



Oxidative Status of Peripheral Blood Mononuclear Cells of Stallions of Different Ages

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ABSTRACT

Background: The aging process causes changes in the structure and functioning of body systems, including the immune system. The aim of this study was to assess the oxidative status of peripheral blood mononuclear cells (PBMCs) of young (3-5 years old), full-aged (6-15 years old) and older stallions (16-25 years old).

Methods: The level of protein carbonyl derivatives, low and medium molecular weight substances, the activity of superoxide dismutase, catalase, γ -glutamyl transpeptidase, acid phosphatase, lactate dehydrogenase were studied in PBMCs of 31 stallions of Arabian breed.

Result: An increase in the total content of carbonyl derivatives was revealed in the group of older stallions compared with young ($p=0.0030$) and full-aged stallions ($p=0.0030$) due to an increase in the content most of all carbonyl derivatives of a neutral and basic nature. An increase in the level of low and medium molecular weight substances was recorded in the group of older animals compared with young ($p=0.0089$) and full-aged stallions ($p=0.0102$). A decrease in γ -glutamyl transpeptidase activity was observed in young stallions compared with full-aged ($p=0.0026$) and older stallions ($p=0.0006$). The data obtained indicate a violation of the redox balance in aged stallions PBMCs.

Key words: Age, Oxidative metabolism, PBMCs, Stallions.

INTRODUCTION

The aging process causes changes in the structure and functioning of body systems, including the immune system. These changes affect both acquired and innate immunity, disrupting their response to internal and external stimuli, which leads to "immune aging" and "inflammatory aging" (Fülöp *et al.*, 2016). The efficiency of oxidative metabolism, *i.e.* the ability of endogenous antioxidants to balance the production of reactive oxygen species (ROS) also decreases with age, which contributes to the creation of an inflammatory environment associated with aging (oxi-inflammaging) (Bullone *et al.*, 2017; De la Fuente and Miquel, 2009). When ROS exceed the antioxidant capacity, it causes oxidative stress, directly related to the development of many diseases preventing healthy aging (Jain and Shakkarpude, 2024; Vatner *et al.*, 2020). Moreover, the horse is a recommended model for the study of some age-related diseases associated with oxi-inflammaging (Bullone *et al.*, 2015).

Peripheral blood mononuclear cells (PBMCs) provide immunological control, resist infection of the body and the development of chronic diseases. Monocytes belong to mononuclear phagocytes producing ROS and reactive nitrogen species, which have powerful oxidative, cytostatic and cytotoxic effects (Baran *et al.*, 2004; Jacinto *et al.*, 2018). The contact of lymphocytes with various endogenous and exogenous factors can also lead to an intensification of the production of ROS, which can act as damaging agents of proteins, lipids and DNA (Nosareva *et al.*, 2015).

Proteins undergo oxidative modification by ROS, reducing sugars and aldehydes obtained as a result of

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lipid peroxidation. Among the various oxidative modifications, the introduction of carbonyl groups such as aldehyde, ketone and lactam into the side chains of amino acids of proteins is the main sign of oxidative damage to proteins and is called "protein carbonylation". The detection and quantification of protein carbonyls is usually carried out to determine the level of oxidative stress in the context of cell damage, aging and a number of age-related disorders (Akagawa, 2021), chronic diseases (Belskikh *et al.*, 2018). The accumulation of protein carbonyls in the cell can have a cytotoxic effect and disrupt cellular metabolism, however, the exact mechanisms of the effect of protein carbonyl derivatives on the aging process require further study (Rudzińska *et al.*, 2020).

Metabolic disorders can also be accompanied by an increase in the blood content of the low and medium

molecular weight substances (LMMWS). LMMWS are compounds of a non-protein nature with a molecular weight from 300 to 5000 Da, formed during metabolism. These include: Urea, ammonia, creatinine, uric acid, glucose, lactic and other organic acids, oligosaccharides, derivatives of glucuronic acid, fatty acids, cholesterol, phospholipids, products of free radical oxidation, intermediate metabolism, nucleotide metabolism, alcohols, aldehydes and other substances present in the blood and in endogenous intoxication, in pathological conditions of the body, their content increases (Edelev, 2018). Quantitative indicators of the content of LMMWS can serve as markers of various pathological conditions of the body. In the early stages of metabolic disorders, there is an increase in the level of LMMWS as a result of a compensatory mechanism, with deeper violations, toxic substances, oligopeptides appear. However, there is no information on the relationship between LMMWS and free radical metabolism in farm animals (Tregubova *et al.*, 2016).

