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Original Article

Evaluation of stress status using the stress map for guide dog candidates in the training stage using variations in the serum cortisol with nerve growth factor and magnesium ions

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ABSTRACT

Most studies on guide dogs for the blind were conducted to investigate the appropriateness of the animals, including in terms of their breeding, constitution, and temperament. However, research to comprehend the stress status of guide dog candidates in response to their training has been unclear.

In this study, the levels of serum cortisol, nerve growth factor (NGF), and magnesium ion (Mg^{2+}) levels of guide dog candidates during the three training stages–the elementary, intermediate, and advanced classes–were examined. Dogs were classified based on the contents of the classes and period during the training in which they were subjected.

Since the dogs in the elementary class had the lowest serum NGF and Mg^{2+} levels, they were understood to be under mental stress and to be unfamiliar with their new surroundings. In contrast, the serum NGF and Mg^{2+} levels were high in the dogs in the advanced class, though they were demonstrated to be mentally stable and acclimated to their environment. Additionally, they were almost free from the stress caused by daily life, since they had the lowest serum cortisol levels.

The status of each dog was plotted on a map consisting of 2 axes representing the serum NGF and Mg^{2+} levels with high or low cortisol levels. Plots could be divided into three domains corresponding to the elementary, intermediate, and advanced classes. Therefore, for working dogs, serum NGF and Mg^{2+} levels in addition to serum cortisol levels may be important factors to comprehend the type of stress situation that each dog was in.

Introduction

The first study on the use of guide dogs for the blind was conducted by J. F. Smithcors (Smithcors, 1958). Another study reported the need to socialize guide dogs and to manage their temperament (Price, 1961). Thereafter, 600 guide dog candidates were monitored from their birth to the end of their training for a detailed survey (Scott & Bielfelt, 1976). Following its publication, research began on the breeding program from a dog's birth, making it easier to eliminate unsuitable physical conditions, such as hip dysplasia. Additionally, since temperament issues were one of critical factors responsible for rejections of guide dog candidates, many researchers have focused on dog temperament (Serpell & Hsu, 2001). There are several articles on behavioral experiments conducted on the appropriateness of guide dogs (Batt, Batt, Baguley, & McGreevy, 2010; Kikkawa, Uchida, Suwa, & Taguchi, 2005; Mizukoshi, Kondo, & Nakamura, 2008). However, we found less research on the stress status of guide dogs during the training stages.

The serum cortisol level is known to be a common stress marker (Beerda, Schilder, van Hoff, de Vriesa, & Mola, 1998; Perego, Proverbio, & Spada, 2014). In dogs, several studies have demonstrated that physical exercise promoted the release of cortisol into the bloodstream (Angle *et al.*, 2009; Huntingford, Kirn, Cramer, Mann, & Wakshlag, 2014; Rathore, Moolchandani, Sareen, & Rajput, 2010; Royer *et al.*, 2005). Increased serum nerve growth factor (NGF) levels due to stress from strenuous exercise were reported in mice (Aloe, Alleva, Böhm, & Levi-Montalcini, 1986), humans (Aloe *et al.*, 1994; Dugué, Leppänen, Teppo, Fyhrquist, & Gräsbeck, 1993; Schedlowski *et al.*,

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1993), horses (Kawamoto *et al.*, 1996; Matsuda, Koyama, Oikawa, Yoshihara, & Kaneko, 1991), and dogs (Ando, Karasawa, Matsuda, & Tanaka, 2016). Moreover, serum NGF levels decreased in patients with depressive disorders (Wiener *et al.*, 2015; Xiong *et al.*, 2011) and in dogs with neurologic disorders (Kordass, Carlson, Stein, & Tipold, 2016). Therefore, emotional strain and anxiety may affect serum NGF levels. We have already reported that blood NGF regulated the immune systems by promoting the functions and survival of neutrophils, macrophages, lymphocytes, and mast cells via their specific receptors (Kannan *et al.*, 1991; Kawamoto *et al.*, 1995; Sawada, Itakura, Tanaka, Furusaka, & Matsuda, 2000; Susaki *et al.*, 1996), demonstrating important involvement of NGF in the maintenance of immune homeostasis.

