



INFLUENCE OF EXHAUSTIVE EXERCISE ON OSMOTIC RESISTANCE OF ERYTHROCYTES AND SOME CLINICAL PARAMETERS IN DOGS

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Summary

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The aim of the experiment was to study the *in vivo* effect of exhaustive aerobic exercise on osmotic resistance of erythrocytes, neutrophil/lymphocyte ratio and some clinical parameters in dogs. We used 12 male, mongrel dogs divided into two groups – animals from experimental group were submitted to exhaustive exercise; animals from control group did no exercise. Minimum osmotic resistance, 5 % haemolysis, 50 % haemolysis, 90 % haemolysis and maximum osmotic resistance were measured using an osmotic fragility test in the following dynamics: before exercise (BE), right after exercise (0 h), on 2nd hour, 4th hour, 24th hour, 48th hour, 72nd hour and on 7th and 14th day after exercise. Neutrophil to lymphocyte ratio (N/L), body temperature (BT), heart rate (HR) and breathing rate (BR) were measured in the same dynamics. We found a decrease of red blood cell osmotic resistance in experimental dogs (measured by 50 % haemolysis) on 24th hour, 48th hour and 72nd hour and on 7th and 14th day after exercise, as compared to control group ($P < 0.05$). Surprisingly in the experimental group maximum osmotic resistance (100 % haemolysis) increased on 24th hour after exercise, compared both to initial level and control group ($P < 0.05$). N/L ratio increased significantly in the experimental group on 2nd hour compared to BE level ($P < 0.05$). In conclusion, exhaustive exercise acts as a stressor and affects adversely the fragility of red blood cells.

Key words: dog, exhaustive exercise, osmotic haemolysis

INTRODUCTION

Exercise has been in the focus of scientific studies for many years. Various experiments involving humans and different animal species have been conducted and many aspects of exercise have been studied. Endurance horse racing and sled dog

racing have been popular for many centuries in some parts of the world. Recently endurance exercise, like marathon running, triathlons and ultramarathons, are becoming very popular among humans. This popularity of endurance sports is

urging science to study more thoroughly the effects of exhaustive exercise on health in all athletic species. Nowadays it is generally accepted that regular moderate exercise has many long-term beneficial effects, while acute exhaustive exercise may have some negative impact. Sustained strenuous exercise has been shown to induce oxidative and physiological stress, which is accompanied by hypercortisolaemia (Royer *et al.*, 2005). Other examples include suppression of immunity, gastrointestinal bleeding, exercise-induced haematuria, intravascular haemolysis, anaemia, trauma and electrolyte balance disorders (Abarbanel *et al.*, 1990; Halvorsen *et al.*, 1990; Robson *et al.*, 2003; Telford *et al.*, 2003; Schott *et al.*, 2006). Sometimes during and after exhaustive exercise, haematologic and biochemical parameters change dramatically. Changes concerning the erythron have been named “sports anemia” – endurance athletes tend to be mildly anaemic. This condition has been described both in humans and animal species (Yamada *et al.*, 1987; Balaban, 1992). Some scientists state this is no true anaemia but it is simply due to expansion of plasma volume. Still several studies have found that exhaustive exercise may lead to intravascular haemolysis that can also contribute to a mildly anaemic state.

The aim of the present experiment was to study the *in vivo* effects of exhaustive aerobic exercise on osmotic resistance of erythrocytes and some clinical parameters in dogs.

MATERIALS AND METHODS

Experimental animals

We used twelve healthy male, mongrel dogs, 1.5–3.5 years of age. Adaptation period continued one month. The dogs were treated against parasites with Bihel-

don (Cheironpharma Europe, each tablet contains praziquantel 50 mg, pyrantel pamoate 150 mg) at a dose of 1 tablet/10 kg. Also they were treated against ectoparasites with antiparasite shampoo, Ectomin and Tapilan (Dorvet, Israel). An antirabies vaccine – Nobivac Rabies (Intervet International B. V.) was also applied. Animals were kept in individual cages (situated indoors, providing constant room temperature) and went for walks twice a day – half an hour in the morning and another walk in the evening. Thus conditions were similar to the way of pet breeding. Dogs were divided into two groups – experimental and control. Dogs of the experimental group weighed 18.67 ± 0.82 kg, and control animals – 20.08 ± 3.29 kg. Only experimental animals were submitted to a prolonged strenuous exercise.

