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Blood plasma antioxidants and dehydration in three-month-long trained Standardbred trotters before and after an intensive race

TADEUSZ OGOŃSKI, RYSZARD PIKUŁA*, GRZEGORZ MAJEWSKI**

Department of Physiological Chemistry, Faculty of Biotechnology and Animal Breeding, University of Agriculture, Doktora Judyma 2, 70-466 Szczecin, Poland
*Department of Horse Breeding, Faculty of Biotechnology and Animal Breeding, University of Agriculture, Doktora Judyma 10, 70-466 Szczecin, Poland
**Horse Training Center, Bonin 31, 73-150 Łobez, Poland

Ogoński T., Pikuła R., Majewski G.

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Summary

The aim of the study was to determine the total antioxidant status in the plasma of horses – Standardbred trotters, exposed to extreme oxygen action during a race. The experiment was carried out on 10 two-year old horses – five stallions and five mares, after three-month-long initial training. On the experimental day, peripheral blood was sampled twice from the horses: at rest and after a race over 3200 m at a pace of 550 m/min. In the blood were assayed: blood platelet, white blood cell and red blood cell counts as well as haematocrit value and haemoglobin concentration. The obtained values were the basis for calculating plasma dehydration factor (D_f). In the plasma was determined the antioxidative ability of plasma (AAP). For assays, a TAS (Total Antioxidant Status) analytical kit of Randox manufacture (UK) was used. In 8/10 horses, the AAP value underwent a reduction after the race. The statistical analysis revealed a significant decrease in AAP value in horses after completing the race ($P_p = 0.0126$). This difference correlates with the value of D_f factor ($R^2 = 0.304$), which may mean that prolonged organism overloading with intensive oxygen work leads to progressive exhaustion of its antioxidative reserves and in consequence, after crossing the critical value, to development of oxygen stress. The largest decrease of AAP value occurred in the horses capable of performing the largest effort ($\Delta TAS = 0.20 \pm 0.14$ mol/dm^3). These animals, i.e. three stallions and three mares, were characterised by a lower heartbeat rate and a lower respiration frequency at rest as well as by the capacity for quick regeneration of these parameters after the completed race.

Keywords: Standardbred trotters, race exercise, blood plasma antioxidants, dehydration

In scientific literature, one can find many references relating to horse exercise tests (1, 9, 14). Their common feature is that physical efficiency assessment is based on a correlation between the rate of movement and the amount of lactic acid produced and the value of the heartbeat rate. These reports, although very helpful in trainer's work, are incomplete. They are practically lacking in information on the effect of oxygen and oxygen-derived reactive oxygen species (ROS) on the horse organism during effort, in particular as concerns horses put to the heaviest physical work. The few reports devoted to this problem (3-5, 12, 13, 16) do not as yet constitute a firm foundation for presenting general conclusions. The scarcity of knowledge on the indicated problem motivated us to conduct an experiment aimed at determining the antioxidant status in the blood plasma of horses – Standardbred trotters – exposed to extreme oxygen action during a race.

Material and methods

Animals. Ten Standardbred horses, five stallions (m) and five mares (f), 2-years-of-age, that were being prepared for a racing season at the Horse Training Center in Bonin in Western Pomerania Province, Poland, were chosen for the experiment. Prior to the onset of training, a control peripheral blood morphology examination was performed in the horses (sample collection 1.1). The proper experiment was carried out after a three-month-long initial training period (82 days) on a day of such conditions (shade temperature 18-20 degrees C; 40-50% relative humidity (%RH); 994 Hpa) that promote water depletion. 3-5 ml of peripheral blood was collected from each horse twice from the

jugular vein into test tubes with Na_4EDTA: 1) at rest – after morning grooming (sample collection 2.1); and 2) immediately (< 2 min.) after the training race over a distance of 3200 m at a pace of 550 m/min (sample collection 2.2). In order to limit the effect of atmospheric oxygen on antioxidant status, immediately after sample collection the blood was placed in a cooler and tightly-closed in filled-to--capacity test tubes. Approval for blood collection was obtained from the Ethical Commission at Agricultural University in Szczecin, Poland. Moreover, in all horses the heartbeat rate (HR, min^{-1}) was measured on the heart with the use of phonendoscope (0.5 min.), as well as respiration frequency (RF, min^{-1}) at rest on costal arch, and after 15, 30, 45 and 60 minutes from the moment of race completion on costal arch or nares.

