



## CHANGES IN SERUM CORTISOL AND SOME INNATE IMMUNITY PARAMETERS AFTER EXHAUSTIVE EXERCISE IN MALE DOGS

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### Summary

Dzhelebov, P. V., D. I. Gundasheva, M. Y. Andonova & E. P. Slavov, 2019. Changes in serum cortisol and some innate immunity parameters after exhaustive exercise in male dogs. *Bulg. J. Vet. Med.*, **22**, No 3, 275–284.

The aim of the present study was to investigate the influence of exhaustive exercise on some innate immunity parameters and cortisol levels. Twelve male, mongrel dogs were divided into an experimental group, submitted to prolonged, strenuous exercise with exhaustion as the end-point, and a control group without any exposure to exercise. Serum cortisol levels were measured before exercise (BE), right after (0 h) and on 2<sup>nd</sup> hour and 4<sup>th</sup> hour after exercise. The neutrophil function (phagocytosis, phagocytic index, hydrogen peroxide production-H<sub>2</sub>O<sub>2</sub>) and classical pathway of complement activation (CPCA) were measured as followed: BE, right after exercise and on 2, 4, 24, 48, 72 h, and 7, 14 day after exercise. In experimental animals cortisol decreased on hour 4 after exercise ( $P < 0.05$ ), compared to BE level, and on hour 2, compared to controls ( $P < 0.05$ ). Percentage of H<sub>2</sub>O<sub>2</sub> producing neutrophils in experimental animals dropped significantly on hour 4 and day 7 after exercise ( $P < 0.05$ ), compared to BE level and on hour 4 ( $P < 0.01$ ) vs control group. Percentage of phagocytising neutrophils decreased slightly on hour 48 ( $P < 0.05$ ), compared to BE level. Phagocytic index and CPCA had an insignificant increase after exercise. Inappropriate changes in cortisol levels could indicate inadequate adaptive response to exercise. Overtraining could make animals more susceptible to infection.

**Key words:** complement system, cortisol, dog, exercise, phagocytosis

### INTRODUCTION

Recently exercise has become a field of intense scientific studies. Many of the studies concerning effects of exercise on endocrine and immune system are highly controversial. Results from such studies are often influenced by the animal species

involved in the study, individual inherent factors (especially in humans), type of exercise and exercise protocol, nutrition, climate and other experimental conditions. Glucocorticoids are the most often investigated hormones during physical exer-

cise. Some studies have found increase in cortisol levels after training in hunting dogs (Preziuso *et al.*, 2000). Cortisol has been also reported to increase pre-exercise, due to anticipation of the exercise event, and after short-duration high-intensity exercise in athletic dogs (Angle *et al.*, 2009). After endurance running conditioned Alaskan sled dogs have showed increase in urinary cortisol, which is indicative of adrenocortical stimulation (Durocher *et al.*, 2007). Other studies have found no changes in serum levels of cortisol after long distance running in female beagle dogs (Arokoski *et al.*, 1993). Another study reports reduced circulating cortisol concentrations after training in rescue dogs (Rovira *et al.*, 2008).

Short-duration strenuous sled pulling activity in dogs caused activation of neutrophils (Moritz *et al.*, 2003). In a study involving humans, prolonged cycling did not cause any change in natural killer cell activity, neutrophil and monocyte phagocytosis (Scharhag *et al.*, 2005). The oxidative burst of neutrophils has been reported by different researches either to decrease or increase after severe exercise like marathon running (Hessel *et al.*, 2000; Chinda *et al.*, 2003). The complement system in humans after severe exercise has been found to activate (Castell *et al.*, 1997). There is very limited information concerning dogs, about the influence of exercise on innate immune mechanisms, such as phagocytosis and complement system.

The aim of the present study was to measure serum cortisol levels after a single bout of prolonged, strenuous exercise with exhaustion as the end-point, and to evaluate short- and long-term changes in neutrophil function (phagocytosis and oxidative burst) and classical pathway of complement activation in experimental canine model.

