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BIOLUMINESCENT SPORT HORSE SALIVA TEST: PROSPECTS FOR USE

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Abstract

Assessment of the physiological state of horses in training is a relevant problem of sport horse breeding worldwide. Existing clinical methods do not provide reliable parameters of the functional state of animals at rest and in physical activity. The reported standards of the physiological state of horses and veterinary guidelines for clinical diagnosis and equestrian sports are contradictory. Therefore, objective tests are necessary to assess the body's response to physical activity. We propose simple, fast, non-invasive method based on an inhibitory effect of saliva on the enzyme activity of luminous bacteria as a screening testing. Changes in the luminescence of the bioluminescent enzymatic system under influence of small amounts of saliva can reveal changes in the body of sport horses as a response to the maximum permissible loads. This study proves for the first time that the bioluminescent enzyme test can track changes in the body condition of sport horses during training. The method uses the integral bioluminescent indicator which depends on the biochemical composition of saliva. The aim of this study is to substantiate suitability of the bioluminescent-based method for testing the functional state of sports horses in training. Trakenen sport horses (*Eguus caballus*) for dressage (n = 12) kept under standard conditions in the Training Center of Horse Breeding Complex (Krasnoyarsk State Agrarian University) were subjected to low, medium and high intensity training before the competition (February-June 2019-2020). Saliva and blood were sampled before (in the morning) and after training. The respiratory rate (RR) and heart rate (HR) were measured. The electrocardiography (ECG) was carried out according to a common method, including assessmsnt of the heart rhythm parameters and the ventricular systolic functional parameters. Hematological test were performed, and blood concentrations of protein and glucose were measured. The saliva was tested by colorimetric, chemi-, and bioluminescent methods. As the intensity of physical activity increased, there was an increase in heart rate, respiration rate, atrial excitation and a decrease in the time of cardiac diastole while hematological and biochemical blood parameters varied within normal limits. The effect of saliva on the intensity of bioluminescence depended on the physical activity. The residual luminescence signal decreased under low and medium intensity training and increased under high intensity training. During low intensity training, a high percentage of luminescence inhibition correlated with an increase in the total blood protein concentration (r = 0.6, p = 0.05) and a decrease in the blood glucose content (r = –0.7, p = 0.05) and the number of erythrocytes (r = –0.6, p = 0.05). Under moderate physical activity, an increase in bioluminescent fluorescence correlated with an increase in RR (r = 0.5, p = 0.1) and in the QRS interval (r = 0.8, p = 0.05). Under high intensity training, a low percentage of luminescence inhibition correlated with the lactate concentration in saliva (r = –0.58, p = 0.1), a reduction in

catalase activity in saliva (r = –0.7, p = 0.05), and a higher amplitude of the P wave on the electrocardiogram (r = 0.8, p = 0.05). Therefore, the bioluminescent analysis of saliva using a coupled enzyme system, NADH:FMN-oxidoreductase and bacterial luciferase can detect the effect of stressful physical activity during horse training of various intensity. The inhibition of bioluminescence can be an indicator of a horse performance in training. The test can be also applicable in sport horse breeding to prevent overtraining.

Keywords: sport horses, saliva, lactate, catalase, NADH:FMN-oxidoreductase, luciferase, bacterial bioluminescence, functional status, hematological parameters, blood biochemistry

Modern domestic horse breeding is a fairly stable, well-structured agricultural sector that can confidently compete both in the global market for horse resources and among livestock industries within the country [1-3]. In the modern world ranking of sports horses, the pets of Russian studs occupy a fairly high position. Russian horses have repeatedly been champions, winners of international equestrian competitions in the USA, UAE [4, 5]. Around the world, the number of horses is rapidly increasing and interest in national breeds is growing [6-8].

Physiological and clinical methods for assessing the functional state of sports horses are objective, but lengthy and difficult to interpret [9-11]. Physiological studies in general do not give a general picture of the condition of the horse [12, 13]. The information presented in the literature on the standards of the physiological state of horses and in veterinary guidelines for clinical diagnostics and equestrian sport is very contradictory. For example, there are significant discrepancies in the values of the main physiological and clinical indicators (temperature, heart rate, number of respiratory movements) and there are no standards for assessing the state of a sports horse both during rest and after muscle work of varying tension [14, 15].

Today, the basic principles of sport horse breeding have undergone a significant transformation based on the principles of Welfare Quality®, according to which the well-being of the horse becomes an object of paramount importance and should never be subordinated to competitive or commercial interests [3, 16]. In this regard, it is of interest to develop non-invasive methods for assessing the functional state of the body [17, 18]. The use of saliva as a material for research removes restrictions on the frequency and availability of measurements during the training or competitive process and allows you to create a convenient tool for the daily work of a rider, trainer, veterinarian. It is also possible to individually control the assessment of the body's response to physical activity and adjust the training process to the response of a sports horse in real time [19, 20].