Laboratory analyses. Blood samples were assayed for blood platelet (PLT), white blood cell (WBC) and red blood cell (RBC) counts as well as haematocrit value (HCT) and haemoglobin concentration (HGB). The obtained values were the basis for calculating the following red cell indices: mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) as well as plasma dehydration factor (D_f). The assays were carried out with the conductometric method by means of the haematological analyser Sysmex F-800 (ICN-Instruments-Poland; accuracy: WBC ± 3.0%, RBC ± 2.0%, PLT ± 5.0%; reproducibility: HGB: C.V. ≤ 1.0%, WBC: C.V. ≤ 1.5%, RBC: C.V. ≤ 1.0%, PLT: C.V. ≤ 4.0%). In the blood plasma (3000 rpm, 10 min.) the antioxidative ability of plasma (AAP) was determined. For assays, a TAS (Total Antioxidant Status) analytical kit of Randox manufacture (UK) was used. Measurements were made using a PYE-Unicam SP1800 double-beam spectrophotometer at 37°C, recording the increase of absorbance in samples containing 20 µl of plasma at a wavelength of 600 nm against water at a time interval of 0 to 5 minutes. Calibration was carried out basing on 4 standard solutions containing 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid at concentrations of 0.206, 0.412, 0.825 and 1.650 $mmol/dm^3$. The lag time of signal recording was between 2 and 4 seconds. The reading of AAP was made exactly after 180 seconds from placing a plasma sample in a measuring cuvette.

Statistical analysis. The collected numerical data were processed statistically using computer software packages MS Excel and MedCalc v4.15a. The structure of examined populations was presented by means of a mean value with population standard deviation $S_{(x)}$. It was assumed that two mean values of an unpaired variable or mean values of differences of a paired variable differ significantly when the calculated probability (P_u – unpaired; P_p – paired) is lower than 0.05. So the reader can make his own interpretation if the real P-values are presented.

Results and discussion

The measurement data and their general statistical evaluation are compared in tab. 1-3. The analysis of the structure of particular populations showed that they fulfil the homogeneity condition.

Blood morphology examination after a three-month--long training period (sample collection 2.1, tab. 2) compared with the initial examination carried out prior to the onset of training (sample collection 1.1, tab. 1) showed a significant growth in the values of both original and derivative parameters of red cell system connected with the oxygen transport in blood: RBC ($P_p < 0.0001$), HGB ($P_p = 0.0002$), HCT ($P_p < 0.0001$), MCV ($P_p = 0.0301$), MCH ($P_p = 0.0118$) and confirmed a significant increase of the degree of physical fitness from training of horses and their readiness for increased oxygen effort. The values of assessed parameters, excluding RBC, were within upper norm limits and evidenced the good state of health of the

Tab. 1. Peripheral blood morphology at rest in two-year old Standardbred trotters prior to starting the training (sample collection 1.1)