## MATERIALS AND METHODS

### *Experimental animals*

The experiment was approved by the Ethics Committee at the Faculty of Veterinary Medicine of Trakia University, Stara Zagora (Licence №17/12.07.2007). A total of twelve healthy male, mongrel dogs, 1.5 – 3.5 years old, were used in this study. Adaptation period continued one month. The dogs were treated against parasites with Biheldon (Cheironpharma Europe, each tablet containing 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 anti-rabies vaccine 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. The animals were fed commercial canine dry food Cotagro adult (Cotécnica S.C.C.L.). Dogs were divided into two groups – experimental group (n=6) and control group (n=6). Dogs of experimental group weighed  $18.67 \pm 0.82$  kg, and control animals –  $20.08 \pm 3.29$  kg. Only experimental animals were submitted to exercise.

### *Exercise protocol*

Adaptation: 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 after a bicycle supplied with a specially constructed bike leash for dogs. Dogs were willing to run and easily performed the exercise. The exercise had a very short duration of

about 10 minutes and can not be considered as endurance training. Runs were performed off road to avoid trauma. During this week dogs were introduced to the exercise and to the environment so they were able to perform the exercise without distracting their attention.

**Exhaustive exercise:** Each dog from experimental group was submitted to a prolonged running at moderate intensity with exhaustion as the end-point. Dogs ran on a leash after a bicycle at an average speed of about 12 km/h or faster depending on dog's abilities. Dogs ran galloping and trotting. When fatigue emerged and they were unwilling to run they were encouraged by the leader verbally and by pulling the leash. Exercise was stopped when dogs could no longer sustain the needed intensity and refused to perform the exercise.

**Explanatory remarks:** Exhaustive exercise began with a 5 min warm up running. 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 instead of using a treadmill, because treadmill running is monotonous and unnatural for the dogs and they easily get bored. On the contrary when running off road they are eager and motivated 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 exercise protocol.

#### *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 h) and on 2, 4,

24, 48, 72 h and 7, 14 day after exercise. We used 0.2 mL heparin (50 units/mL) for each sample as anticoagulant. For measuring cortisol and CPCA blood was collected in tubes and was allowed to clot and then serum was separated. Blood samples for BE level and on the following days were taken at 8 a.m.

#### *Blood analysis*

- Nitroblue tetrazolium reduction test  
Count of H<sub>2</sub>O<sub>2</sub> producing neutrophils was calculated as percentage of total neutrophil count (100) on a safranin stained blood smear (Baehner & Nathan, 1968).
- Percentage of phagocytising neutrophils  
Percentage of phagocytising neutrophils in whole blood samples was defined by the immunofluorescent method of Samnaliev (Samnaliev *et al.*, 1995). A total of 150 neutrophils are counted on the smear. The parameter is defined by the formula: % phagocytising cells = (count of phagocytising cells/150) × 100.
- Phagocytic index  
Phagocytic index shows the mean number of engulfed bacteria by a single phagocytising cell: Phagocytic index = total count of engulfed bacteria / 150
- CPCA  
The classical pathway complement activation was determined according to the method of Stelzner & Stein (1971).
- Cortisol assay  
The serum cortisol was determined by a competitive immunoassay using direct chemiluminescent technology (Bayer ACS:180<sup>®</sup> Automated Chemiluminescence Systems, Bayer Vital GmbH&Co. KG, Geschäftsbereich Diagnostics, Germany).

#### *Statistical analysis*

Results are presented as means±SD. Data was submitted to Shapiro-Wilk's test (Sta-

tistica 6.0, by StatSoft), standard F-test and t-test (StatMost, version 2.5, provided by DataMost corporation). Differences were considered statistically significant at the  $P < 0.05$  level.

RESULTS

Two of the dogs completed 24 km of running, two of them ran 25 km and the other two covered a distance of 30 km. All dogs completed the exercise for about 2 hours (1h 58 min – 2h 9 min). Mean running distance and time were respectively  $26.33 \pm 2.88$  km and  $122.83 \pm 4.58$  min.