The ability to adapt in conditions of impaired metabolism is provided by an antioxidant protection system, which includes an enzymatic (superoxide dismutase (SOD), catalase, glutathione peroxidase, *etc.*) and a non-enzymatic component (Nikitina *et al.*, 2022). Trofimov *et al.* (2018) indicate that the level of viability of PBMCs depends both on the availability of antioxidants and on the activity of key antioxidant enzymes (Trofimov *et al.*, 2018). Therefore, it is of particular interest to study the activity of SOD and catalase in the age aspect of animals. In mammals, there are three isoforms of SOD (SOD1, SOD2, SOD3) and all of them require the presence of an oxidative-reducing active transition metal in the active center to complete the decomposition of the superoxide anion. Among the three isoforms, SOD1 requires copper-zinc as a cofactor and is located in the cytosol and mitochondrial intermembrane space, SOD2 uses manganese and is located in the mitochondrial matrix and SOD3 requires copper-zinc and is located in the extracellular space. Hydrogen peroxide is enzymatically neutralized by catalase to water and molecular oxygen. Catalase is also an important antioxidant enzyme, hemoprotein and is widely distributed throughout the cell (Adwas *et al.*, 2019; Powers and Jackson, 2008).

The analysis of the enzymatic activity of the peripheral blood of stallions allows to identify multidirectional shifts reflecting the reaction of leukocytes to general metabolic changes, as well as the participation of these cells in immune and regenerative processes (Dolgushin and Sobolev, 2009). Determination of lactate dehydrogenase (LDH) activity in horses is one of the methods for determining stress reactivity (Evsyukova, 2016). LDH is an important enzyme of the anaerobic metabolic pathway. When cells are exposed to anaerobic or hypoxic conditions, ATP production by oxidative phosphorylation is disrupted. Consequently, LDH is activated in conditions involving the use of alternative energy production routes (Bahridinov *et al.*, 2023). There is reliable data on the

relationship between oxidative stress and increased LDH activity with age (Rashidova and Gashimova, 2019).

Acid phosphatase (AP) is a hydrolytic enzyme of lysosomes that catalyzes the reaction of phosphoproteins dephosphorylation in an acidic environment, while researchers are interested in studying the activity of the enzyme in lymphocytes (Novitsky *et al.*, 2011). Hydrolytic lysosomal enzymes, including acid phosphatase, are directly related to the processes of phagocytosis, affecting the activity of cellular immunity (North, 1966). In addition, phagocytosis is considered as one of the cell's ways to resist oxidative stress (Filomeni *et al.*, 2015).

Gamma-glutamyltransferase (GGT) is a member of the structural superfamily of N-terminal nucleophilic hydrolases expressed by a wide number of cell types, including PBMCs (Grisk *et al.*, 1993); it was later discovered that peripheral blood monocytes, after activation, are able to release the high-molecular form "b-GGT" associated with exosomes (Belcastro *et al.*, 2015). Previously, it was believed that the main role of GGT is to restore cysteine level, but in recent years a lot of experimental material has been accumulated indicating the important role of GGT-mediated cleavage of gamma-glutamyl bonds, triggering a cascade of reactions modulating the redox balance inside and outside the cell (Corti *et al.*, 2020).

The aim of this study was to investigate the oxidative status of peripheral blood mononuclear cells of young stallions (3-5 years old), full-aged animals (6-15 years old) and older stallions (16-25 years old).

MATERIALS AND METHODS

Animals

The study was performed on the livestock of the All-Russian Research Institute for Horse Breeding (ARRIH, Ryazan Region, Russia) and the Tersk Stud Farm N169 (Stavropol Region, Russia). All procedures were carried out in accordance with the "European Convention for the protection of vertebrates used for experimental and other scientific purposes" ETS No. 123 (18 March 1986) and the Law of the Russia Federation on Veterinary Medicine No. 4979-1 (14 May 1993). All research involving animals was conducted according to the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). The animal study protocol was approved by the commission for the control of the keeping and use of experimental animals (Commission on Bioethics) of the All-Russian Research Institute for Horse Breeding (Protocol Number: 2022/11/3).

31 stallions of Arabian breed were used in the experiments, aged 3 to 25 years. During the experimental studies, the conditions of keeping and feeding stallions corresponded to the established standards. The stallions were kept in individual stalls. The stallions received hay, oats and granulated compound feed with the addition of minerals according to established norms and were subjected to physical activity for at least 1 hour a day.

