змін в показниках червоної крові у коней після пробігу на 32 км, що свідчить про хороший фізичний стан та адаптованість коней до довготривалої їзди. Натомість осмотична резистентність еритроцитів була достовірно нижчою після пробігу, що свідчить про порушення цілісності еритроцитарних мембран внаслідок фізичних навантажень. **Висновки.** Вивчення динаміки показників крові у поєднанні з біохімічними параметрами та стійкістю еритроцитів дає змогу оцінити процеси адаптації до фізичних навантажень на витривалість в організмі коней, а також проаналізувати рівень їхного фізіологічного резерву.

Ключові слова: дистанційні кінні пробіги, коні, гематологічні показники, резистентність еритроцитів, фізичні навантаження.

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