In experimental group cortisol levels after exercise decreased, as compared to BE levels. Changes were statistically significant ( $P < 0.05$ ) on 4<sup>th</sup> hour after exercise (Fig. 1). Comparison between experimental and control group in dynamics showed statistically significant lower cortisol levels in experimental group on 2<sup>nd</sup> hour after exercise ( $P < 0.05$ ).

In experimental group, compared to BE levels, percentage of  $H_2O_2$  producing neutrophils decreased after exercise, with the lowest number detected on hour 4 ( $P < 0.05$ ). Number of ROS producing neutrophils was also significantly lower on day 7 after exercise ( $P < 0.05$ ). Comparison between groups showed a marked decrease in experimental dogs as compared to controls on hour 4 ( $P < 0.01$ ), (Fig. 2).

Percentage of phagocytising neutrophils did not show any dramatic changes. In experimental group this parameter decreased on hour 48 after exercise ( $P < 0.05$ ). Comparison between groups did not show any significant differences (Table 1).

Both phagocytic index and CPCA showed a trend to increase after exercise with a maximum respectively on 24<sup>th</sup> and 48<sup>th</sup> hour, but changes were not statistically significant. Comparison between the two groups in dynamics also had no significant differences (Table 1).

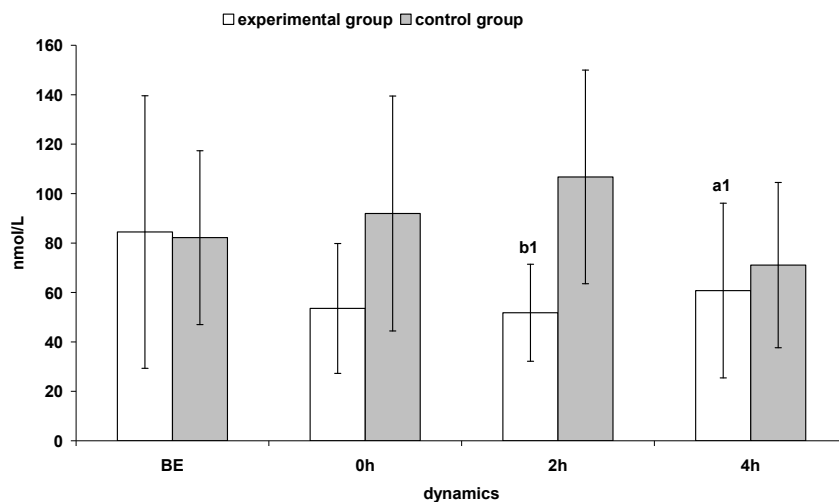
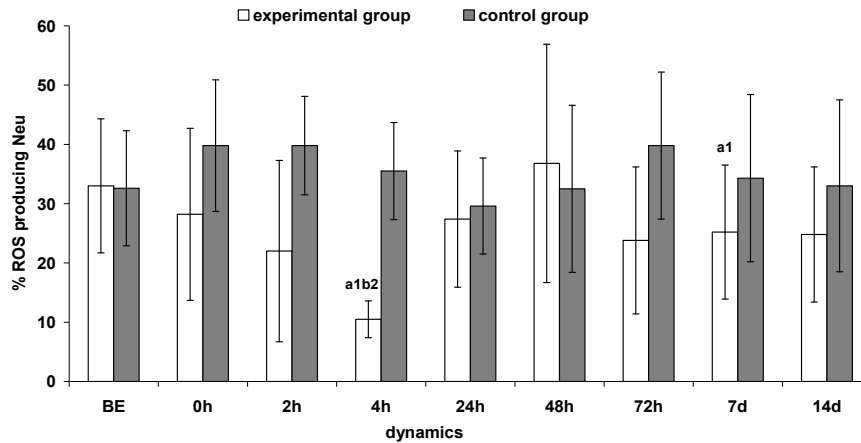


Fig. 1. Serum cortisol levels (mean±SD) in experimental group (n=6) and control group (n=6); a<sup>1</sup>  $P < 0.05$  within experimental group, as compared to BE level; b<sup>1</sup>  $P < 0.05$  experimental vs. control group, compared at the same point of dynamics.



