

## **INFLUENCE OF PHYSICAL ACTIVITY OF RACEHORSES ON LACTATE DEHYDROGENASE AND CREATINE KINASE ACTIVITIES, AND PROTEIN SYNTHESIS**

JOVIĆ S, STEVANOVIĆ JELKA, BOROZAN SUNČICA, DIMITRIJEVIĆ B  
and MILOSAVLJEVIĆ P

*University of Belgrade, Faculty of Veterinary Medicine, Serbia*

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*The aim of the research was to assess the effects of physical activity of various intensity on the degree of damage to certain organs resulting from increased free radical production, as well as the adaptability of the organism to physical exercise.*

*Two groups of healthy 3-5-year-old full-blooded racehorses were assessed. The first one ran a 2400-meter gallop race, which is considered a short-lasting, intense physical activity; lipid status was assessed prior to, and 48 and 72 h after the race. The second group ran a forty-kilometer endurance ride, which is a long-lasting moderate physical activity; the lipid status was assessed immediately before, on finishing and 48, 72, 96, 120 and 144 h after the race.*

*The total activity of LDH changed 72 h and 96 h following the gallop race ( $p>0.05$ ), whilst the maximum activity was measured immediately after the endurance ride. By means of electrophoresis LDH in all the horses 5 isoforms were detected. The activity of LDH1 72 h after the gallop race significantly rose in comparison to the one before the race ( $p<0.05$ ), whilst the activity of all other isoenzymes, LDH2-LDH5, did not change significantly ( $p>0.05$ ). Following the endurance ride LDH1 activity rose at all sampling times, reaching the maximum at 96 h and 144 h in comparison to the values both before and on finishing the ride ( $p<0.01$ ). The increase in the activity of LDH2 was significantly higher 48 h, 72 h, 96 h and 120 h ( $p<0.05$ ) after the race in comparison to that before the race, and at 48 h, 72 h, 96 h, 120 h and 144 h ( $p<0.05$ ) in comparison to the values on finishing the race. LDH3 activity significantly decreased and LDH5 rose immediately after the endurance ride ( $p<0.01$ ), whilst LDH4 significantly rose at all times following the endurance ride ( $p<0.01$ ). The CK activity pointed to high, medium and low degree of adaptation of horses to physical activity.*

*The concentrations of total proteins, albumins and globulins remained within the physiological range at all sampling times, with the exception of 96 h after the endurance ride, when the fall in albumin concentration was significant ( $p < 0.01$ ).*

*Long-lasting physical activity of low intensity leads to cellular damage in the myocardium, muscles, liver and to hypoalbuminaemia, which is a consequence of free radical production.*

*Key words: creatine kinase, endurance ride, gallop race, horses, lactate dehydrogenase, proteins*

## INTRODUCTION

Numerous studies confirmed that reactive oxygen species (ROS) may contribute to altered homeostasis due to increased physical activity, i.e. muscle fatigue and damage (Pyne, 1994; Powers and Lennon, 1999; Marlin *et al.*, 2002; Cheung *et al.*, 2003; Close *et al.*, 2005). Oxydative stress may occur as a result of physical activity itself, or myopathies due to exercise and intense haemolysis resulting from increased lipid peroxidation. In aerobic endurance stress there is an increase in the production of ROS. Mitochondria in active muscles are presumably the main source of ROS, although free radicals are produced by erythrocytes, as well as in the inflammatory response which accompanies the increased muscular activity.

ROS may be synthesised during or after physical activity in active muscles and in ischemic tissues. *In vivo* they are produced in various tissues, in particular in skeletal muscles especially during physical activity. All ROS-producing cells, especially those in skeletal muscles, are involved in intense activity during and after long-lasting muscle contractions (Close *et al.*, 2005). The main culprit for the rise in production in these conditions is the increased respiration rate, since the need for oxygen is then enormously increased. Thus, the use of oxygen in muscles in strenuous exercise may be even 100-200 times as active as at rest (Davies *et al.*, 1982; Sjodin, 1990; Chevion *et al.*, 2003).

It is certain that oxidative stress induced by exercise contributes to accelerated development of muscle fatigue and damage (Hargreaves *et al.*, 2002; Marlin *et al.*, 2002; Williams *et al.*, 2004; Gondim *et al.*, 2009) leading to intolerance towards exercise and diminished its performance in various animal species, as well as to a decline in the body's defense capacity.

Myoglobin released from damaged skeletal muscle cells and haemoglobin from erythrocytes, which undergo intense haemolysis during significant blood pressure increase or due to peroxidation of membrane lipids in physical activity, both may increase lipid peroxidation. Lipid peroxidation inevitably leads to reactive aldehydes, which may be transported from the place of initial damage to other tissues. It is found that ROS may cause damage to skeletal muscles not only at

the places where produced (Close *et al.*, 2005). Oxidative damage resulting from exercise is especially severe in skeletal muscles and leads to poor work capacity (Reid *et al.*, 1992; Pyne, 1994; Ramzan and Palmer, 2010). Plasma contains proteins, such as haptoglobin haemopexin, which bind free haemoglobin, haeme iron, and thus neutralize the possible induction of lipid peroxidation.

Abeni *et al.* (2013) detected increased creatine kinase (CK) activity in 10-15-year old horses compared with other ages. To assess apoptosis resulting from training, Boffi *et al.* (2002) performed three-month-long research on full-blooded horses on a treadmill. In samples obtained by muscle biopsy they found increased activity of CK in comparison to untrained horses. DNA fragmentation was also proven, which both indicate the existence of a training-induced apoptosis in skeletal muscle cells. Apoptosis or programmed cell death is a genetically controlled response in terms of suicide provoked by DNA fragmentation. Common drivers of apoptosis are  $Ca^{2+}$  and ROS, which are present in the pathogenesis of exercise-induced myopathy. It is very likely that this activated apoptosis, in which the injured muscle cells trigger their own death and are replaced by new stronger ones, is to start the damage-repair cycle, which is actually the mechanism of training-induced increase in muscle mass.

