NOTE *Internal Medicine*

Changes in serum NGF levels after the exercise load in dogs: a pilot study

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ABSTRACT. Serum nerve growth factor (NGF) levels are increased by the external stress in mice, humans and horses; however, similar variations have been unclear in dogs. Since dogs are usually subjected to conditions of work, exercise and activity as important partners of humans, we measured serum NGF levels post-exercise and compared them with serum cortisol levels, as a biomarker of physical stress. Serum cortisol levels were immediately elevated post-exercise and returned to basal levels within 1 hr. On the other hand, serum NGF levels were significantly increased 1 hr post-exercise and gradually returned to basal levels. Further research is necessary; nevertheless, we have demonstrated for the first time that serum NGF levels respond to exercise stress in dogs.
KEY WORDS: cortisol, dog, external load, nerve growth factor (NGF), serum level

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The earliest evidence of the release of nerve growth factor (NGF) into the bloodstream following the stress of territorial fighting in male mice was reported 30 years ago [2]. In humans, levels of NGF in the plasma of young soldiers were increased following emotional excitation and physical stress [8, 27] associated with their first parachute jump from an aircraft [4]. Moreover, levels of blood NGF in horses were increased under stressful conditions induced by transportation [13], as well as by exercise loads [20]. Recent investigations suggest that the release of NGF might be involved in the activation of immune cells and associated with homeostatic adaptation. However, there has been little information on the kinetics of blood NGF in dogs, following exposure to external stressors. Dogs have a long history as being important companions of humans [29]. Some of them work as guide dogs for the blind and deaf, as well as police and rescue dogs, while others support our quality of life as companion dogs. Thus, an evaluation of the stress status of dogs could inform the effective care and management of working and companion dogs.

The aim of the present study was to investigate the influence of external loads on serum NGF levels in dogs and compare those levels with serum cortisol levels, a common biomarker of stress [7, 22]. For the first study, we tried to detect serum NGF levels before and after free exercise as a simple and noninvasive condition in dogs. Cortisol levels are assumed to be reliable, sensitive markers of stress [7, 14]. Therefore, to confirm whether exercise could induce exercise stress to dogs, we simultaneously measured serum cortisol levels.

All animal experiments complied with both the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology and the guidelines for the use of laboratory animals provided by the Science Council of Japan. The experimental procedures were approved by the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology (No. 27–62; July 27, 2015).

Three healthy mixed breed dogs (hybrid dogs that were generated by crossbreeding Beagle dogs with F1 of Greyhound dogs × Labrador retriever dogs) and one Beagle dog (all dogs used were purchased from Oriental Yeast Co., Ltd., Tokyo, Japan) were subjected to the study. Of the four dogs, two were neutered males, and the other two, neutered females, ranging from 5–6 years of age. They were housed in individual cages in a room illuminated daily from 7:00–19:00, with a temperature of 23 ± 1°C and a humidity of 45–65%. The animals were fed with dog food once a day at 19:00. Experiments were performed from 9 to 10 o'clock in the morning.

The dogs were subjected to exercise loads for fifty min, until the heart rate showed significant increase comparing to that of pre-exercise. Exercise loads consisted of free running, playing with balls and playing tag with experimenters. These three types of the loads were mixed at random, depending on the interests of dogs, trying not to stop their movements. We measured the heart rate of the dogs before and after exercise. The heart rates were significantly increased following the

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Table 1. Mean heart rate

Group	Pre-exercise	Hours after exercise				
		0	1	3	6	9
non-Exercise (n=4)	91.5 ± 5.1	103.5 ± 7.5	106.5 ± 6.2	99.0 ± 3.9	99.0 ± 5.7	105.0 ± 5.2
Exercise (n=4)	92.4 ± 1.5	126.0 ± 6.4[a)]	109.0 ± 5.9	101.0 ± 5.5	104.2 ± 6.0	99.0 ± 10.5

Each value is expressed as mean ± SE. a) P<0.05, when compared to pre-exercise.

Table 2. Serum cortisol and NGF levels

Items	Group	Pre-exercise	Hours after exercise				
			0	1	3	6	9
Cortisol (*ng*/m*l*)	non-Exercise (n=4)	5.93 ± 0.50	6.51 ± 0.65	4.75 ± 0.88	6.41 ± 0.23	7.32 ± 0.32	6.20 ± 0.55
	Exercise (n=4)	6.43 ± 1.54	16.74 ± 6.91[a)]	9.04 ± 4.30	7.45 ± 2.81	9.54 ± 3.58	8.21 ± 2.94
NGF (*ng*/m*l*)	non-Exercise (n=4)	22.44 ± 2.49	20.43 ± 2.22	19.68 ± 2.14	19.44 ± 2.42	19.47 ± 3.13	18.90 ± 3.42
	Exercise (n=4)	24.35 ± 3.17	25.40 ± 2.82	28.06 ± 4.35[a)]	24.96 ± 3.44	23.85 ± 3.79	23.63 ± 3.23

Each value is expressed as mean ± SE. a) P<0.05, when compared to pre-exercise.

completion of exercise (Table 1). An increase in heart rate is the most reliable index to evaluate exercise loads [6]; thus, sample collection was done following the aforementioned exercise procedures.