Blood levels of magnesium ions (Mg^{2+}) are known to decrease during enzymatic reactions (Cowan, 2002), including protein synthesis, energy metabolism (He *et al.*, 2006; Pfeiffer & Barnes, 1981), muscles contractions (Altura & Altura, 1981), and blood pressure regulation (He, Yao, Savoia, & Touyz, 2005; Resnick *et al.*, 2000). Additionally, we have reported that serum Mg^{2+} levels in dogs decreased associating with external stress including stress caused by environmental changes, such as air temperature changes, and by nervousness and unaccustomed situations (Ando *et al.*, 2017). Therefore, serum Mg^{2+} levels might fluctuate not only due to enzyme reactions but also due to the strain resulting from environmental changes.

The aim of this study was to quantitatively evaluate stress levels in guide dog candidates by measuring the variations in serum cortisol, NGF, and Mg^{2+} levels, and to comprehend the physical and mental status in the training process.

Materials and methods

Animals

The animal experiments complied with the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology and those for the use of laboratory animals by the Science Council of Japan. The procedures that we performed were approved by the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology (No. 27–62; July, 27, 2015). Further, all procedures involved in the blood collection from the dogs were approved by the animal care committee of The Eye Mate Inc. (Tokyo, Japan).

Twenty Labrador retrievers (19–30 months, mean age: 22 ± 0.7 months) selected from the training school for guide dogs were subjected to tests to measure their serum cortisol, NGF, and Mg^{2+} levels. They were housed in individual cage in a room that was illuminated daily from 6:00–21:00. The temperature of the kennel was controlled to 15–25 ± 3°C to match the outside temperature, since the training was carried out within city areas. They were fed appropriate food once daily at 7:00 am and were provided water ad libitum. Further, they were neutered and belonged to The Eye Mate Inc., which has the longest history of training capable guide dogs in Japan and provides the largest number of well-trained guide dogs. They were fed the same food and raised under the same conditions.

Dogs used in the experiment

Twenty guide dog candidates were used to measure serum cortisol, NGF, and Mg^{2+} levels. The training phases of guide dog candidates consist of three classes based on the number of training hours and days. The elementary class includes dogs that have just begun training to walk with their instructor for 10–15 min. The intermediate class includes dogs that have just started to wear a harness and that walk for 20–30 min on an empty street by following simple commands from their instructor. The advanced class includes dogs in the final stage of training that wear harnesses and walk on busy streets for 40–50 min by following complex commands from their instructor. Six dogs from the elementary class (mean age: 19.8 ± 0.8 months; one neutered male; five neutered females), eight from the intermediate class (mean age: 22.0 ± 0.8 months; two neutered males; six neutered females), and six from the advanced class (mean age: 24.8 ± 1.1 months; one neutered male; five neutered females) were included in our study.

Blood sampling

Blood samples (1.5 mL/dog) were collected from the cephalic vein by experienced veterinarians during May, July, and August prior to (pre), immediately after (0 h), and 1 and 3 h following each training load. They were collected into serum-separator tubes (SST II; Becton, Dickinson & Co., Franklin Lakes, NJ, USA) and were allowed to stand for 30 min at room temperature, following which they were centrifuged for 10 min at 425 x *g*. The separated serum was collected and stored at -30°C until further analysis.