Physical activity leads to increased production of ROS in athletes, to the point that may exceed antioxidant defenses and lead to oxidative stress. Oxidative stress resulting from physical exercise does not lead to changes in liver function, but increases the level of thiobarbituric reactive species (TBARS) in skeletal muscle and red blood cells (Soares *et al.*, 2011) which may lead to increased levels of lactate dehydrogenase (LDH), whose activity increases in the presence of hydroxyl radicals that cause muscle damage (Watson *et al.*, 2005). Two-to-six-year-old horses have a higher activity of LDH than older ones according to Kirschvink *et al.* (2006). However, Abeni *et al.* (2013) found the lowest activity in those between 10 and 15 years of age. Continuous high-intensity training increases the activity of LDH in racehorses and influences the activity of enzymes involved in the metabolism of glucose and lipids (Arai *et al.*, 2002, 2003; Li *et al.*, 2012).

Strenuous exercise leads to certain increase in the intensity of metabolism and therefore increases the production of ROS and threatens the antioxidant defense system. Research on the effects of oxidative stress during strenuous exercise, performed by Chevion *et al.* (2003) was performed on 31 males loaded with 35kg of extra weight, who walked extreme marches of 50 and 80 km at sea level. The 50km-long march was completed by 29 participants but only 16 completed the 80km march. Both marches led to an increase in plasma levels of uric acid by 25% and 37%, respectively, which was a result of intensified metabolic rate including the metabolism of purine nucleotides. In addition, approximately a 10-fold increase in creatine phosphokinase activity was detected in the plasma. Plasma protein carbonyl content, markers of protein oxidative damage, decreased significantly during both marches. These results are discussed taking into account

that the increase in breathing rate during physical activity leads to a production of ROS over the one which can be removed by the antioxidant system.

LDH plays an important role in glycolysis catalysing its last stage. Since it is in the cytoplasm, when cell damage occurs, LDH easily escapes into the extracellular space, resulting in its increased plasma activity. Measurement of total serum LDH has little diagnostic value because its location in the cells of different tissues. Given the distribution of LDH isoenzymes in various organs, it is important to specify them.

The increased activities of CK and LDH in serum immediately after exercise is due to a selective increase in the permeability of muscle membranes as a result of increased peroxidation which occurred during exhausting exercise. The degree of peroxidation was determined by the increase in TBARS (Anderson, 1975; Soares *et al.*, 2011). In the research conducted by Morillas-Ruiz *et al.* (2006), an increase of CK and TBARS was detected after exercise, which was confirmed by the results obtained in horses. However, it is also possible that in exhausting exercise there is an adaptation of muscles followed by changes in the CK activity (Piccione *et al.*, 2009; Lawan *et al.*, 2010; Soares *et al.*, 2011). High CK levels may correlate with the status of physical training, whilst persistently high levels indicate subclinical myopathy (Piccione *et al.*, 2009).

Besides protein whose concentration increases significantly during the acute phase, there are so-called "negative acute phase proteins," which decrease in the acute phase of infection and stress. Such proteins include albumins, whose concentrations decrease by 10-50% (Toussaint, 2005; Abeni *et al.*, 2013). The reason for these changes in protein concentrations in the acute phase of infection is their reduced production or increased degradation. In the case of albumins, there may not be a real decline in concentration, but it often seems so as a reflection of increased synthesis of other acute-phase proteins, which are all plasma globulins, and thus alter the albumin-globulin ratio.

The decrease in albumins and transferrin in humans after finishing exercise and a 1,600-km ultramarathon was proven by Fallon and Sivyer (1999) and Fallon (2001).

ROS are able to attack all the major classes of biological macromolecules, proteins, DNA (Sen, 1995; Tauler *et al.*, 2004; Aguilo *et al.*, 2005; Tauler *et al.*, 2006) and especially lipids (McBride and Kraemer, 1999; Close *et al.*, 2005). Increased oxygen consumption, accompanied by a sharp rise in production of oxygen derivatives, is responsible for lipid peroxidation of cell membranes. It leads to a degradation of proteins and enzymes and the consequent development of oedema in tissues. Saturated fatty acids and those with one double bond are much more resistant to ROS than polyunsaturated fatty acids (PUFAs), which are abundant in cell membranes and thus easily attacked by ROS (Close *et al.*, 2005), which is inevitably accompanied by impairment of cell functions.

ROS generated during increased muscle activity are potent inducers of oxidative damage to various cellular molecules by means of protein oxidation

(Chevion *et al.*, 2003). Oxidative damage to cellular proteins involves modification of amino acids, changes in their secondary (due to the interaction of radicals with the skeleton of the polypeptide chain) and tertiary structures, indirect damage by the activation of proteolytic enzymes with hypochloric acid and products of lipid peroxidation. All these changes result in an alteration in membrane permeability due to altered activity of enzyme molecules in the cell membrane, aggregation and inactivation of receptor proteins or dysfunction of transport proteins and, consequently, lead to disrupted cell signaling, ionic transport and homeostasis. The circuit of damage does not complete here but continues, so that non-specific permeability to ions, especially  $\text{Ca}^{2+}$ , further destabilise biomembranes, by activation of calcium-dependent proteases, including phospholipase A2. Its release initiates a cascade of reactions which lead to the production of by-products in peroxidation processes, lipid mediators and neuropeptides, which exacerbated the inflammatory response (Slater, 1987; Halliwell and Gutteridge, 1990).

Enzyme components of the primary antioxidant defense are comprised of antioxidant molecules which collect free radicals, such as the family peroxiredoxin proteins (function as collectors of superoxide and hydrogen peroxide). All these molecules block the initiation of the chain reaction of ROS and associated lipid peroxidation (Margaritis *et al.*, 2003). Each one detoxifies some of the ROS, and protein antioxidants are supplemented with nonprotein ones (intracellular ascorbate, glutathione etc.). It is very important that vitamin E reacts with peroxy radicals faster than they may react with unsaturated fatty acids and proteins. Vitamin E in blood plasma protects lipoproteins from oxidation (Halliwell, 1991), and is a major antioxidant of LDL molecules.

Sources of ROS during exercise are enhanced purine oxidation, damage to iron-containing proteins, disruption of homeostasis of  $\text{Ca}^{2+}$  and ROS production induced in endothelial cells. In addition, activated neutrophils, which infiltrate the muscles after damage caused by exercise, can also produce increased ROS (Chevion *et al.*, 2003; Close *et al.*, 2005).