No	Sex	PLT1.1 × $10^9/dm^3$	WBC1.1 × $10^9/dm^3$	RBC1.1 × $10^{12}/dm^3$	HGB1.1 × 10^{-2} kg/dm^3	HCT1.1 % (v/v)	MCV1.1 × $10^{-15}/dm^3$	MCH1.1 × 10^{-15}/kg	MCHC1.1 × 10^{-2} kg/dm^3
1	m	162	6.9	7.1	10.9	30.1	42.4	15.4	36.2
2	m	212	8.1	7.2	10.0	26.6	37.0	13.9	37.6
3	m	206	8.4	7.9	11.5	32.1	40.8	14.6	35.8
4	m	171	9.2	6.2	10.0	31.1	50.0	16.1	32.2
5	m	180	11.0	6.8	10.6	27.3	40.0	15.5	38.8
6	f	171	10.9	6.8	9.9	32.3	47.7	14.6	30.7
7	f	165	11.3	7.6	10.9	33.4	43.9	14.3	32.6
8	f	153	11.9	7.5	11.1	33.1	44.0	14.7	33.5
9	f	190	10.4	7.3	10.5	30.5	41.7	14.3	34.4
10	f	191	8.6	5.9	9.1	24.5	41.3	15.3	37.1
Mean		180	9.7	7.0	10.5	30.1	42.9	14.9	34.9
$S_{(x)}$		18.3	1.57	0.58	0.67	2.85	3.57	0.63	2.51

Explanation: $S_{(x)}$ – population standard deviation

animals qualified for the experiment. The number of erythrocytes (RBC) surpassed the norm and pointed to the readiness of horses for very intensive work under full oxygen access. Statistically higher values of HCT2.1 ($P_u = 0.0555$), MCV2.1 ($P_u = 0.0512$) and MCHC2.1 ($P_u = 0.0704$) indicated a slightly higher efficiency of oxygen transporting system in mares.

The increase of erythrocyte number and haemoglobin amount contained in them enables a more efficient distribution of oxygen throughout the organism and thus a prolongation of work time under full oxygen saturation. According to Kędzierski and Podolak (10), the increase of haemoglobin concentration under the influence of training is evidence of organism adaptation to effort. According to Szarska (17, 19, 20), there are considerable differences in haemoglobin concentration depending on horse breed, sex, age and training type. A properly conducted training program leads to an increase of haemoglobin concentration at rest and of haematocrit value, with a growth in these parameters being well evident in young horses which are just starting the training program. This author points out that the increase of erythrocyte number and haemoglobin amount contained in them is one of the effects of properly conducted training. Overloading or stress brings about a worsening of these parameters in horses.

On the experimental day two successive blood morphology examinations were made in each horse: the

Tab. 2. Peripheral blood morphology at rest in two-year old Standardbred trotters after three-month long training (sample collection 2.1)

No	Sex	PLT2.1 $\times 10^9/dm^3$	WBC2.1 $\times 10^9/dm^3$	RBC2.1 $\times 10^{12}/dm^3$	HGB2.1 $\times 10^{-2}$ kg/dm^3	HCT2.1 % (v/v)	MCV2.1 $\times 10^{-15}/dm^3$	MCH2.1 $\times 10^{-15}$/kg	MCHC2.1 $\times 10^{-2}$ kg/dm^3
1	m	156	10.8	13.4	19.4	50.1	37.3	14.4	38.7
2	m	280	10.3	10.2	10.6	29.9	29.3	10.4	35.5
3	m	196	9.0	11.4	15.7	46.0	40.5	13.8	34.1
4	m	210	14.3	12.8	15.4	40.1	31.3	12.0	38.4
5	m	180	14.4	11.3	17.5	43.0	38.0	15.5	40.7
6	f	340	13.0	11.5	16.4	47.3	41.1	14.3	34.7
7	f	340	8.5	13.3	11.9	55.8	41.8	8.9	21.3
8	f	150	13.3	15.6	17.4	60.2	38.5	11.1	28.9
9	f	168	12.4	13.5	19.9	51.3	38.1	14.8	38.8
10	f	150	8.5	9.9	13.6	44.3	44.9	13.8	30.7
Mean		217	11.5	12.3	15.8	46.8	38.1	12.9	34.2
$S_{(x)}$		71.5	2.21	1.67	2.88	8.02	4.46	2.05	5.56

Explanation: as in tab. 1.