Blood samples (1.5 m*l*/dog) were collected by experienced veterinarians at each time point from the cephalic vein. Samples were collected prior to (pre), immediately after (0 hr) and 1, 3, 6 and 9 hr after each exercise load. The samples were collected into serum separator tubes (SST II; Becton, Dickinson & Co., Franklin Lakes, NJ, U.S.A.) and allowed to stand for 30 min at room temperature, and then centrifuged for 10 min at 425 ×*g*. Separated serum was collected and stored at −30°C until further analysis.

Serum cortisol levels were assayed using the sandwich ELISA and Parameter™ Cortisol Assay (R&D Systems, Minneapolis, MN, U.S.A.), according to the manufacturer's instructions. The samples were assayed in duplicate, and values were expressed as means.

We used a NGF ELISA kit (NGF Emax Immuno Assay System: Promega, Madison, WI, U.S.A.) for detecting canine NGF as reported previously [16]. All assays were performed in flat bottom 96-well plates (Nunc MaxiSorp®; Thermo Fisher Scientific, Inc., Tokyo, Japan). All measurements were performed in duplicate according to the manufacturer's protocol, and values were expressed as means.

The paired *t*-test was applied for comparisons between groups. Serum NGF and cortisol levels were normalized to pre-exercise values. Data were analyzed using IBM SPSS Statistics version 22.0. Following tests for normality, the one-way layout analysis of variance (ANOVA) Dunnett's test was used to detect differences between pre-exercise and post-exercise data. The level of significance was declared at P<0.05. The results were presented as means ± SE.

For the measurement of serum NGF and cortisol levels, the experiments were carried out with one-week interval on 4 dogs at the same time as follows; 1st-exercise experiment, non-exercise experiment (collection of blood at each time point corresponding to the exercise experiment without exercise) and 2nd-exercise experiment. Actual measurement data are shown in Table 2. Cortisol levels were elevated immediately after exercise. On the other hand, NGF levels were significantly increased 1 hr later. Significant differences between the exercised and non-exercised groups were identified in serum cortisol levels (t=−2.23, df=23, P<0.05) and in serum NGF levels (t=−3.42, df=23, P<0.05). To minimize the individual differences and evaluate the kinetics of serum cortisol and NGF, the value of each measurement was normalized to the corresponding pre-exercise value as the basal level. Increase in serum cortisol levels at 0 hr was obvious (2.6 times) in the exercise group, though those of the non-exercise group were unchanged (Fig. 1). However, increase in serum NGF levels was confirmed (1.2 times) at 1 hr post-exercise in the exercise group, though those of the non-exercise group were unchanged (Fig. 2).

Our findings of elevated serum cortisol levels post-exercise (Fig. 1) were consistent with those of previous studies that have demonstrated that physical exercise promotes the release of cortisol in dogs [5, 12, 24, 26]. Cortisol levels increased 1.2 times by the treadmill exercise (9 km/hr) for 60 min in German shepherd dogs [24]. In this study, cortisol levels increased 2.6 times by the exercise loads for 50 min. From these results, the exercise load applied in this study must become a certain stressor that induced release of cortisol for the dogs. Serum cortisol levels were significantly increased immediately after exercise and then returned to basal levels shortly thereafter (P<0.05). In contrast, serum cortisol levels were not significantly altered in the same dogs when they were not subjected to exercise. No significant differences in serum cortisol levels were noted between groups at 1 hr and thereafter.

In another study on aggressive behavior in mice, serum NGF levels showed a rapid increase after 1 hr and a sharp reduction 6 hr later [2]. In the present study, we obtained similar results (Fig. 2). Serum NGF levels were significantly increased within the first 1 hr and then returned to basal levels post-exercise (P<0.05). After reaching a peak, serum NGF levels were stabilized for the next 6 hr. On the other hand, when the same dogs were not subjected to any exer-