## MATERIAL AND METHODS

### *Animals and blood sampling*

The research was performed on two groups of 3-5-year old, male, full-blooded racehorses in the Republic of Serbia. The first one comprised 12 clinically healthy animals which ran a 2400-meter-long gallop race and were kept in stables at the Belgrade racecourse. The second group comprised 13 racehorses which ran the 40-kilometer-long endurance ride. These were kept in stables at the Belgrade or Pančevo racecourse.

All blood samples were taken from the jugular vein without adding any anticoagulant, from gallopers before, and 48 and 72 h after the race, and from those who ran the endurance ride immediately before and after the activity, as well as 48, 72, 96, 120 and 144 h after the completion of the ride.

Serum was separated from blood which coagulated spontaneously at room temperature, centrifuged at 3000 rpm for 10 minutes, frozen and kept at -20°C until analyzed.

### *Biochemical analysis*

#### *Relative isoenzyme distribution of lactate dehydrogenase*

LDH isoenzymes (LDH1, LDH2, LDH3, LDH4 and LDH5) were detected by vertical electrophoresis, on denaturing 7.5% PAG gel, using TRIS - glycine buffer, according to the method of Laemmli (HOEFFER MINI VE, Amersham, LKB, 2117, Bromma, Uppsala Sweden). The detection of bands was performed with sodium lactate (substrate) in the presence of NADH and tetrazolium blue. The colour intensity of the formazan is measured by densitometry using Scion Image Beta 4.02 software for Windows. The activity of each isoenzyme is expressed as a percentage of total LDH activity.

#### *Creatine kinase activity*

The serum activity of creatine kinase was detected by an enzymatic method, with an assay kit (Bioanalytica, BioSystem).

#### *Total protein concentration*

The concentration of total proteins in the serum was determined by a spectrophotometric, biuret method – Kerr's modification of the method described by Gornall. Absorbances were read on a spectrophotometer CECIL CE 2021 UV/VIS, 540 nm. Protein concentrations were calculated from the standard curve ( $y=0.0022x+0.0121$ ), adjusted to solutions of bovine serum albumins (BSA) in concentrations from 10 g/L to 100 g/L.

#### *Albumine concentrations*

The concentrations of serum albumins were determined by spectrophotometric analysis, using the method with bromocresol green (BCG). The results were read on a spectrophotometer CECIL CE 2021 UV/VIS, measuring the adsorbance at 637 nm, using BSA as a standard. Albumin concentrations were calculated from the standard curve ( $y=0.0022x+0.0121$ ), adjusted to solutions of BSA in concentrations ranging from 10 g/L to 100 g/L.

#### *Statistical analysis*

Statistical analysis of the results was performed with GraphPad Prism 5. Statistic significance was determined using ANOVA (Tukey's test and Dunnett's test) and t-test. The minimum level of statistical significance was set at  $p<0.05$ .

The correlation between the parameters tested was examined according to Person's correlation coefficient (*r*), the regression line (*Fy*) and testing the significance of correlation and regression coefficients.

## RESULTS

### *Activity of lactate dehydrogenase and its isoenzyme forms (LDH1, LDH2, LDH3, LDH4 and LDH5)*

The results of the determination of total LDH activity in the serum of horses which ran the gallop race and endurance ride are given in Table 1.

Table 1. Activity of total plasma LDH in horses which ran 2400-meter gallop race and 40-kilometer endurance ride (TA – total area).

Sampling time	$\bar{X} \pm SD$ (TA)	min	max
<i>Gallop race</i>			
before race	3136 ± 771.8	2051	4479
72 h after the race	3078 ± 707.8	2049	4432
96 h after the race	3256 ± 800.8	2379	4937
<i>Endurance ride</i>			
before ride	2530 ± 672.4	1528	3450
immediately after the ride	4208 ± 1421**	2539	7642
48 h after the ride	2941 ± 1132#	1652	4833
72 h after the ride	2349 ± 847.1###	1104	3794
96 h after the ride	2151 ± 801.1###	1365	4235
120 h after the ride	3012 ± 1137	1136	4992
144 h after the ride	2718 ± 998##	1480	4367

Data: means ± SD

\**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001 The levels of statistical significance before and after the ride

#*p*<0.05, ## *p*<0.01, ### *p*<0.001 The levels of statistical significance immediately after the ride and 48h to 144h after the ride

The results showed that total LDH activity changed 72 h and 96 h after the gallop race (*p*>0.05) and correlated positively with the concentrations of total proteins before (*r* = 0.7037, *p*<0.05), as well as 72 h (*r* = 0.6096, *p*<0.05) and 96 h (*r* = 0.8205, *p*<0.01) after the race. The analysis of activity following the endurance ride revealed highest values on finishing the ride, which was significant in comparison to those before (*p*<0.01), and 48 h (*p*<0.05), 72 h (*p*<0.001), 96 h (*p*<0.001) and 144 h (*p*<0.01) after the ride.

In all the horses in the experiment five isoenzyme forms of LDH were detected by means of electrophoresis (Figure 1).

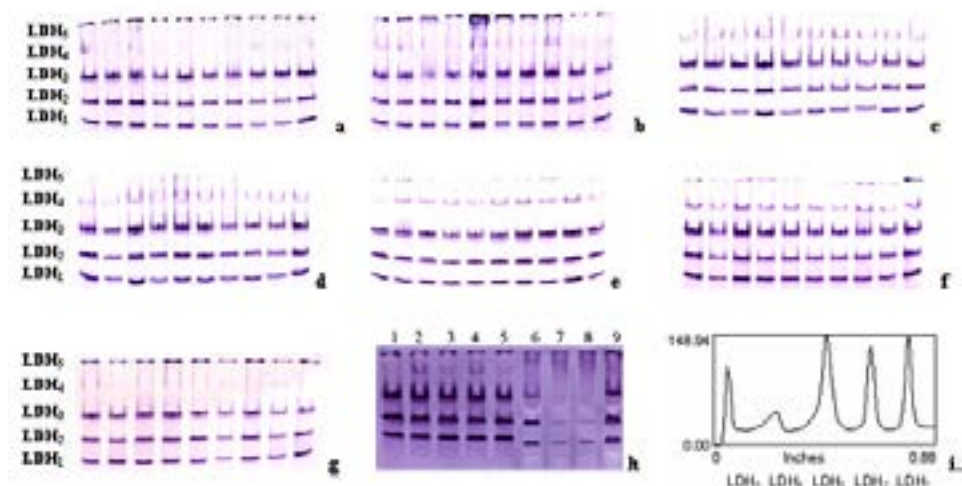


Figure 1. Electrophoresgrams of isoenzymatic distribution of total LDH (LDH5, LDH4, LDH3, LDH2 and LDH1) in horse plasma on 7.5% PAGE-gel in non-denaturing conditions before endurance ride (a), immediately after (b), 48 h (c), 72 h (d), 96 h (e), 120 h (f) and 144 h after the ride (g), before gallop race 1,4,7; 72 h after the race 2,5,8; and 96 h after the race 3,6,9 (h) and densitogram LDH1-5 (i)