Collection of PBMCs

A blood sample from each clinically healthy stallion from the jugular vein was taken once before morning feeding during the sperm collection period during the breeding season (February- April) of the year 2023 and 2024.

Blood sampling was carried out as described in the article (Belskikh *et al.*, 2020) using test tubes containing sodium heparin, separation gel and ficoll solution to create a density gradient (Vacutainer® CPT™-BD, USA). Lymphocytes were isolated from the blood by centrifugation of the collected blood in BD CPT tubes at a relative centrifugal acceleration of 1.600 for 16 minutes in accordance with the manufacturer's instructions. After centrifugation, plasma with lymphocytes and monocytes was taken from the contents of the test tube above the separation gel. Mononuclear leukocytes were separated from plasma by centrifugation at 3.000 rpm for 10 minutes. The resulting cells were washed with 0.9% NaCl, followed by centrifugation at 3.000 rpm for 5 minutes three times. The isolated mononuclear leukocytes were resuspended in 1 ml of distilled water to obtain a suspension. Detergent (10 µl Triton X-100) was added to 1 ml of the suspension and it was frozen. After defrosting, the suspension was used to determine the protein concentration, products of protein oxidative modification, LMMWS and enzyme activity.

Biochemical analysis of PBMCs

The assessment of the content of protein carbonyl derivatives was performed as described in the article (Shitikova *et al.*, 2023). The assessment of the total content of protein carbonyl derivatives was evaluated on the spectrophotometer SF-2000 (St. Petersburg) using the R.L. Levine method modified by Dubinina (Dubinina *et al.*, 1995). The area under the absorption spectrum curve of the products of protein oxidative modification consists of the areas under the curve of aldehyde-dinitrophenylhydrazones (ADNPH) and ketone-dinitrophenylhydrazones (KDNPH) recorded in the UV and visible part of the spectrum, as described in the patent (Fomina *et al.*, 2014).

The protein content was determined by the Lowry method using a commercial kit from the Eco-Service Scientific and Practical Center (St. Petersburg).

The determination of the LMMWS was carried out according to the method of Malakhova (Malakhova, 1995). The method is based on the protein precipitation of biological fluids with a solution of 15% trichloroacetic acid. LMMWS remain in the supernatant and are examined at wavelengths of 238-298 nm in 4 nm increments. The calculation of the final result is carried out using an integral measurement of the area of the figure formed by the obtained extinction values by multiplying the sum of the extinction values by the wavelength step in conventional units per gram of protein (c.u. /g of protein):

$$\text{LMMWS} = (\text{E238} + \text{E242} + \text{E246} + \dots + \text{E298}) \times 4$$

Registration of the spectrum in this ultraviolet zone allows for a comprehensive assessment of more than 200

names of substances formed during normal metabolism and impaired metabolic processes.

The activity of SOD was determined photometrically by inhibition of the quercetin autooxidation reaction (Kostyuk *et al.*, 1990). The activity of catalase was determined spectrophotometrically, the method is based on the ability of hydrogen peroxide to form a stable colored complex with molybdenum salts (Koroljuk *et al.*, 1988). The activity of GGT, LDH and AP was determined on a semi-automatic analyzer Stat Fax 1904+ (Awareness Technology Inc, USA) using diagnostic kits (BioSystems, Spain).

Statistical analysis

Depending on the age, the animals were divided into 3 groups: The first included young stallions aged 3-5 years (n=7), the second-full-aged stallions aged 6 to 15 years (n= 15) and the third-older stallions aged 16-25 years (n=9). Statistical processing was performed using GraphPad Prism 9 and Microsoft Office Excel 2016 (StatSoft Inc., USA). The Shapiro-Wilk test was used to assess the distribution in groups. In the case of an abnormal distribution, the nonparametric Kraskell-Wallis criterion was used to assess statistical significance, the two-stage Benjamini-Krieger-Yecutieli method was used to correct for multiple comparisons, the results were provided in the form of a median and upper and lower quartiles. If the distribution was normal, the Welch's test and Dunnett's test were used to assess statistical significance and the results were provided in the form of Min and Max. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

A comparative analysis of the absorption spectrum of carbonyl derivatives in stallions' PBMCs of three age groups showed that the highest level of protein oxidative modification products is observed in the group of older stallions (Fig 1). There is a statistically significant increase in the total content of carbonyl derivatives in the group of older stallions (16-25 years) compared with full-aged stallions (6-15 years old) and young stallions (3-5 years) (Table 1). This increase is due to an increase in the content of ADNPH of a basic and neutral nature and KDNPH of a neutral nature in comparison with full-aged and young stallions and KDNPH of a basic nature in comparison with young stallions (Table 1).