Exercise protocol

Adaptation. The aim of the study was to submit experimental dogs to a prolonged strenuous exercise until exhaustion. During adaptation period only one person took care of the dogs so that they can get used to him and accept him as “the leader of the pack”. In each day of the last week of adapting period, that person trained them to run on a leash after him riding a bicycle. Dogs were willing to run and easily performed the exercise, which had a very short duration of about 10 min and can not be considered as endurance training. Runs were performed off road to avoid trauma. During this week dogs got used to the environment so they were able to run without distracting their attention.

Exhaustive exercise. In the day of the experiment each dog was submitted to a prolonged submaximal exercise until exhaustion. Dogs ran on a leash following the “leader” riding bicycle at an average

speed of about 12 km/h or faster depending on dog's abilities. Dogs ran galloping and trotting. Heart rate during exercise exceeded resting heart rate by 77%. When fatigue emerged and they were unwilling to run they were encouraged by the leader verbally and by pulling gently the leash. Exercise was stopped when dogs demonstrated inability to perform and refused to continue.

Explanatory remarks. Exhaustive exercise began with a 5 min warm up running. To avoid trauma dogs performed off road and to prevent dehydration in every five kilometers of running dogs had access to water. Experiment was conducted under the conditions of moderate climate in spring and autumn, and ambient temperature did not exceed 20 °C. We preferred this exercise protocol to running on a treadmill, because treadmill running is less difficult and dogs get easily bored. On the contrary when running off road they were eager to follow their "leader" as long as they can run. Dogs had no endurance training before conducting the experiment so they reached exhaustion easier through this strenuous and prolonged exercise.

The experiment complies with the current laws of the Republic of Bulgaria. It was approved by the Ethics Committee of the Faculty of Veterinary Medicine of Trakia University.

Blood samples

Blood samples were collected in sterile glass tubes by puncture of *vena cephalica* in the following dynamics – before exercise, right after exercise (0 hour) and on 2nd hour, 4th hour, 24th hour, 48th hour, 72nd hour, 7th day and 14th day after exercise. We used 0.2 mL heparin (50 units/mL) for each sample as anticoagu-

lant. Blood samples for BE level and on the following days were taken at 8 a.m.

Blood analysis

- Osmotic fragility test

The test is used to measure red blood cell resistance to haemolysis when exposed to a series of increasingly dilute saline solutions (from 0.85 % to 0.10 % NaCl, step of dilution – 0.05 %). A row of 16 tubes is prepared. In each tube 5 mL of the needed saline solution are pipetted. Then 50 µL of blood are added. Tubes were kept for 30 min at room temperature, then were centrifuged for 10 min at 2000 rpm. Absorbance of each sample (A) is measured using spectrophotometer (wavelength 541 nm; against distilled water). Then percentage of haemolysis is calculated by the formula:

$$\% \text{ haemolysis} = \frac{A \text{ test supernatant}}{A \text{ 0.10\% supernatant}} \times 100$$

- Neutrophil to lymphocyte (N/L) ratio – it was calculated by dividing the count of neutrophils by the count of lymphocytes.

Clinical parameters

Body temperature (BT), heart rate (HR) and breathing rate (BR) were measured to monitor overall clinical status.

Statistical analysis

Results are presented as means ± SD. Data was submitted to standard F-test and t-test (StatMost, v.2.5, DataMost Corporation). Differences were considered statistically significant at the P<0.05 level.

RESULTS

Minimum osmotic resistance and osmotic resistance, measured by 5% haemolysis, did not differ significantly within experi-

Table 1. Changes in osmotic resistance of erythrocytes in experimental (n=6) and control group (n=6). Results are presented as mean ± SD