Measurement of serum cortisol, NGF, and Mg^{2+} levels

Serum cortisol levels were assayed in duplicate with a sandwich enzyme-linked immunosorbent assay (ELISA) using the Parameter Cortisol Assay kit (R&D Systems, Minneapolis, MN, USA) per the manufacturer's instructions, and the results were expressed as means. We used the NGF ELISA kit (NGF Emax Immuno Assay System: Promega, Madison, WI, USA) to detect the NGF levels, as reported previously (Kordass *et al.*, 2016). All the assays were performed in flat bottom 96-well plates (Nunc MaxiSorp®; Thermo Fisher Scientific, Inc., Tokyo, Japan). The measurements were performed in duplicate per the manufacturer's protocol, and the values were expressed as means. Serum Mg^{2+} levels were measured by the quantitative colorimetric determination method using the QuantiChrom Magnesium Assay Kit (DIMG-250; BioAssay Systems, Hayward, CA, USA) per the manufacturer's protocol. These assays were performed in flat-bottom 96 well plates (Nunc PolySorp®; Thermo Fisher Scientific, Inc., Tokyo, Japan). Absorbance values were measured at 500 nm using a micro plate reader (ImmunoMini (NJ-2300); BioTec, Suffolk, UK). Serum Mg^{2+} values were expressed as the mean of triplicate measurements.

Statistical analysis

Data were analyzed using IBM SPSS Statistics ver. 24.0. A one-way analysis of variance (ANOVA) Dunnett's test was used to confirm differences between pre-training and post-training data. P values of $<$ 0.05 were considered statistically significant. The comparisons among the three groups were analyzed via multiple comparisons with Bonferroni correction ($P < 0.05/3$ were estimated as levels of significance) when Levene's test was not statistically significant and via multiple comparisons with the Kruskal Wallis test when Levene's test was statistically significant. The results are reported as means ± standard error.

Results

The serum cortisol levels of guide dog trainees during the different training phases were presented in Fig. 1. In the three groups, the serum cortisol levels increased markedly, with statistical significance, immediately following the training load and returned to basic levels within a short period of time ($P < 0.05$ vs. pre.). The levels following the trainings involved in the elementary, intermediate, and advanced classes were 26.8 ± 9.6, 40.2 ± 10.0, and 19.3 ± 5.2 mg/dL, respectively, and were almost double or more than double the values measured while the dogs were at a steady state (S-state) before the training. Further, these levels changed along with the phase of the training stage. During the intermediate class, when the full-scale training just begins, the levels were high, and during the advanced class, when the dogs

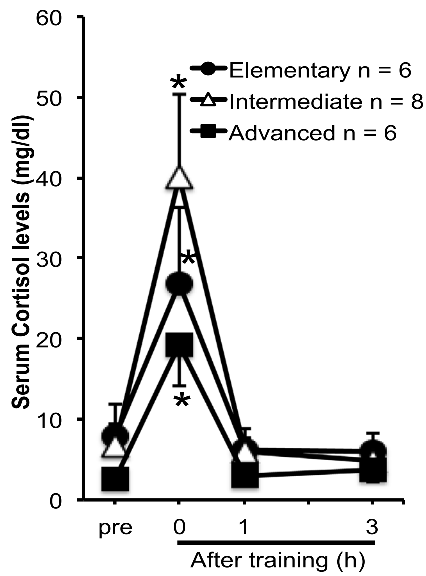

Fig. 1. Time course of the variations in the serum cortisol levels of the guide dog trainees during different training phases
Each point represents the means ± SE associated with the dogs in the elementary, intermediate, and advanced classes. A one-way layout analysis of variance (ANOVA) Dunnett's test was used to test the differences between the pre-exercise and post-exercise data. * $P < 0.05$ vs. pre-exercise. The statistical significance of the differences among the three groups was tested using the Kruskal-Wallis test. ($P < 0.05$).

almost finish their training, they were low. During the elementary class, when the dogs just start their preliminary training, the levels were almost equal to those in the intermediate class and were located between the domains of the intermediate and advanced classes on the map.