The data derived from the assessment of the activity of isoenzyme forms LDH1 LDH2, LDH3, LDH4 and LDH5 in the plasma of horses which ran the gallop race and the endurance ride are presented in Table 2 and Figure 1.

The analysis of relative isoenzyme distribution of LDH proved a significant increase in the activity of LDH1 72 h after the gallop race in comparison to the one before the race ( $p < 0.05$ ), with a tendency to rise 96 h after finishing the ride ( $p > 0.05$ ). The activity of other isoenzymes, LDH2 - LDH5, varied 72 h and 96 h after the race in comparison to the one which preceded, but without a statistically significant difference ( $p > 0.05$ ).

After the endurance ride the LDH1 activity had a tendency to grow at all sampling times compared to the period before and on finishing the ride. This isoenzyme exhibited maximum activity 96 h and 144 h after the ride, significantly higher than before and immediately after it ( $p < 0.01$ ). The activity of LDH2 showed a similar pattern after the endurance ride as did LDH1: in all tested intervals the activity increased in comparison to the period before and immediately after the race, and was significantly higher 48 h ( $p < 0.01$ ), 72 h ( $p < 0.001$ ), 96 h ( $p < 0.01$ ) and 120 h ( $p < 0.05$ ) after the ride than prior to it and 48 h, 72 h, 96 h, 120 h ( $p < 0.001$ ) and 144 h after the ride ( $p < 0.05$ ) than on completing the ride.

However, the activity of isoenzyme LDH3, which points to possible damage to the lungs, a significant decrease was proven on finishing the ride ( $p < 0.01$ ) in comparison to the preceding value, whilst later there was no significant



Table 2. Activities of LDH1, LDH2, LDH3, LDH4, LDH5 (%) in plasma in horses which ran 2400-meter-long gallop race and 40-kilometer-long endurance ride.

Sampling time	± SD (%)				
	LDH1	LDH2	LDH3	LDH4	LDH5
Gallop race					
before race	25.43 ± 5.58	20.34 ± 4.05	32.86 ± 4.92	13.19 ± 3.88	7.64 ± 2.77
72 h after the race	32.65 ± 9.12*	20.36 ± 4.60	32.17 ± 5.17	11.02 ± 3.54	6.52 ± 3.17
96 h after the race	29.90 ± 5.47	21.59 ± 4.14	31.29 ± 5.83	11.36 ± 3.41	7.31 ± 3.78
Endurance ride					
before ride	19.74 ± 3.31	22.82 ± 2.63	40.85 ± 4.22	3.36 ± 1.81	12.12 ± 4.34
immediately after the ride	19.88 ± 3.18	21.59 ± 2.87	33.17 ± 4.51**	6.39 ± 2.61**	19.94 ± 7.78**
48 h after the ride	22.64 ± 3.99	26.60 ± 3.30** ,###	36.87 ± 9.49	6.81 ± 2.46**	9.64 ± 3.44###
72 h after the ride	23.57 ± 3.94	27.38 ± 2.82*** ,###	37.07 ± 6.50	6.35 ± 3.29**	6.45 ± 2.92* ,###
96 h after the ride	24.86 ± 3.50** ,##	26.48 ± 2.25** ,###	37.22 ± 2.10	5.91 ± 2.20**	6.33 ± 3.30* ,###
120 h after the ride	23.28 ± 3.51	25.92 ± 2.11* ,###	38.93 ± 3.70	6.01 ± 2.05**	8.05 ± 3.39###
144 h after the ride	25.34 ± 3.05** ,##	24.84 ± 1.19#	34.52 ± 4.00	5.66 ± 1.50**	9.86 ± 4.93###

Data: means ± SD

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 The levels of statistical significance before and after the race/ride

#p<0.05, ## p<0.01, ### p<0.001 The levels of statistical significance immediately after the race and 48h to 144h after the ride

difference ( $p > 0.05$ ). By contrast, LDH4, which indicates the degree of muscular damage, significantly increased at all sampling times following the endurance ride ( $p < 0.01$ ). Similarly, LDH5, which points to liver tissue damage, showed a statistically significant increase of activity, but only immediately on completing the ride ( $p < 0.01$ ), compared to its activity before the ride, as well as after 48 h to 144 h after the ride (Table 2). In comparison to the value before the ride, the activity of LDH5 was roughly similar as soon as 48 h after the ride and remained so until the end of the trial. In addition, there was a significant decrease of LDH5 activity 72 h, 96 h after the ride in comparison to the value which preceded the activity ( $p < 0.05$ ).

*Comparison the activity of lactate dehydrogenase and its isoenzyme forms (LDH1-LDH5) in horses which took part in gallop race and endurance ride*

The comparison of total LDH activity in horses engaged in physical activity of various degree at the same sampling times (Figure 2), it is clear that the gallop ride led to more severe cell membrane damage and release of this enzyme into the extracellular space. This is confirmed by the statistical significance at 72 h ( $p < 0.05$ ) and 96 h ( $p < 0.01$ ) after the race/ride.