Tab. 3. Post-exercise peripheral blood morphology in two-year old Standardbred trotters after three-month long training and after the race over a distance of 3200 m at a pace of 550 m/s (sample collection 2.2)

No	Sex	PLT2.2 $\times 10^9/dm^3$	WBC2.2 $\times 10^9/dm^3$	RBC2.2 $\times 10^{12}/dm^3$	HGB2.2 $\times 10^{-2}$ kg/dm^3	HCT2.2 % (v/v)	MCV2.2 $\times 10^{-15}/dm^3$	MCH2.2 $\times 10^{-15}$/kg	MCHC2.2 $\times 10^{-2}$ kg/dm^3
1	m	157	11.7	17.3	25.0	74.4	43.1	14.5	33.6
2	m	281	10.3	14.2	10.2	28.6	20.1	7.2	35.7
3	m	198	9.2	16.1	23.6	66.8	41.4	14.6	35.3
4	m	210	14.6	14.2	17.1	41.2	29.0	12.0	41.5
5	m	180	14.4	14.1	20.7	54.9	38.9	14.7	37.7
6	f	377	13.0	14.2	27.1	55.8	39.4	19.1	48.6
7	f	340	8.5	14.4	19.0	60.1	41.9	13.2	31.6
8	f	168	13.6	17.1	29.8	88.7	52.0	17.5	33.6
9	f	190	12.8	16.2	21.0	47.6	29.4	13.0	44.1
10	f	156	8.5	11.2	27.0	43.2	38.6	24.1	62.5
Mean		226	11.7	14.9	22.1	56.1	37.4	15.0	40.4
$S_{(x)}$		75.0	2.26	1.72	5.46	16.57	8.50	4.29	8.92

Explanation: as in tab. 1.

first at rest (sample collection 2.1, tab. 2), while the second immediately after the race (sample collection 2.2. tab. 3). The comparison of assay results showed a statistically significant increase in the values of: PLT ($P_p = 0.0592$), WBC ($P_p = 0.0468$), RBC ($P_p = 0.0001$), HGB ($P_p = 0.0028$), HCT ($P_p = 0.0317$), as well as no statistically significant changes in the values of: MCV ($P_p = 0.7493$), MCH ($P_p = 0.1521$) and MCHC ($P_p = 0.0964$). The post-exercise increase of RBC and HCT was consistent with observations of other authors. Szarska (21) reported a 40% increase in haematocrit value in relation to its value at rest in horses starting in long-distance horse rallies over 120-160 km. On the other hand, interpretation of the absence of changes in MCV, MCH and MCHC necessitates taking into account the simultaneous occurrence of two physiological mechanisms and the effect of applied measurement method on the numerical values obtained. During an intensive effort the organism undergoes rapid dehydration. Large energy expenditure and metabolic conversions accompany this, as well as the necessity of the efficient removal from the organism of considerable amounts of thermal energy, entailing intensive water depletion, the effect of which is an increase of sodium ion concentration in plasma and extracellular fluid, connected with increased respiration frequency and depth (exhaled air water practically does not contain electrolytes). This leads to a drop in the volume of extracellular fluid and consequently to the development of hypernatraemia. A consequence of dehydration and hypernatraemia in blood is an increase in haematocrit value, concentration of morphotic components and haemoglobin, as well as an increase in mean haemoglobin corpuscular concentration and a decrease in mean haemoglobin volume. Thus, the increase of PLT, WBC, RBC, HGB and HCT values in the blood of the examined horses may be evidence of blood dehydration due to water depletion during the race. However, the increase of RBC, HGB and HCT values connected with dehydration can be difficult to assess, as they may be connected to the outburst of erythrocytes amassed in the spleen and to the increase of their concentration in peripheral blood in horses during exercise (17, 20). Moreover, due to the measurement method of blood cell volume applied in the experiment, consisting in introduction of a sample to a standard isoosmotic environment, the red blood cell indices (MCV, MCH and MCHC) calculated from experimental data cannot be the basis for drawing conclusions about the dehydration and hypernatraemia connected with it.