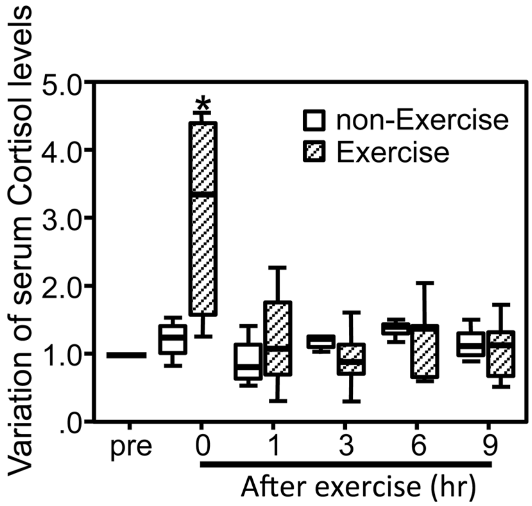

Fig. 1. Variation in serum cortisol levels. Each value was normalized to the pre-exercise value of 1. Data were shown using a box plot that is composed of the median (solid line in each column), upper hinge, lower hinge and whiskers representing upper adjacent value or lower adjacent value. Open boxes indicate cortisol variations of dogs in the non-exercise group (n=4), and striped boxes indicate cortisol variations of those with exercise (n=4). An ANOVA Dunnett's test was used to analyze differences between pre-exercise and post-exercise data. *: $P<0.05$ versus pre-exercise.

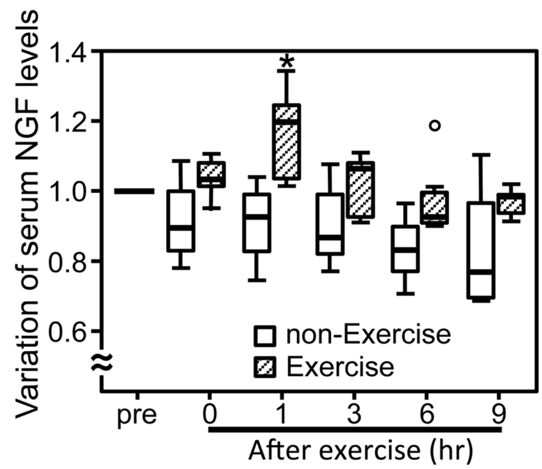

Fig. 2. Variation in serum NGF and cortisol levels. Each value was normalized to the pre-exercise value of 1. Data were shown using a box plot that is composed of the median (solid line in each column), upper hinge, lower hinge and whiskers representing upper adjacent value or lower adjacent value, and far out values (open dot at 6 hr of the exercise group). Open boxes indicate NGF variations of dogs in the non-exercise group (n=4), and striped boxes indicate NGF variations of those with exercise (n=4). An ANOVA Dunnett's test was used to test differences between pre-exercise and post-exercise data. *: $P<0.05$ versus pre-exercise.

cise, similar increases were not observed.

Human serum NGF levels have been reported to range from 50–200 *p*g/m*l* [15, 18]; those of rats, from 10–40 *n*g/m*l* [9, 28]; and those of mice, from 10–100 *n*g/m*l* [1, 21]. Serum NGF levels in goats, pigs and horses are reportedly higher than those in humans [25]. In addition, serum NGF levels in male mice are higher than those of female mice, particularly after the stress of fighting [2]. Basal serum NGF levels of dogs used in this study ranged from 15–35 *n*g/m*l*, which was approximately equal to those of rodents.

Here, we first described the increase in serum NGF levels in dogs following exercise. Since serum NGF levels become elevated after external stress [2, 3, 19], NGF has been proposed as one of the important markers that indicate the stress status of humans and animals. In addition, serum cortisol levels are regarded as an appropriate biomarker of stress [6, 22]; thus, we evaluated whether or not dogs subjected to exercise were also under stress, by evaluating the changes in their cortisol levels. Figure 1 shows that serum cortisol levels increased immediately after exercise, suggesting that the dogs were subjected to some form of exercise-induced stress. Since serum cortisol levels in humans and animals are immediately increased following the application of stressors [17, 23], the exercise load applied in the present study might have elicited a stress response in the dogs. On the other hand, serum NGF levels increased within the first hour (Fig. 2), exhibiting different dynamics from that of cortisol. Since NGF release is affected by the tonic activity of sympathetic neurons [10, 11], serum NGF levels in dogs might become elevated via sympathetic activation during exercise. Previous studies have focused on the association between adrenal gland activation and NGF upregulation. However, following the stress of territorial fighting, serum NGF levels were also elevated in male mice that had undergone adrenalectomy, suggesting that the increases in blood cortisol and NGF levels might be unrelated events [2]. In mice, NGF release predominantly depends on the salivary gland; however, the possible major source of serum NGF in dogs remains unexplored. In this study, we showed that serum NGF levels responded to free exercise loads with less emotional stress in dogs for the first time. Since serum NGF levels are affected by emotional stress including fear and anger [3, 4], to investigate whether serum NGF levels reflect emotional stress or not in dogs must be important for evaluating their mental status. Although further investigation is also necessary to define the interactions between cortisol and NGF in dogs, serum NGF levels could potentially become another stress biomarker for working dogs.

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