The kinetics of increased LDH1 activity differed between the two groups of horses, depending on the intensity of the physical activity. After gallop, the 72-h-post-race increase was 28.39% and in the next 24 h was 17.58%, while the endurance accounted for 19.40% and 25.93% increase in comparison to the basic level. Thus, the gallop race resulted in a higher degree of damage to cell membranes and LDH1 release into the extracellular space, which was demonstrated by high statistical significance of the difference in the LDH1 activity between the two groups of horses 72 h ( $p < 0.01$ ) and 96 h ( $p < 0.05$ ) following the race. Comparison of the LDH2 activity at the same time intervals after both races revealed a substantial increase in horses participating in the endurance ride in comparison to those which galloped, both 72 h ( $p < 0.001$ ) and 96 h ( $p < 0.01$ ) after the race. Similarly, the activity of LDH3 was significantly higher in horses completing the endurance ride than galloping race both 72 h ( $p < 0.05$ ) and 96 h ( $p < 0.01$ ) after the run. By contrast, the LDH4 activity at the same time intervals was significantly higher in galloping horses than those which participated in the endurance ride both 72 h ( $p < 0.01$ ) and 96 h ( $p < 0.001$ ) after crossing the finishing line. However, the activity of LDH5 remained roughly unchanged ( $p > 0.05$ ).

*Creatine kinase activity*

The results of the analysis of creatine kinase activity in horses following the gallop race and endurance ride are presented in Table 3 and Figure 3.

The activity of creatine kinase increased significantly 72 h ( $p < 0.001$ ) and 96 h ( $p < 0.01$ ) after the gallop race.

Following the endurance ride in horses highly adapted to physical activity creatine kinase did not vary significantly, whilst in those moderately adapted there was a significant difference on finishing the ride in comparison to the values both

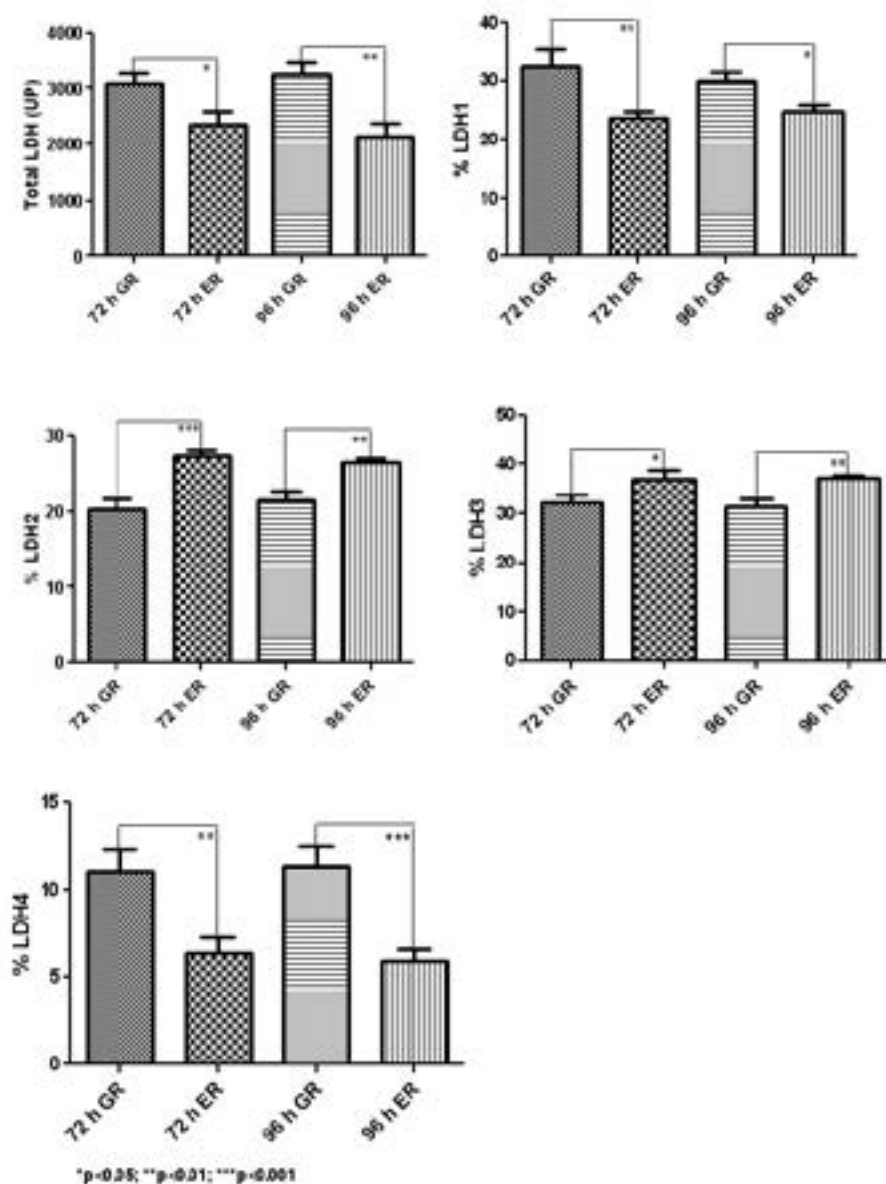


Figure 2. Activity of total LDH (UP), LDH1, LDH2, LDH3, LDH4 (%) in plasma of horses which ran a 2400-metre long gallop race (GR) and those which ran 40-kilometre-long endurance ride (ER) at same time intervals - 72 h and 96 h after the end of the race/ride.

before ( $p < 0.01$ ) and 48 h–144 h after the ride ( $p < 0.001$ ). In horses with low-degree of adaptation to physical activity maximum enzyme activity was noticed on completing the ride ( $p < 0.001$  in comparison to all sampling times).