**Fig. 2.** Percentage of H<sub>2</sub>O<sub>2</sub> producing neutrophils (mean±SD) in experimental group (n=6) and control group (n=6); a<sup>1</sup> P<0.05 within experimental group, compared to BE level; b<sup>2</sup> P<0.01 experimental vs. control group, compared at the same point of dynamics.

In control group measured parameters did not show any significant differences.

## DISCUSSION

Metabolic effects of cortisol provide normal glucose blood levels needed for the brain and serve to adapt the body when being put under extreme conditions. Surprisingly, the present study found a decrease in cortisol levels after experimental exhaustive running. This finding is in disagreement with most studies investigating the effect of different types of physical exercise (Desmecht *et al.*, 1996; Gundasheva *et al.*, 2005). Generally increase in cortisol levels depends both on intensity and duration, but correlates most positively with duration of exercise (Hyypä, 2005). Field studies involving humans, horses and dogs participating in different races based on prolonged running report that levels of cortisol elevate after the race (Cook *et al.*, 1987; Durocher *et al.*, 2007;

Fergestad *et al.*, 2016). The present study, although based on prolonged running, used untrained experimental animals that were not adapted to endurance exercise. If these untrained dogs were involved in endurance race event they would probably become non-finishers or would perform extremely bad. Presumably the present experimental exercise protocol had an overreaching effect and led to a short-term overload. The dogs also demonstrated a decrease in blood glucose levels (unpublished data). Some studies point that severe overtraining can lead to inappropriately low levels of cortisol (McKeever & Gordon, 2008). Mechanisms of decrease in cortisol levels under such conditions are not well understood. Some authors state that overreaching may be reflected by reduced responsiveness to ACTH, which is initially compensated for by an increased pituitary ACTH response, but decreased adrenal cortisol response (Wittert *et al.*, 1996; Lehmann *et al.*, 1997).

**Table 1.** Percentage of phagocytising cells, phagocytic index, CPCA in experimental group (n=6) and control group (n=6). Results are presented as mean±SD.

Group	BE level	Right after exercise	2h	4h	24h	48h	72h	7d	14d
<i>Percentage of phagocytising cells (%)</i>									
Experimental group	19.3±4.9	19.4±10.3	19.2±9.2	20.5±13	24±11.3	18±4.7 a <sup>1</sup>	22.4±3.4	20.2±12.6	23.5±9.2
Control group	18.7±2.4	19.2±5.5	17.5±3.4	18.8±6.8	19±7	18.5±4.7	19.3±5	18.2±3.4	20.4±7.6
<i>Phagocytic index</i>									
Experimental group	0.40±0.13	0.32±0.16	0.39±0.15	0.47±0.35	0.60±0.27	0.40±0.06	0.50±0.11	0.50±0.38	0.57±0.22
Control group	0.28±0.05	0.37±0.16	0.38±0.10	0.39±0.18	0.39±0.15	0.36±0.14	0.33±0.19	0.29±0.12	0.39±0.20
<i>CPCA (CH50)</i>									
Experimental group	309±87.4	302±72.2	363.5±105.4	324.2±72.4	353±66	377.7±119.2	347±105.7	299.2±70.3	333.7±72.6
Control group	287.8±79.4	288±48.8	333.8±57.1	347.4±93	349.2±62.2	326±79.2	356.6±70	304±50	322±73.9

a<sup>1</sup> P<0.05 within experimental group, as compared to BE level.

Other investigators suggest that imbalance between secretion and clearance may decrease cortisol. At lower work rates cortisol clearance increases disproportionately to secretion and plasma cortisol concentration may actually decrease (Cashmore *et al.*, 1977). Increased hepatic clearance is probably the leading reason for cortisol decrease. Changes in globulin levels may also be suspected to impact cortisol clearance. However, hypothalamus and pituitary or adrenal dysfunction after overtraining seems to be a more reasonable explanation for eventual decrease in cortisol (Barron *et al.*, 1985).