As a screening test for the saliva of a sports horse, we propose to use a bioluminescent method using a bacterial enzyme system [21, 22], which has proven effective in testing the condition of the human body [23, 24]. A change in the luminescence of a bioluminescent test system when exposed to a small amount of saliva can indicate deviations in the body of sports horses that occur in response to maximum permissible loads, and allow the rider to restructure the training process. An important characteristic of bioluminescent testing of horse saliva is non-invasiveness, which allows painless and quick testing during training.

In the present work, it is shown for the first time that changes in the state of the body of a sports horse can be controlled by an integral bioluminescent indicator, which depends on the biochemical composition of saliva.

The purpose of the work is to evaluate the possibility of using the bioluminescent method for testing the functional state of sports horses in training.

Materials and methods. The study was performed on a group of sports horses (*Eguus caballus*) of the Trakehner breed (n = 12) with a specialization in dressage. The horses were kept under standard conditions of the horse breeding training and sports complex of the Krasnoyarsk State Agrarian University. Testing

of each horse, collecting saliva and blood was carried out before and after training at low, medium and high intensity during the preparation for the competition (February-June 2019-2020).

Low-intensity physical activity included horse training for 1 hour, medium-intensity exercise for 1.5 hours, and high-intensity exercise for 2 hours on a lunge or under saddle. The training program consisted of the following stages: on a free rein, in collection at a trot with the inclusion of lateral elements, shortening and spreading of the gaits and transitions from one gait to another, a hitch at a trot on a long rein, a step.

Saliva samples (1.0-1.5 ml) were collected in disposable sterile plastic tubes. Sampling before exercise was carried out in the morning (before feeding). After physical exercise, saliva, which was formed in sufficient quantities, was taken immediately after the completion of training. Basically, this procedure did not affect the emotional state of the horses.

The functional state of the animal was assessed by respiratory rate (RR), heart rate (HR), and electrocardiogram (ECG). Respiratory rate was determined visually, heart rate was determined by ECG on an EK3T-01-R-D electrocardiograph (Monitor, Russia). ECG was recorded in three standard and three enhanced limb leads. ECG analysis was carried out according to the generally accepted method, including the determination of the nature of the heart rhythm: ventricular systolic index (AVS), the height and width of the teeth, and the duration of the intervals (25).

Hematological studies were performed according to the generally accepted method with the counting of erythrocytes and leukocytes in the Goryaev chamber, the hemoglobin content was determined by the method of Sahli [25]. Biochemical analysis of blood serum was carried out according to the generally accepted method, the content of protein and glucose was measured [25].

Before studying saliva, the samples were centrifuged for 15 min at 5000 rpm (Eppendorf Centrifuge 5810 r, Eppendorf, Germany).

The concentration of lactate (lactic acid) in saliva samples was measured by the photometric method (colorimetry) (a UV-1800 spectrophotometer, Shimadzu, Japan) in accordance with the description [26, 27].

Chemiluminescent and bioluminescent testing of saliva was performed on a TriStar LB 941 plate luminometer (Berthold Technologies, Germany).

Catalase activity in saliva was determined by the H_2O_2-luminol-dependent chemiluminescent method using 25 rl of luminol (AppliChem, Germany) and 25 μl of 3% hydrogen peroxide (H_2O_2) (MCD Chemical, Russia) [28]. The dynamics of luminescence was recorded in the presence of saliva for 5 min. When analyzing the activity of the enzyme, the time of onset of the chemiluminescent reaction (t_0), the maximum intensity of the chemiluminescent reaction (I_{max}), and the maximum area of the chemiluminescent curve (S_{max}) were accounted.

For bioluminescent testing of saliva, a bienzymatic system NADH:FMN-oxidoreductase + luciferase was used, which is included in the kit of analytical bioluminescence reagents (CRAB) (Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk). The kit contains lyophilized preparations of highly purified luciferase enzymes EC 1.14.14.3 (0.4 mg/ml) from a recombinant strain of Escherichia coli and NADH:FMN-oxidoreductase EC 1.5.1.29 (*Photobacterium leiognathi*) (0.18 units of activity). The composition of the reaction mixture for the bioluminescent reaction was as follows: 80 μl of 0.05 M potassium phosphate buffer (pH 6.8-7.0), 5 μl of CRAB solution, 10 μl of 0.0025% tetradecanal solution (Merck, Germany), 50 μl of 0.4 mM NADH solution (Sigma, USA), 10 μl of 0.5 mM FMN solution (Serva, Germany). The reaction mixture was added to the cell of the tablet, and the value of the maximum luminescence

intensity was recorded (control measurement). In the experimental measurement, 40 μl of buffer was replaced by 40 μl of saliva diluted 60 times in buffer. The luminescence intensity was measured in duplicate. The ratio of the average maximum bioluminescence intensities of the experimental measurement (I) to the control (I0) was used to calculate the value of the residual luminescence (T, %).