In addition, the group of older stallions was characterized by the highest level of endogenous intoxication (Table 2). A significant increase in the level of LMMWS was recorded in the group of older animals compared with full-aged stallions and young stallions (Table 2). These data suggest that during aging in older stallions, not only free radical processes affecting protein molecules occur in the PBMCs, but also products of impaired metabolism of non-protein origin are formed. Apparently, with age, there is a decrease in the ability of the animal's body to withstand oxidative stress. Thus, in a

study by Jacinto *et al.* (2018), increased ROS production was found in PBMCs of old rats compared with a group of young animals (Jacinto *et al.*, 2018). Previously, we observed a significant increase in the content of protein carbonylation products in the seminal plasma of aged stallions compared with younger ones, mainly due to aldehyde derivatives of a neutral nature (Atroshchenko *et al.*, 2023).

There was a tendency to increase the activity of antioxidant enzymes SOD and catalase in the group of older stallions, but no significant differences were found (Table 2). There is various information in the literature about age-related differences in antioxidant capacity. Thus, in blood plasma according to Czech *et al.* (2016), lower SOD activity was observed in the blood of aged Lesser Polish horses compared with young horses (Czech *et al.*, 2016) and Kirschvink *et al.* (2006) revealed a significant decrease in SOD levels in horses older than 6 years (Kirschvink 2006). However, according to Mendoza-Nunez *et al.* (2007) with increasing age, an increase in the content of lipoperoxides is observed in humans, associated with a decrease in the overall antioxidant capacity and activity of

glutathione peroxidase, while the activity of SOD remained unchanged (Mendoza-Nunez *et al.*, 2007), similar data were obtained in horses (Molinari *et al.*, 2020). Williams *et al.* (2008) show that antioxidant levels and blood lipid peroxidation were similar in old and in mature horses (Williams *et al.*, 2008). In human lymphocytes with age, an increase in the content of carbonyl derivatives of proteins was observed, as well as a decrease in the activity of SOD and catalase (Gautam *et al.*, 2010). Similar data were obtained in a study on PBMCs of humans and mice, which fits into the theory of oxi-inflammaging (De la Fuente and Miquel, 2009). However, in mononuclear leukocytes of peripheral blood of healthy ponies of different age groups, greater endogenous DNA damage is associated with increased RBC glutathione concentrations, which indicates that antioxidant protection is not suppressed in older animals (Marlin *et al.*, 2004). An increase in catalase activity in blood cells with age may occur, apparently, due to increased formation of hydrogen peroxide and be adaptive in nature (İnal *et al.*, 2001). Kasapoglu and Özben (2001) revealed an increase in the activity of

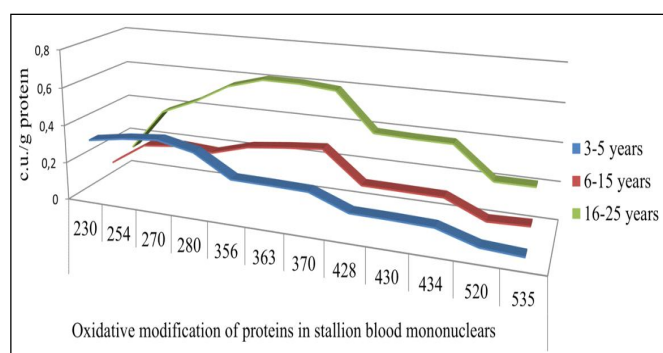


Fig 1: Absorption spectrum of protein oxidative modification products in PBMCs of stallions of different ages (c.u./g protein), Me.

Table 1: Comparative analysis of the absorption spectrum of protein oxidative modification products in PBMCs of stallions of different ages (c.u./g protein).

Indicator	Age of stallions, years		
	3-5	6-15	16-25
$S_{ADNPH}^{neutral}$	41.55 [39.07;46.40]* p=0.0107	33.90 [18.18;46.15]* p=0.0082	69.41 [50.25;224.44]
S_{ADNPH}^{basic}	11.73 [7.01;17.90]* p=0.0015	15.42 [7.94;20.67]* p=0.0015	35.79 [30.74;65.18]
$S_{KDNPH}^{neutral}$	15.56 [9.83;20.08] p=0.0063	19.59 [13.35;21.91] p=0.0069	34.74 [23.04;46.15]
S_{KDNPH}^{basic}	2.10±1.37* p=0.0361	2.30±1.36	7.46±5.33
Stotal	70.97 [55.97;87.73]* p=0.0030	67.41 [42.27;93.64]* p=0.0030	147.46 [91.66;360.62]

*-Statistically significant differences from the 16-25 years group, p<0.05.