The insignificant elevation of cortisol in control dogs is probably the result of the excitement during blood sampling. Actually any kind of stressor leads to elevation in cortisol levels. So it is difficult to determine normal blood levels in animals, because restraint and puncture of veins always act as a stressor. And for that reason recently some less invasive methods for measuring cortisol have been developed (Bayazit, 2009; Bennett & Haysen, 2010).

Alterations in immune functions after exercise, which are to some extent due to hormonal changes, have also been a matter of great interest. It is generally accepted that moderate exercise is beneficial for health. On the contrary acute bouts of exercise are thought to suppress immunity. Accordingly the present study found a transient decrease in phagocytic activity after running until exhaustion. However there is little evidence linking exert exercise to long-term health problems. Many aspects of immunity alterations after exercise have been studied. While increase in WBC count is consistent in different studies based on prolonged exercise (Nieman, 2000; Davis *et al.*, 2008; Cywińska *et al.*, 2010), changes in leukocyte functions are

sometimes controversial. Endurance race in horses led to a long-term decrease in neutrophil and monocyte oxidative burst activity (Robson *et al.*, 2003). Total phagocytic activity of neutrophils also has been reported to decrease in humans after marathon race (Chinda *et al.*, 2003). On the contrary swimming until exhaustion in guinea pigs led to a global stimulation of phagocytic function in peritoneal macrophages (Ortega *et al.*, 1992). Other similar rodent studies exist (Su *et al.*, 2001). Although leukocyte activity in blood and tissues may be different, some consistency about blood neutrophils exists – both phagocytic capacity and oxidative burst are decreased after endurance exercise. In most studies mechanisms underlying changes in innate immunity remain unclear. *In vitro* studies report influence of some cytokines and hormones on phagocytic activity of neutrophils, but *in vivo* phagocytosis is influenced by a variety of factors and the overall effect may differ. Prolonged running is associated with muscle damage (Kuipers, 1994), which sounds a reasonable factor for inflammatory and acute-phase response. It is expected that neutrophil phagocytosis will be enhanced under such conditions, but actually studies report reduced phagocytic capacity and reduced killing capacity per cell (Chinda *et al.*, 2003; Robson *et al.*, 2003). Such changes may be due to stress and overload during prolonged exercise (Nieman, 2000). It is also suggested that in the early stages of prolonged exercise phagocytic activity may be increased. Such statements seem to be logic, but quite simple and do not reveal any exact mechanism. According to some researchers decrease in oxidative burst of neutrophils may be the result of activation of antioxidant systems or down-regulation of oxidases, which aims to prevent further

muscle damage by oxide radicals during period after exert exercise (Tiidus, 1998). Some data exist that stress-induced suppression of immunity is seen even in adrenal-ectomized animals, which is due to the presence of immunosuppressive proteins in the blood of stressed animals (Tizard, 1996). However, with respect to immunity, decreased phagocytic activity is transient and is compensated by the marked neutrophilia (Chinda *et al.*, 2003).

Our investigation failed to find activation of the complement system. On the contrary, some studies concerning humans participating in marathon races report increase in some components of complement system, which is probably due to muscle damage (Castell *et al.*, 1997). Other studies did not find changes in complement system (Risøy *et al.*, 2003), which is in agreement with our results. Presumably the complement system is reacting mainly upon antigen stimulation, but not upon muscle inflammation due to exert training. This can not totally exclude the possibility that exert exercise induces local complement activation in muscle, leading to local inflammation (Risøy *et al.*, 2003).

## CONCLUSION

In conclusion, running at moderate intensity until exhaustion in untrained dogs led to decrease in cortisol levels and transient decrease in phagocytic activity of blood neutrophils, but did not alter CPCA. Decrease of cortisol could be a marker of inadequate adaptive response to endurance exercise and severe exercise could make animals more susceptible to infection. Working, hunting and sports dogs should be gradually introduced to exhaustive exercise.

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Paper received 09.11.2017; accepted for publication 30.11.2017

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