Table 3. Activity of creatine kinase (U/L) in serum of horses which ran 2400-meter-long gallop race and 40-kilometer-long endurance ride

Sampling time	$\bar{X} \pm SD$ (U/L)	min	max
<i>Gallop race</i>			
before race	62.30 $\pm$ 16.10	46.30	78.00
72 h after the race	165.00 $\pm$ 37.28***	121.00	211.00
96 h after the race	124.60 $\pm$ 26.20**,+	98.00	150.01

Endurance ride			
Sampling time	$\bar{X} \pm SD$ (U/L)		
	Highly adapted	Moderately adapted	Slightly adapted
before ride	22.00 $\pm$ 4.69	100.5 $\pm$ 42.02	44.60 $\pm$ 17.64
immediately after the ride	68.00 $\pm$ 10.07	169.0 $\pm$ 14.07**	500.4 $\pm$ 76.45***
48 h after the ride	60.50 $\pm$ 35.45	52.00 $\pm$ 22.38###	75.80 $\pm$ 30.34###
72 h after the ride	50.00 $\pm$ 7.62	49.00 $\pm$ 16.25###	56.00 $\pm$ 21.85###
96 h after the ride	46.25 $\pm$ 19.47	70.50 $\pm$ 29.24###	51.80 $\pm$ 24.65###
120 h after the ride	62.50 $\pm$ 29.10	59.50 $\pm$ 13.77###	63.40 $\pm$ 22.21###
144 h after the ride	66.25 $\pm$ 32.62	57.00 $\pm$ 18.17###	54.80 $\pm$ 18.32###

Data: means  $\pm$  SD

\*\* $p < 0.01$ , \*\*\* $p < 0.001$  The levels of statistical significance before and after the race/ride

#  $p < 0.05$ , ###  $p < 0.001$  The levels of statistical significance immediately after the ride and 48h to 144h after the ride

+ $p < 0.05$  statistically significant difference between 72h to 96h after the race

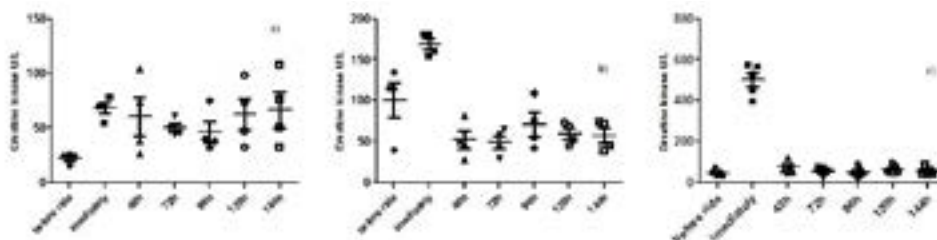


Figure 3. Creatine kinase activity (U/L) in horse serum before 40-kilometre-long endurance ride, on finishing the ride, 48 h, 72 h, 96 h, 120 h and 144 h after the ride; (a) highly adapted (b) moderately adapted (c) horses with low degree of adaptation

*Concentrations of total proteins, albumins, globulins and A/G ratio*

In order to assess the influence of physical activity on the synthesis of blood proteins, in racehorses serum concentrations of total proteins, albumins and globulins, as well as the A/G ratio was determined (Table 4).

Table 4. Concentrations of total proteins, albumins, globulins (g/L) and A/G ratio in serum of horses which ran 2400-meter-long gallop ride and 40-kilometer-long endurance ride

Sampling time	$\bar{X} \pm SD$ (g/L)			A/G Ratio
	Total proteins	Albumins	Globulins	
<i>Gallop race</i>				
before race	71.75 ± 10.93	44.48 ± 5.80	27.27 ± 11.73	1.63
72 h after the race	72.65 ± 9.64	45.42 ± 5.85	27.23 ± 11.67	1.67
96 h after the race	71.77 ± 12.42	47.04 ± 4.50	24.73 ± 13.52	1.90
<i>Endurance ride</i>				
before ride	79.01 ± 9.14	44.17 ± 5.19	34.84 ± 8.69	1.27
immediately after the ride	83.52 ± 9.30	46.50 ± 3.76	37.02 ± 11.02	1.26
48 h after the ride	76.56 ± 9.93	45.43 ± 4.91	31.13 ± 8.05	1.46
72 h after the ride	74.39 ± 6.70	43.85 ± 4.84	30.54 ± 8.67	1.43
96 h after the ride	74.50 ± 10.11	41.75 ± 3.08 <sup>###+</sup>	32.75 ± 9.79	1.27
120 h after the ride	73.45 ± 8.04	43.82 ± 3.69	29.63 ± 8.20	1.48
144 h after the ride	75.62 ± 7.15	43.25 ± 4.30	32.37 ± 8.71	1.34

Data: means ± SD

<sup>###</sup> p<0.01 The levels of statistical significance immediately after the ride and 48h to 144h after the ride

<sup>+</sup>p<0.05 statistically significant difference between 48h to 144h after the ride

It was observed that the concentration of total serum proteins in each group of horses was similar at all test times (p>0.05). In addition, the mean values of total proteins in the serum of the horses participating in both activities did not differ significantly (p=0.0840), neither did the protein content regardless of the level of physical activity and the time interval after the race (p>0.05). The concentration of total protein in galloping horses positively correlated with total LDH activity before (r=0.7037, p<0.05), and 72 h (r=0.6096, p<0.05) and 96 h (r=0.8205, p<0.01) after the race, whilst in the participants of the endurance ride correlates with albumin concentration 48 h after the ride (r=0.5927, p<0.05).

The results revealed no significant difference in the content of albumins 72 h and 96 h after the gallop race, in comparison to the concentration before the race (p>0.05). By contrast, the endurance ride led to statistically significant differences in albumin concentrations 96 h after the race in comparison with the values both on completion (p<0.01), and 48 h after the ride (p<0.05). The mean values of serum albumin concentration in both groups of horses before the race did not differ significantly (p=0.884). However, the difference was significant 96 h after the completion of activities (p<0.01).

## DISCUSSION

Oxidative stress resulting from physical exercise is responsible for increasing levels of certain enzymes in skeletal muscles. Particularly interesting is the increase in LDH levels, caused by the increased production of hydroxyl radicals, which do damage muscle cells and hepatocytes (Kayatekin *et al.*, 2002; Águila *et al.*, 2005). Since LDH is located in the cytoplasm, if the cells damaged it easily passes into the extracellular space, resulting in increased activity in the blood plasma. After prolonged physical exercise the MDA content increases in the blood, which reflects the activity of the corresponding LDH isoenzymes and suggests possible damage of the tissues other than muscles (Chiaradia *et al.*, 1998). Given that, in our studies (unpublished results) an increased lipid peroxidation, which accompanies the overproduction of ROS and may lead to myopathy and hemolysis induced by physical exercise, was proven, LDH was assessed in horses. It was shown that there was no change in the total activity of LDH after the gallop race, but a slight increase was observed 96 h on its completion in comparison to the basic level ( $p>0.05$ ). A significant increase in the total activity of LDH was proven immediately after the endurance ride ( $p<0.01$ ), which tends to return to basic values afterwards. The difference between the activities of total LDH in horses after physical exercise of various intensity was significant both 72 h ( $p<0.05$ ) and 96 h after the races ( $p<0.01$ ).