Water loss in the blood of a given horse can be calculated by comparing the concentration of selected blood components before and after intensive exercise. When adopting the assumption about non-significance of changes in the volume of blood cells during the course of the experiment, it is possible to use haematological data for that purpose. In the experiment being described, plasma dehydration factor D_f, defined as a quotient of the concentrations of respective components on blood before and after the race, was adopted as a measure of water content reduction. The following values were obtained: PLT ($D_f = 0.96 \pm 0.047$; $n = 10$), WBC ($D_f = 0.98 \pm 0.023$; $n = 10$), RBC ($D_f = 0.83 \pm 0.075$; $n = 10$), HGB ($D_f = 0.75 \pm 0.169$; $n = 10$), HTC ($D_f = 0.87 \pm 0.151$; $n = 10$). Since the values obtained for RBC, HGB and HCT are significantly lower than those obtained for PLT and WBC, which may be connected with the outburst of erythrocytes to the blood during the race, mean D_f (PLT, WBC) value calculated based on the values of PLT and WBC concentrations was adopted as a measure of plasma dehydration. The calculation results are presented in tab. 4. However, since one should be prepared for a decrease in the cell volume in hypertonic blood, the numerical values of the D_f factor presented in the tab. 4 should be treated as maximum values. This means that in case of an individual horse the amount of water lost during the race may be assessed at $\geq$ 3% of plasma volume at rest.

In order to confirm a high exposure to oxygen action during the experiment, the heartbeat rate and the respiration frequency were measured after race completion. It is believed that 5 to 10 minutes after

Tab. 4. Antioxidative ability of peripheral blood plasma (AAP) in two-year old Standardbred trotters: prior to (sample collection 2.1), and after (sample collection 2.2) the race over a distance of 3200 m at a pace of 550 m/s and a corresponding values of plasma dehydration factor (D_f) resulted by the race

No	Sex	TAS × 10^{-3} mol/dm^3		D_f (PLT. WBC)	TAS × D_f × 10^{-3} mol/dm^3
		2.1	2.2	2.2	2.2
1	m	1.11	0.85	0.96 ± 0.035 (2)	0.81 ± 0.030 (2)
2	m	1.02	0.91	1.00 ± 0.002 (2)	0.91 ± 0.002 (2)
3	m	0.93	0.89	0.98 ± 0.006 (2)	0.87 ± 0.005 (2)
4	m	0.97	0.95	0.99 ± 0.010 (2)	0.94 ± 0.010 (2)
5	m	0.96	1.00	1.00 ± 0.000 (2)	1.00 ± 0.000 (2)
6	f	1.29	0.85	0.95 ± 0.049 (2)	0.81 ± 0.042 (2)
7	f	1.05	0.90	1.00 ± 0.000 (2)	0.90 ± 0.000 (2)
8	f	1.02	0.97	0.94 ± 0.043 (2)	0.91 ± 0.041 (2)
9	f	0.97	0.94	0.93 ± 0.042 (2)	0.87 ± 0.040 (2)
10	f	0.90	0.93	0.98 ± 0.019 (2)	0.91 ± 0.018 (2)
Mean ± $S_{(x)}$ (n)		1.02 ± 0.107 (10)	0.92 ± 0.046 (10)	0.97 ± 0.038 (20)	0.89 ± 0.053 (20)

Explanations: $S_{(x)}$ – population standard deviation; n – number of independent observations

