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.

KEYWORDS: 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
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 ml/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 centrifuged for 10 min at 425 × g. Separated serum was collected and stored at −30°C until further analysis. 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 instructions. The samples were assayed in duplicate, and values were expressed as means.

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 in dogs. Serum cortisol levels were significantly increased 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).

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) Dunnet’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 levels post-exercise. The level of significance was declared at P<0.05, when compared to pre-exercise.

| Items         | Group        | Pre-exercise | Hours after exercise |
|---------------|--------------|--------------|---------------------|
|               |              |              | 0       | 1       | 3       | 6       | 9       |
| Cortisol (ng/ml) | 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<sup>a</sup> | 9.04 ± 4.30 | 7.45 ± 2.81 | 9.54 ± 3.58 | 8.21 ± 2.94 |
| NGF (ng/ml)   | 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<sup>a</sup> | 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.

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<sup>a</sup> | 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
cise, similar increases were not observed.

Human serum NGF levels have been reported to range from 50–200 pg/ml [15, 18]; those of rats, from 10–40 ng/ml [9, 28]; and those of mice, from 10–100 ng/ml [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 ng/ml, 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 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 ng/ml, which was approximately equal to those of rodents.

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.

ous 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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