Parameter	Groups	Dynamics											
		BE	0h	2h	4h	24h	48h	72h	7d	14d			
Minimum osmotic resistance	Experimental	0.53±0.04	0.51±0.02	0.52±0.03	0.51±0.02	0.52±0.03	0.51±0.02	0.52±0.03	0.51±0.02	0.52±0.03	0.51±0.02	0.51±0.02	0.51±0.02
	Control	0.51±0.02	0.51±0.02	0.52±0.03	0.51±0.02	0.51±0.02	0.53±0.03	0.51±0.02	0.50±0	0.51±0.02	0.51±0.02	0.51±0.02	0.51±0.02
5% haemolysis	Experimental	0.55±0.02	0.56±0.03	0.58±0.08	0.56±0.05	0.57±0.05	0.55±0.04	0.55±0.03	0.56±0.04	0.55±0.03	0.55±0.03	0.56±0.04	0.59±0.08
	Control	0.55±0.03	0.54±0.01	0.53±0.01	0.54±0.01	0.53±0.01	0.53±0.02	0.53±0.02	0.53±0.02	0.53±0.02	0.53±0.02	0.53±0.02	0.54±0.02
50% haemolysis	Experimental	0.46±0.02	0.47±0.02	0.47±0.03	0.47±0.03	0.48±0.02	0.47±0.02	0.49±0.03	0.48±0.02	0.47±0.02	0.49±0.03	0.48±0.02	0.47±0.01
	Control	0.45±0.01	0.46±0.03	0.45±0.02	0.45±0.02	0.46±0.02	0.45±0.01	0.46±0.01	0.45±0.02	0.45±0.01	0.46±0.01	0.45±0.02	0.45±0.02
90% haemolysis	Experimental	0.39±0.05	0.35±0.06	0.38±0.05	0.38±0.04	0.38±0.06	0.37±0.08	0.42±0.05	0.41±0.01	0.39±0.04	0.42±0.05	0.41±0.01	0.39±0.04
	Control	0.37±0.06	0.38±0.03	0.41±0.03	0.37±0.07	0.37±0.04	0.39±0.02	0.35±0.09	0.39±0.04	0.39±0.04	0.35±0.09	0.39±0.04	0.35±0.09
Maximum osmotic resistance	Experimental	0.31±0.05	0.28±0.06	0.28±0.07	0.28±0.07	0.26±0.04	0.28±0.08	0.31±0.05	0.32±0.04	0.28±0.08	0.31±0.05	0.32±0.04	0.31±0.04
	Control	0.31±0.04	0.34±0.05	0.3±0.04	0.31±0.05	0.32±0.03	0.32±0.04	0.31±0.06	0.33±0.03	0.32±0.04	0.31±0.06	0.33±0.03	0.33±0.03

Statistically significant differences: a¹ P<0.05 within the experimental group as compared to BE level; b¹ P<0.05 experimental vs. control group, at the same point of dynamics.

Table 2. Changes in neutrophil/lymphocyte ratio, body temperature, heart rate and breathing rate in experimental (n=6) and control group (n=6). Results are presented as mean ± SD.

Parameter	Groups	Dynamics													
		BE	0h	2h	4h	24h	48h	72h	7d	14d					
N/L ratio	Experimental	2.28±1.20	2.67±1.31	3.25±1.14	2.63±1.33	2.15±1.55	1.82±0.73	1.80±0.80	1.68±0.41	1.47±0.23					
	Control	2.38±1.10	2.68±1.52	2.2±0.91 ^{a1}	2.08±1.03	2.15±1.15	2.25±1.13	2.38±1.28	2.25±0.90	1.8±0.36					
Body temperature, °C	Experimental	38.47±0.32	39.63±0.64	38.02±0.33	37.98±0.39	38.22±0.37	38.32±0.35	38.18±0.33	38.23±0.35	38.50±0.35					
	Control	38.50±0.39	38.40±0.28	38.22±0.44	37.98±0.40	38.30±0.32	38.35±0.24	38.35±0.33	38.28±0.23	38.23±0.3					
Heart rate, min ⁻¹	Experimental	81.17±8.95	143.67±31.51	96.5±17.18	80.67±19.92	78.83±4.66	78.33±7.55	80.67±12.04	81.33±6.15	83.00±11.01					
	Control	83.50±14.53	89.17±10.82	100.0±14.09	88.00±9.12	91.67±5.72	92.67±6.89	86.00±6.20	86.67±4.68	86.67±5.32					
Respiratory rate, min ⁻¹	Experimental	31.33±7.87	135.83±26.29	34.00±12.26	31.50±9.95	29.83±8.30	27.83±6.40	29.33±7.55	28.17±7.96	28.33±9.16					
	Control	25.17±2.93	23.83±7.47	24.50±5.86	23.50±1.22	25.00±3.29	23.50±2.66	26.00±3.10	25.0±5.48	25.00±4.15					

Statistically significant differences: ^{a1} P<0.05; ^{a2} P<0.01; ^{a3} P<0.001 within experimental group as compared to BE level; ^{b1} P<0.05; ^{b2} P<0.01; ^{b3} P<0.001 experimental vs. control group, compared at the same point of dynamics.

mental and control groups. Comparison between groups, at the same points of dynamics, also showed no significant differences (Table 1).

The comparison between groups showed a decrease in osmotic resistance of erythrocytes measured by 50 % haemolysis in experimental animals, compared to controls on hours 24, 48, 72 and days 7 and 14 after exercise ($P < 0.05$).

Osmotic resistance, measured by 90% haemolysis, did not vary statistically significantly in experimental and control animals. Differences between groups were not observed at each time interval.

Maximum osmotic resistance in the experimental group increased on hour 24 after exercise, as compared both to BE level and to control group ($P < 0.05$).