LDH can be found in the cells of various tissues, the measurement of its total activity has little diagnostic value; thus, it is important to define its isoenzyme forms, having in mind their distribution in certain tissues and organs. Our results showed a significant increase in the activity of LDH1: 28.39% 72 h after the gallop race ( $p<0.05$ ). The increasing trend remained after 96 h, when it was 17.58% higher than the basic value ( $p>0.05$ ). However, the activity of other isoenzyme forms (LDH2 - LDH5) did not differ significantly ( $p>0.05$ ). The revealed isoenzyme distribution indicates higher levels of LDH originating from the myocardium. This finding is in accordance with the data that in horses after physical activity besides enzymes from muscles, there were some LDH isoenzyme forms originating from other tissues (Chiaradia *et al.*, 1998).

After the completion of the 40-kilometer-long endurance ride a tendency of rise in the LDH1 activity at all sampling times was noticed in comparison to the values before and on crossing the finishing line ( $p<0.01$ ). This activity was 19.40% higher 72 h and 25.93% higher than the basic level 96 h after the ride. The kinetics of the LDH2 activity was similar to that of LDH1, significantly increasing at all time intervals in comparison to the values which preceded the ride and were measured immediately after it ( $p<0.05$  to  $p<0.001$ ). However, a significant rise was present in the activity of LDH4, the marker of skeletal muscle damage, all the time after the race ( $p<0.01$ ). LDH5, which points to the degree of liver damage, significantly rose only immediately after the endurance ride ( $p<0.01$ ), declining at all other time intervals in comparison to the basic level ( $p<0.05$  to  $p<0.001$ ), which indicates that there was a quick adaptation of hepatocytes to the increased needs resulting from prolonged physical exercise.

The available data on the activities of LDH and its isoforms in horses are limited, and are difficult to compare. Our results are in agreement with the findings of Balogh *et al.* (2001), who demonstrated a significant increase in LDH activity ( $p < 0.05$ ) immediately after exercise in horses which participated in prolonged physical exercise (Pentathlon). After a 24-hour rest LDH activity returned to the approximate value prior to the race. This type of testing was performed more frequently on people. Thus, Watson *et al.* (2005) confirmed that oxidative stress developed in athletes due to physical exercise does not lead to changes in the functionality of the liver, but increases the level of TBARS in skeletal muscles. This may cause the rise in the concentrations of LDH and it in turn can increase the production of hydroxyl radicals, which cause muscle damage. However, tests of serum LDH activity in the maximum and submaximum cycloergometric test performed on amateur athletes and cyclists by Tauler *et al.* (2004) indicate that both tests do not lead to increased LDH activity, which is in contradiction with our results.

Analysis of LDH isoenzyme forms revealed that due to long low intensity physical exercise and development of oxidative stress, in the 40-kilometer endurance ride, damage to the myocardium, skeletal muscles and hepatocytes occurs, in contrast to the 2400-meter gallop race, which causes changes only in the myocardium. These may correlate with the duration and intensity of exercise.

The activity of CK showed various degrees (high, medium and low) of adaptation of horses to exercise, with consequently different levels of its activity immediately after the race and the speed and dynamics of returning to basic levels, depending on the the degree of oxidative stress.

Strenuous and/or long-lasting exercise creates stress to the body and therefore raises the activity of the adrenal cortex, resulting in at least 2-3 fold increased cortisol levels (Freestone *et al.*, 1991; Lassourd *et al.*, 1996; Nogueira and Barnabas, 1997; Horohov *et al.*, 1999). It is physiologically justified because this hormone has a strong influence on metabolism, increases glycogenolysis and gluconeogenesis, promotes the mobilization of fat, stimulates the synthesis of proteins needed for damage repair, stimulates the transformation of noradrenaline into adrenaline, increases the sensitivity of adrenaline receptors etc. Except for these metabolic effects, cortisol induces the increase of blood concentrations of some cytokines, in particular, a significant increase in IL6, IL8, IL10 and TNF. At the same time, the very damaged muscle and other tissues during intense and/or long-term exercise trigger the secretion of cytokines (Pyne, 1994; Fallon and Sivyer, 1999; Fallon, 2001). There are many explanations for their increased production. They primarily stimulate the activity of the immune system, which is necessary in increased physical activity due to intensive contact with foreign agents which could lead to negative consequences for the organism. In addition, these cytokines, particularly IL6, are powerful stimulators of synthesis of acute phase proteins and their release into the blood (Pyne, 1994; Fallon, 2001).

Total protein concentrations did not change significantly in the tested time intervals in the horses completing the gallop race or endurance ride. Specifically, 72 h and 96 h after galloping their concentration slightly increased, as well as

immediately after the endurance ride ( $p > 0.05$ ), whilst in all tested intervals after the ride it was slightly lower than before the race ( $p > 0.05$ ). These results suggest that, regardless of the different intensity and duration of exercise, both races did not lead to significant changes in protein concentrations. This finding is quite expected, given that in racing animals changes in proteins are mainly due to haemoconcentration (Rose *et al.*, 1977; Rose and Hadgson, 1982; Poso, 1983; Jablonska *et al.*, 1991; Stevanovic *et al.*, 1996; White *et al.*, 2001; Manohar *et al.*, 2004; de Miranda *et al.*, 2009) and that water was available to horses *ad libitum*. Galloping horses consumed water after the race and the participants of the endurance ride drank during break and veterinary supervision. During endurance weather conditions (high humidity and low temperature) contributed to the absence of haemoconcentration. These results are in agreement with the findings of Nostell *et al.* (2006) who, comparing the physiological response of the standardized exercise test on a treadmill used to simulate the race on the track in thoroughbred racing trotters, also failed to observe differences in total plasma protein concentration after exercise and during recovery. However, our findings disagree with those presented by Balogh *et al.* (2001), who in horses jumping over hurdles and participating in pentathlon, on finishing the activities and 24 h later found a significant increase in total protein concentration. These differences in protein concentration can be due to different degrees and intensity of exercise and, consequently, different energy requirements for different types of physical activity, as well as a variety of environmental conditions.