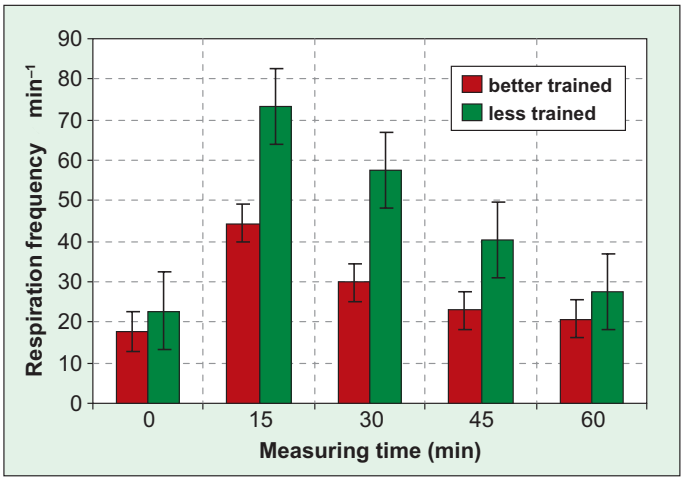

Fig. 1. Race adaptation degree after three-month long training of Standarbred stallions measured by respiration frequency before and after the performed race over a distance of 3200 m at a pace of 550 m/min. In better trained horses, capable of performing a more intensive effort (no 1-3, tab. 4) when compared with less trained ones (no 4-5, tab. 4), a higher degree of plasma antioxidants consumption during the race was found

completing the aerobic exercise, the heartbeat rate should not exceed 60-64 heart beats per minute, with a value of 64 heart beats per minute after 15 minutes from exercise completion being evidence of horse overloading. The increase of heartbeat rate over 150 heart beats per minute indicates a transition from aerobic metabolism to the anaerobic one (17). The heartbeat rate of the examined horses after 15 minutes had elapsed from race completion was 71.7 ± 12.88 (n = 9) heart beats per minute, which means that these horses were loaded during the experiment with maximum oxygen work.

In tab. 4 are presented the results of antioxidant content (AC) determination in the plasma of examined horses with the TAS method (total antioxidative ability meant as a resultant ability of examined material towards a counteraction of specific oxidation reaction). The application of Randox TAS test for measuring the AC of equine plasma has important limitations. The numerical values, read from the calibration curve exactly after 180 seconds according to manufacturer's recommendations, does not correspond with total antioxidant concentration in the examined sample. They are only proportional to them and should be regarded to be too high with reference to the values set as a standard.

The analysis of AAP values in each horse before and after race completion (columns 3 and 4, tab. 4) showed a recurrent downward tendency. In 8/10 cases, the AAP value underwent a decrease after the race. Since a natural consequence of hyperosmotic organism dehydration is a drop in glomerular filtration and an increase of metabolite concentration in plasma, water loss in the blood sampled after race completion (sample collection 2.2) should have been taken into account when comparing the values assayed before and after the race. The statistical analysis of differences of pairs of the values obtained when allowing for plasma dehydration factor D_f (PLT, WRB) demonstrated a significant reduction of AC in the plasma of horses after race completion ($P_p = 0.0371$). This difference correlates with the value of D_f factor ($R^2 = 0.461$), which can mean that a prolonged loading of the organism with intensive oxygen work, and a progressing dehydration degree being adopted as its measure, leads to a gradual depletion of its antioxidative reserves and consequently, after surpassing the critical value, to the development of oxidation stress. In the group of horses taking part in the experiment, the largest drop of AAP values occurred in the horses that were capable of accomplishing the maximal effort. These animals, i.e. three stallions (no 1-3, tab. 4) and three mares (no 6-8, tab. 4), were characterised by lower heartbeat rate and lower respiration frequency at rest as well as by the capacity for quick regeneration of these parameters after the performed race (fig. 1).

Conclusions

It is believed that race endurance of the organism is higher to the degree its oxygen limit is higher, and in particular the higher the oxygen consumption per 1 kg body weight during one minute. However, the increase of oxygen consumption during a race inevitably gives rise to an increase in the production of ROS in tissues which leads to the development of physiological oxidative stress.