Table 2: Enzyme activity and LMMWS content in PBMCs of stallions of different ages, Me [Q1;Q3].

Indicator	Age of stallions, years		
	3-5	6-15	16-25
SOD, c.u./mg protein	1.44 [1.34;1.57]	2.04 [1.10;3.03]	5.63 [1.05;8.62]
Catalase, mcmol/min/g protein	46.98 [37.28;72.15]	56.90 [47.85;87.16]	121.30 [91.03;152.90]
AP, mccat/g protein	0.50 [0.39;0.62]	0.60 [0.40;0.64]	0.31 [0.25;0.50]
GGT, mccat/g protein	0.54 [0.43;0.61]	1.15 [0.91;1.25] p=0.0026	1.17 [1.15;1.33] p=0.0006
LDH, mccat/g protein	3.47 [1.68;6.69]	4.90 [3.00;5.55]	4.50 [2.32;5.23]
LMMWS, c.u./g protein	13.82 [12.27;15.75]** p=0.0089	16.01 [11.25;24.42]** p=0.0102	38.05 [18.09;54.78]

*-Statistically significant differences from group 3-5 years, $p < 0.05$.

** -Statistically significant differences from group 16-25 years, $p < 0.05$.

SOD and catalase with age, the authors of the study emphasize the need for a more complete investigation of the links of antioxidant protection, since an increase in the activity of some antioxidants may be compensatorily accompanied by a decrease in the activity of others not included in the study (Kasapoglu and Özben, 2001). It is possible that the tendency we discovered to increase the activity of SOD and catalase may be associated with the intensification of oxidative processes with age, which leads to the tension of antioxidant system in the protective cells of stallions, but this assumption requires confirmation and research of additional participants in antioxidant protection in stallions' PBMCs.

In addition, the lowest level of GGT activity was detected in the group of younger stallions, statistically significant differences were recorded in comparison with the group of full-aged and older stallions (Table 2). Along with its key role in the preserving intracellular glutathione level by maintaining cysteine homeostasis, GGT-mediated cleavage of GSH could cause the reduction of ferric iron Fe(III) to ferrous Fe(II), thus starting a redox cycling process liable to result in the production of superoxide radical and hydrogen peroxide, which can cause lipid peroxidation and protein carbonylation. It should be noted that to start this process, the presence of free iron in the cell is necessary, which can appear *in vivo* under conditions of necrosis and inflammation, especially in the presence of activated phagocytes producing prooxidants (reviewed by Corti *et al.*, 2020). GGT can also generate ROS in monocytes due to the cytokine-like mechanism through activation TLR4/ NF- κ B redox-signaling pathway (Sanguinetti *et al.*, 2022). These data are in good agreement with the theory of oxi-inflammaging (Bullon *et al.*, 2017; De la *et al.*, 2009) and may partly explain the observed lowest GGT activity in the PBMCs of young stallions.

In connection with the above, further more complete studies of the redox metabolism and redox regulation of PBMCs in aged stallions are needed, as well as the search for preventive measures with the use of specific antioxidants.

CONCLUSION

The highest level of protein oxidative modification products and products of endogenous intoxication of non-protein origin is observed in PBMCs of older stallions in comparison with full-aged and young stallions. Statistically significant increase in the total content of OMP in PBMCs of older stallions it is due to an increase in the content most of all carbonyl derivatives: both KDNPH and ADNPH are of a neutral and basic nature. These data suggest that during aging in older stallions free radical processes affecting protein molecules occur in the PBMCs, as well as products of impaired metabolism of non-protein origin are formed.

We also found a tendency to increase the activity of SOD and catalase in the group of older stallions, which may indicate the tension of antioxidant protection due to increased ROS formation and be adaptive in nature. At the same time, the lowest activity of GGT- an enzyme that potentially triggers a cascade of oxidative reactions in mononuclear cells was detected in PBMCs of young stallions in comparison with full-aged and older stallions.

The data obtained indicate a violation of oxidative metabolism in PBMCs of aged stallions and indicate the need for antioxidant therapy in the group of older stallions.

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Conflict of interest

The authors declare that there is no conflict of interest.

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