Although the total protein content did not change significantly, we assessed the concentration of the major protein fractions, i.e. serum albumins, because these belong to the so-called negative acute-phase proteins and their concentration points to the possible development of dysproteinaemia. Both 72 and 96 h after high-intensity physical activity (2400-meter gallop race) a significant difference in the concentration of albumin was detected in comparison with the values before the race ( $p > 0.05$ ). However, 96 h after the long-standing low-intensity physical activity (40-kilometer endurance ride) there was a statistically significant decrease in serum albumin concentration in comparison to the value immediately after the race and 48 h later ( $p < 0.01$  and  $p < 0.05$ ). The concentration of albumin in both groups of racing horses before the race was roughly the same. Comparing the values after the activities, it was found that 96 h after the endurance ride the content of albumin was significantly lower than at the same time after the gallop race ( $p < 0.01$ ). However, the decline in serum albumin after endurance is in agreement with literature data obtained in humans, which show that the concentration of albumin in athletes decreased after exercise (Fallon and Sivyer, 1999) and ultramarathons (Fallon, 2001). In the acute phase albumin concentration decreases by 10-50% (Toussaint, 2005). However, this may not be due to a real decline in serum albumin due to the development of acute phase, but is often a reflection of increased synthesis of other acute-phase proteins which are all plasma globulins (Pritchard *et al.*, 2009).

Regarding LDH, its total activity in the serum after the gallop race was not significantly altered, but the determination of isozyme distribution demonstrated a



significant increase in activity LDH1 72 h after the race ( $p < 0.05$ ), indicating that this type of exercise leads to cell damage in the myocardium. Total LDH activity in horses participating in the endurance ride significantly increased on finishing the activity ( $p < 0.01$ ). Significant increase in LDH1 144 h after the endurance ride and LDH2 at all observed intervals ( $p < 0.05$ ) refers to the damage of myocardium cells. Significant increase in LDH4 in all tested intervals after endurance ride ( $p < 0.01$ ) points to skeletal muscle cell damage, whilst the increased LDH5 activity immediately after the race ( $p < 0.01$ ) indicates hepatocyte damage caused by this type of exercise.

The activity of CK pointed to high, moderate and low degree of adaptability of the horses to exercise. The 2400-meter gallop race and the 40-kilometer endurance ride did not influence the concentration of total proteins and globulins. Dysproteinemia was proven, precisely hypoalbuminemia 96 h after the endurance ride, in comparison to the value on crossing the finishing line ( $p < 0.01$ ) and 48 h later ( $p < 0.05$ ).

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Adress for correspondence:

Slavoljub Jović, MSc, PhD

Department of Physiology,

Faculty of Veterinary Medicine, University of Belgrade,

Bulevar oslobođenja 18,

11000 Belgrade, Serbia

E-mail: romanov@vet.bg.ac.rs

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## UTICAJ INTENZITETA OPTEREĆENJA TRKAČKIH KONJA NA AKTIVNOST LAKTAT DEHIDROGENAZE, KREATIN KINAZE I SINTEZU PROTEINA

JOVIĆ S, STEVANOVIĆ JELKA, BOROZAN SUNČICA, DIMITRIJEVIĆ B  
i MILOSAVLJEVIĆ P

### SADRŽAJ

Cilj rada je bio utvrđivanje efekata fizičkog opterećenja različitog intenziteta na stepen oštećenja ćelija pojedinih organa usled pojačane produkcije slobodnih radikala, kao i adaptiranosti organizma na fizičko opterećenje.

U ispitivanju su učestvovali zdravi punokrvni trkački konji, starosti 3-5 godina, podeljeni u dve grupe. Prva grupa je podvrgnuta kratkotrajnom fizičkom opterećenju visokog intenziteta tokom galopske trke na 2400 m, a ispitivani parametri su određivani pre trke, 48 h i 72 h posle trke. Druga grupa je bila podvrgnuta prolongiranom fizičkom opterećenju niskog intenziteta tokom endjurans trke na 40 km, a ispitivani parametri su određivani pre trke, neposredno nakon trke, 48 h, 72 h, 96 h, 120 h i 144 h posle trke.

Ukupna aktivnost LDH se menjala 72 h i 96 h nakon galopske trke ( $p > 0,05$ ), dok najveću aktivnost dostiže neposredno nakon endjurans trke. Elektroforetskim razdvajanjem LDH kod svih ispitivanih konja ustanovljeno je 5 izoformi. Aktivnost LDH1 72 h nakon galopske trke se značajno povećala u odnosu na aktivnost pre trke ( $p < 0,05$ ), dok se aktivnost ostalih izoenzimskih oblika, LDH2-LDH5, nije statistički značajno razlikovala ( $p > 0,05$ ). Nakon endjuransa uočen je porast aktivnosti LDH1 u svim ispitivanim vremenskim intervalima, sa maksimalnom aktivnošću 96 h i 144 h u odnosu na period pre i neposredno posle trke ( $p < 0,01$ ). Porast aktivnosti LDH2 je bio statistički značajno veći 48 h, 72 h, 96 h i 120 h ( $p < 0,05$ ) u poređenju sa vrednostima pre trke i 48 h, 72 h, 96 h, 120 h i 144 h ( $p < 0,05$ ) u odnosu na vrednost neposredno posle trke. Aktivnosti izoforme LDH3 ispoljava statistički značajan pad, a LDH5 povećanje neposredno nakon endjuransa ( $p < 0,01$ ), dok LDH4 značajno raste u svim ispitivanim vremenskim intervalima posle endjuransa ( $p < 0,01$ ). Aktivnost CK je imala visoki, srednji i nizak stepen adaptiranosti konja na opterećenje.

Koncentracija ukupnih proteina, albumina i globulina se u svim analiziranim intervalima kretala u fiziološkim granicama, izuzev endjuransa kada je ustanovljen statistički značajan pad koncentracije albumina 96 h nakon trke ( $p < 0,01$ ).

Prolongirano fizičko opterećenje niskog intenziteta dovodi do oštećenja ćelija miokarda, mišićnog tkiva, hepatocita i hipoalbuminemije kao posledice stvaranja slobodnih radikala.