Statistical data processing was performed using the Statistica 10 program (StatSoft, Inc., USA) with the calculation of the median (*Me*) and interquartile ranges (C_{25}-C_{75} percentiles). Differences between the indicators of dependent samples were assessed using the nonparametric Mann-Whitney U test, and correlation was assessed using Spearman's rank correlation coefficient; significance level of $p \leq 0.1$.

Results. Testing the functional parameters of the body of sports horses in training revealed trends to a significant increase in NPV ($p = 0.1$) and heart rate ($p = 0.1$) with an increase in physical activity from low to high relative to the values obtained before training (Fig. 1).

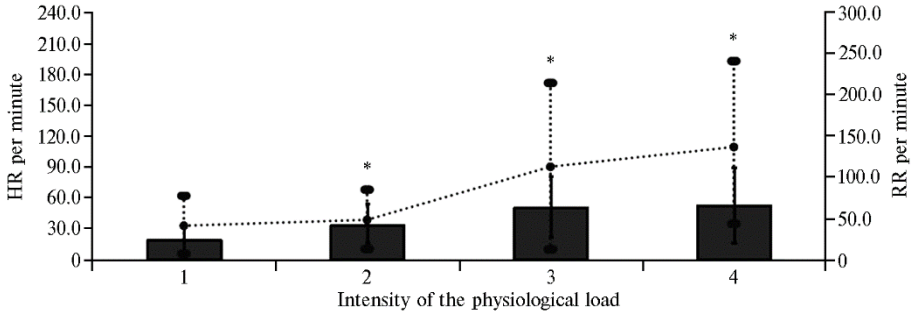

Fig. 1. Heart rate (HR, graph) **and respiratory rate** (RR, diagram) **in Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity:** 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given ($n = 12$).
* Differences with pre-training value ($p = 0.1$).

The heart rate of the studied horses remained sinus regular. Prior to training, electrocardiological parameters corresponded to the average standard.

Low-intensity exercise increased P wave amplitudes to 4.0 mm (3.0-4.0 mm) and R wave amplitudes to 13.0 mm (12.0-14.0 mm) relative to pre-training values. There was also a shortening of the P-Q intervals up to 0.07 s (0.06-0.1 s), QRS up to 0.06 s (0.06-0.08 s), QPST up to 0.5 s (0.5-0.5 s), TP up to 0.6 s (0.5-0.8 s), RR up to 1.3 s (1.0-1.5 s), life expectancy up to 36.1% (31.1-46.2%). Physical activity of moderate intensity did not change the P wave amplitude, which was 2.0 mm (2.0-2.0 mm), and increased the R wave amplitude up to 11.0 mm (7.0-12.0 mm) compared to the values up to workouts. Shortening of QPST intervals to 0.4 s (0.4-0.5 s), TP to 0.4 s (0.01-0.7 s), RR to 1.2 s (1.1-1.5 s) were recorded. Life expectancy increased to 35.9% (33.3-37.0%). High-intensity physical activity increased P wave amplitudes up to 4.0 mm (4.0-4.0 mm) and R wave amplitudes up to 24.0 mm (23.0-26.0 mm) compared to pre-training values. The intervals TP were also shortened to 0.2 s (0.2-0.2 s), RR to 0.9 s (0.8-0.9 s). Life expectancy increased to 49.3% (31.0-46.9%).

With low-intensity exercise, the systolic index (P wave) in horses increased ($p = 0.004$) relative to that with moderate-intensity exercise. Physical activity of great intensity affected systolic and diastolic parameters. There was a statistically significant increase in the amplitude of the P ($p = 0.0043$) and R ($p = 0.0043$) waves and a decrease in the intervals P-Q ($p = 0.0043$), TP ($p = 0.017$) and RR ($p = 0.017$) relative to the corresponding values at loading of average intensity.