The effort of race horses is short but very intensive, usually bringing about an extreme loading of the equine organism. The number of heart beats per one minute can increase in them even seven times in relation to resting values, while the amount of oxygen taken in by trained horses under extreme conditions can exceed 35 times the resting level (18). That means approximately a 35-fold increase of superoxide anion radical production, which is much more reactive than oxygen (8, 11, 22). A high concentration of endogenous ROS is particularly dangerous for skeletal muscles and the cardiac muscle, because antioxidative enzyme activities and antioxidant concentrations are low in them (7). It was determined that intensive oxygen effort leads to a reduction of the tissue antioxidant pool. In human muscles after a sub-maximal effort, an increase in the ratio of GSSG/GSH concentrations was demonstrated as well as of allantoin concentration, which originates from uric acid as a result of non-enzymatic reaction with ROS (6). Whereas in relation to horses it was only confirmed that intensive exercise can exert an influence on systemic redox balance, and that the restoration of the normal state takes time. In sport pentathlon horses' two 1-minute-runs of intense exercise over jumps caused biochemical and lipid peroxidative changes in their plasma and RBCs (RBC GSH and TBARS concentrations did not

change immediately after exercise, but decreased after 24 hours of rest) (3), and in horses competing in a 140 km endurance race prolonged low-medium intensity exercise induced detectable changes in circulatory antioxidants and produced systemic oxidative stress (increases in TBARS and a loss of TGSH) (12).

During the race a gradual depletion of the energy potential of the organism and growing weariness may be attained. Moreover, the defence potential of the organism undergoes depletion. In animals with different trained physical fitness levels, these two incidences can occur at different stages of a race. In horses with an insufficiently developed antioxidative defence system, in the final stage of a race one should expect an increase of ROS concentration, surpassing of antioxidative defence barrier and progressive damage of cell structures (13). Since the basic assumption of rational training is to prepare an organism for maximal effort in the future (during a contest), surpassing the organism oxidation safety threshold during training is equivalent to lowering its current total efficiency and to regressing the training effects previously achieved. Thus, the energy potential and the defence potential of a race horse organism should be built simultaneously, while, apart from the assessment of degree of trained physical fitness, selection of animals that are ready for intensive aerobic effort should include an examination of the current state of the antioxidative system. This refers in particular to horses that have achieved a very high level of racing efficiency. These animals are capable of achieving a very high oxygen limit during a race and should be exploited very cautiously due to a high risk of exceeding the antioxidative defence barrier and development of oxidation stress.

Finally, one cannot rule out that the current redox status of organism depends on diet type. Deficiencies of antioxidative substances e.g. of ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), sulphur amino acids essential for glutathione synthesis, or deficiency of riboflavin necessary for FAD synthesis as well as of microelements: zinc, copper, manganese and selenium, can have an effect on lowering the capacity of antioxidative defence system of organism. Frankiewicz-Jóźko and Szarska (5) stated that a deficiency of cofactors of antioxidation triad enzymes: Zn, Cu and Se in the diet of horses decreased the level of total antioxidant status, ascorbic acid, as well as of superoxide dismutase activity in blood. Avellini et al. (2) showed that a combination of training with vitamin E and selenium supplementation in the diet for horses intensified the system of antioxidative defence and reduced effort-induced peroxidation processes in blood cells and extracellular fluids. Antioxidants, in particular the natural ones which are supplied with food can be of significant preventive importance in the whole training cycle, notably in its final stage. However, it is worth keeping in mind that practically for all administered exogenous antioxidants one can find a threshold (high) concentration at which an antioxidant will be toxic – most antioxidants have prooxidant properties at very high concentrations, which is an exceptionally unfavourable phenomenon everywhere oxygen shock has already inflicted damages (15).

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Author's address: dr Tadeusz Ogoński, ul. Orawska 22/13, 70-131 Szczecin; e-mail: tadeusz.ogonski@biot.ar.szczecin.pl