N/L ratio increased significantly on 2nd hour after exercise in experimental dogs, as compared to BE level ($P < 0.05$) (Table 2). The increase in N/L ratio was not statistically significant compared to control group at the same point of dynamics.

The measured clinical parameters changed in a similar pattern – body temperature increased right after exercise in experimental dogs, compared both to BE level ($P < 0.05$) and to control group ($P < 0.01$); heart rate increased right after exercise within the experimental group ($P < 0.01$) and compared to controls increase was significant on 0 hour ($P < 0.01$), 24th hour ($P < 0.05$) and 48th hour ($P < 0.01$) after exercise; respiratory rate increased right after exercise in experimental group, as compared to BE level and to controls ($P < 0.001$).

DISCUSSION

Experimental animals demonstrated dramatic changes in body temperature, heart rate and respiratory rate. These changes

are the result of the increased energy expenditure, needed to sustain prolonged strenuous contraction of skeletal muscles. It is interesting to note that experimental animals had a lower heart rate in the later hours after exercise. This could be the result of central cardiovascular adaptation to exercise – improvement of coronary circulation may lead to increased cardiac output, which results in decrease in heart rate. Such adaptive response is typical not only for humans, but has been described also in athletic dogs (Stepien *et al.*, 1998). The marked increase in body temperature during exercise is due to the activated metabolism. Moreover, in such conditions it is difficult for the canine species to maintain normal body temperature, because of insufficient perspiration. One possible negative effect of overheating is discussed below. Prolonged strenuous exercise has been shown to increase levels of serum cortisol in dogs (Royer *et al.*, 2005) and hypercortisolaemia has been identified as a stress marker. Some scientists have stated that neutrophil to lymphocyte ratio is also an applicable marker of stress response (Stull *et al.*, 1999; Gundasheva *et al.*, 2005). Moreover, the increase in N/L ratio is directly linked to high levels of cortisol, which is known to induce leucocytosis accompanied by neutrophilia and lymphopenia (Gleeson, 2006). Thus, we consider that the documented increase in N/L in our study is indicative for exercise induced stress.

Nevertheless, our experimental single bout of exhaustive exercise had only short-term effects on body temperature, heart rate, respiratory rate and neutrophil to lymphocyte ratio. Surprisingly, we found that exercise had a long-term negative effect on erythrocyte fragility. Though osmotic resistance, measured by 5% haemolysis, did not change signifi-

cantly in experimental animals, it is obvious from results that red blood cells tended to be more fragile. It is also seen that standard deviations increased in the experimental group, proving that prolonged exercise has an impact on erythrocyte fragility. Osmotic resistance, measured by 50% haemolysis, showed an even more definitive trend for decrease of erythrocyte resistance in experimental group. Moreover, decrease was statistically significant when compared to control group at the same points of dynamics. It is considered that measuring 50% haemolysis reflects most sensitively changes in erythrocyte fragility. Surprisingly, we found an increase in maximum osmotic resistance on hour 24 after exhaustive exercise, which could be due to increased number of more resistant young erythrocytes or reticulocytes released from the spleen. It is known that spleen is a reservoir of red blood cells, which can enter the circulation during exercise (Vatner *et al.*, 1974). As the fraction of these more resistant erythrocytes is very small, changes are seen only when the maximum osmotic resistance is measured.

Prolonged exercise is well known to induce intravascular haemolysis in humans and some animal species (Cywinska *et al.*, 2011). The exact mechanisms leading to changes in erythrocyte fragility have not been fully understood. Many factors have been suspected to contribute to exercise-induced haemolysis. Mechanical compression of erythrocytes in capillaries by footstrike can lead to haemolysis during some types of exercise (Hanzawa *et al.*, 2000; Telford *et al.*, 2003). Mechanical compression of erythrocytes in capillaries in the contracting muscle can also lead to haemolysis during exercise or in the recovery period. Shiraki (1968) has suggested that strenuous exercise, by

means of promoting adrenaline secretion, causes the spleen to contract, which increases sensitivity of erythrocyte membrane after exercise stress. Other factors include: high body temperature, electrolyte disturbances or acidosis (Cywinska *et al.*, 2011). Exercise-induced oxidative stress has also been defined as a factor inducing haemolysis (Senturk *et al.*, 2005). Further investigations are needed to define whether the increase in erythrocyte fragility after exercise can contribute to development of anaemia in this animal species.

CONCLUSION

We can conclude that exhaustive exercise in dogs acts as a stressor and may lead to increase in N/L ratio, BT, HR, BR and erythrocyte osmotic fragility. Changes in fragility of red blood cells can be indicative for the severity of exercise stress.

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