Hematological and biochemical parameters were in the range of the

physiological norm both before training and after exercise. The amount of hemoglobin increased to 14.0 g% (11.8-15.6 g%) at low intensity exercise and up to 13.1 g% (11.8-13.8 g%) at high intensity. The content of erythrocytes decreased with increasing load up to 10.0 million/μl (8.6-11.8 million/μl) at its low intensity and up to 7.5 million/μl (7.2-9.0 million/μl) at medium and high intensity. The number of leukocytes, on the contrary, increased to 5.7 thousand/μl (4.1-5.8 thousand/μl) with a low load, up to 6.8 thousand/μl (5.9-8.1 thousand/μl) with an average and up to 8.3 thousand/μl (7.9-9.8 thousand/μl) with high intensity. There was an increase in the concentration of total protein and a decrease in glucose with increasing physical activity. The index of total protein after exercise of low, medium and high intensity was 64.4 g/l (61.8-65.1 g/l), 62.5 g/l (60.9-63.6 g/l), and 66.9 g/l (63.9-66.9 g/l), respectively, For glucose, it was 4.9 mmol/l (4.8-5.1 mmol/l) for low and 4.4 mmol/l (4.1-4.8 mmol/l) for medium and high intensity. We did not find statistically significant differences in changes in the quantitative composition of cells, concentrations of total protein and glucose, depending on the intensity of the loads.

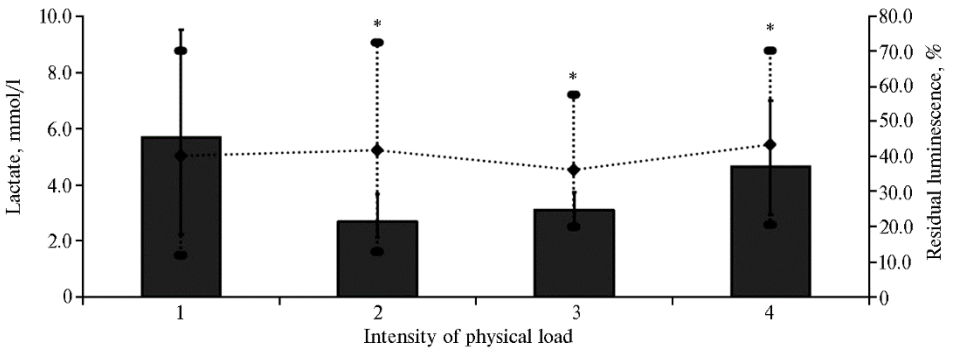

Fig. 2. Residual luminescence (diagram) and lactate concentration (graph) **in saliva of Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity:** 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given (n = 12).
* Differences with pre-training value (p = 0.1).

The concentration of lactate in saliva tended to increase during physical activity of low and high intensity and amounted to 5.2 mmol/l (4.7-5.9 mmol/l) and 5.4 mmol/l (3.8-7.2 mmol/l), and at medium load it corresponded to the indicators obtained before training, 4.5 mmol/l (3.8-5.0 mmol/l) and 5.0 mmol/l (4.1-5.4 mmol/l) (Fig. 2).

The results of saliva testing also showed the dependence of the bioluminescent glow on physical activity. There was a tendency to its decrease relative to the indicators before training with low-intensity physical activity, followed by an increase with increasing load (see Fig. 2). We believe that the quenching of the bioluminescent glow during physical activity of different intensities is due to a change in the metabolic composition of saliva, which is caused by the functional state of the horse's body during exercise.

At low, medium and high physical activity, catalase production was activated relative to pre-workout levels. The highest intensity of the luminol-dependent chemiluminescent luminescence was observed at a low load, while the minimum intensity was observed at a high load. A statistically significant increase in the intensity of the luminol-dependent chemiluminescent luminescence of saliva indicated an increase in free radical oxidation processes (Fig. 3).

Therefore, in horses with an increase in physical activity, the functional indicators of the body increased, which indicated the activation of the respiratory, cardiovascular and enzymatic systems during training. Excitation of the atria and a

reduction in the time of cardiac diastole during physical exertion indicated a rapid blood filling of the ventricular volumes of the heart, which was explained by the intensive work of the skeletal muscles. A high physical load, in contrast to a low one, increased the rate of propagation of excitation through the muscles of the right and left ventricles, which characterized the intensive work of a healthy horse's heart in a state of active training [29].

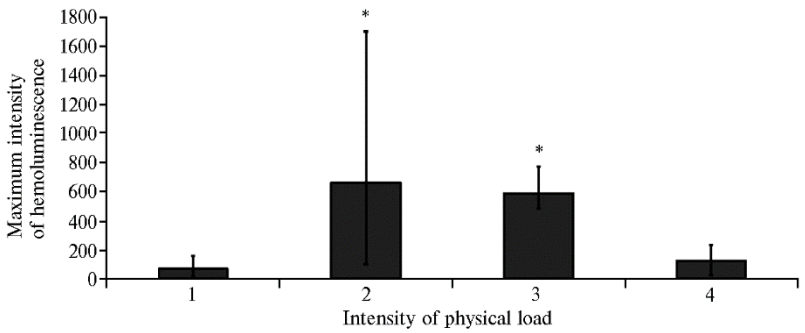

Fig. 3. Intensity of luminol-dependent hemoluminescence in the presence of H_2O_2) in saliva of Trakehner sports horses (*Eguus caballus*) depending on the intensity of physical activity: 1 — before training, 2 — low, 3 — medium, 4 — high intensity. The median (*Me*) and percentiles (C_{25}-C_{75}) are given (n = 12). * Differences with pre-training value (p = 0.1).

As reported, in endurance sport horses, haematological parameters and salivary chemistry are associated with heart rate [30, 31]. Our results of the clinical analysis of the blood of Trakehner sports horses confirmed the change in hematological parameters in connection with the heart rate or NPV during exercise. However, the identified changes remained within the normal range, which indicated a good functional state of the horses [30] or, possibly, a high degree of their preparedness. The latter was not noted in other studies, since they considered the effect of physical activity of the same intensity.

Studies of racehorses with low performance in Italy and Ukraine showed that increased exercise intensity affected the change in cardiomyocyte permeability and the release of enzymes into the blood [32, 33]. In our experiments, physical activity of different intensity caused a change in the concentration of glucose and total protein in the blood serum, that is, they influenced the dynamics of carbohydrate metabolism [30].

In similar studies of horses of various sports specializations, an increase in the concentrations of lactate and pyruvate in the blood was noted at different periods of the training cycle [11, 12, 32]. Our data show that lactate concentration in horse saliva remained stable at low and high levels of exercise. An increase in the maximum concentration of lactate with increasing load may be due to insufficient oxygen saturation of the body, which indicates a poor performance of the horse [8, 11, 32]. Therefore, the stable increase in lactate concentration observed by us during low and high intensity training compared to the pre-training index indicated the absence of hypoxia and indicated a high fitness of the horses.

The ability of horses to overcome high physical loads is confirmed by changes in catalase activity, which characterizes the state of oxidative systems and aerobic oxidation processes [14]. On horses of different breeds, it was found that the current physical load could be assessed by the reduced, increased or multidirectional dynamics of the catalase content [17-19]. We found an increase in the production of salivary catalase in horses with an increase in the intensity of physical activity, which can be explained by the activation of free radical processes due to oxidative stress.

According to the presented data, horse saliva is as informative for the analysis of the functional state of the body as blood. Saliva, as a dynamic biological fluid, rapidly changes its composition depending on the increase in physical activity [14, 31]. The bioluminescent index collectively takes into account such changes. It is of importance to identify the factors that determine the intensity of the bioluminescence of saliva in physical exertion and act as the reasons for its change. For these data, we analyzed the correlation between the magnitude of the residual luminescence and the functional parameters, cardiac hemodynamics, blood biochemical parameters, the content of lactate and catalase in saliva.

With low-intensity exercise, a high percentage of luminescence inhibition correlated with an increase in total protein concentration ($r = 0.6$, $p = 0.05$), a decrease in glucose content ($r = -0.7$, $p = 0.05$), and blood erythrocyte counts ($r = -0.6$, $p = 0.05$). During moderate exercise, the increase in bioluminescence correlated with an increase in RR ($r = 0.5$, $p = 0.1$) and lengthening of the QRS interval ($r = 0.8$, $p = 0.05$). During high-intensity exercise, a low percentage of luminescence inhibition correlated with salivary lactate ($r = -0.58$, $p = 0.1$), decreased salivary catalase ($r = -0.7$, $p = 0.05$), and increase in P wave amplitude ($r = 0.8$, $p = 0.05$).

So, bioluminescent testing of saliva of horse in training using the NADH:FMN-oxidoreductase + luciferase bienzyme system showed that the observed luminescence intensity is related to changes in the concentration of metabolites in saliva and depends on the physical load experienced by the animal. The value of the residual glow decreases with low physical activity and increases with high physical activity. It was found that the intensity of the luminescence correlates with the concentration of catalase and lactate in saliva. Thus, our finding have proved potential use of non-invasive saliva testing to assess the impact of physical activity on sports horses in training. The obtained preliminary results make it possible to assess the physiological state of horses under physical loads of various volume and intensity.

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