Fig. 2 illustrates that the variations in the serum NGF levels during the different training phases were quite different from those in the serum cortisol levels in Fig. 1. The variations in the serum NGF levels owing to the training load were not statistically significant, but they

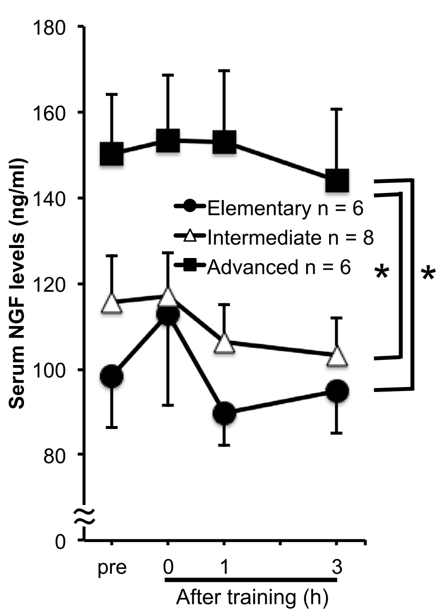

Fig. 2. Time course of the variations in the serum NGF levels of the guide dog trainees during different training phases
Each point represents the means ± SE associated with the dogs in the elementary, intermediate, and advanced classes. The statistical significance of the differences among the three groups was tested using Bonferroni correction. * $P < 0.05/3$.

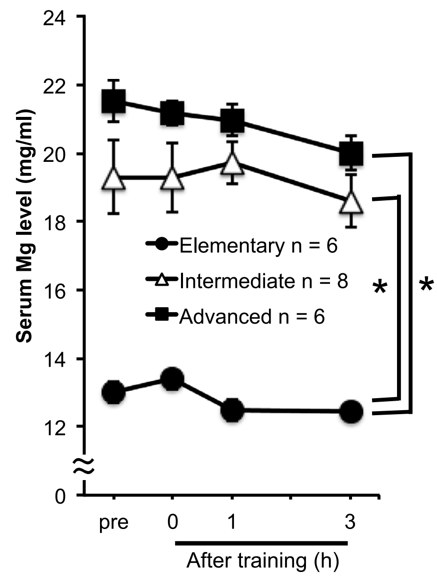

Fig. 3. Time course of the variations in the serum Mg^{2+} levels of the guide dog trainees during different training phases
Each point represents the means ± SE associated with the dogs in the elementary, intermediate, and advanced classes. The statistical significance of the differences among the three groups was tested using Bonferroni correction. * $P < 0.05/3$.

were statistically significant when compared among the three groups in the elementary, intermediate, and advanced classes ($P < 0.05 / 3$). The S-state level of serum NGF during the advanced class was significantly high at 150.2 ± 7.3 ng/mL, and that in the elementary class was low at 99.1 ± 6.7 ng/mL. The intermediate class NGF levels were located near the elementary class domain between the domains of the elementary and advanced classes on the map.

Fig.3 shows the serum Mg^{2+} levels measured during the different training phases. Similar to the variation in the serum NGF levels, those in the serum Mg^{2+} levels were statistically significant when comparing among the three groups in the elementary, intermediate, and advanced classes ($P < 0.05 / 3$). The S-state serum Mg^{2+} levels during the elementary class were the lowest at 13.0 ± 0.3 μg/mL, whereas the S-state serum Mg^{2+} levels during the advanced class were the highest at 21.5 ± 0.6 μg/mL. The serum Mg^{2+} levels measured during each of the classes were almost constant between the S-state levels and those measured 3 h after the training. Unlike in the case of serum NGF levels, the intermediate class serum Mg^{2+} levels were located near the advanced class domain on the map.

Discussion

Cortisol is one of the representative stress markers, and an increase in cortisol levels in response to exercise has been reported in dogs (Angle *et al.*, 2009; Huntingford *et al.*, 2014; Rathore *et al.*, 2010; Royer *et al.*, 2005). As shown in Fig. 1, serum cortisol levels elevated with increases in the training load and returned to S-state levels within a few hours, indicating that the training load gave stress to the dogs. The exercises in both elementary and intermediate classes caused each dog a great deal of stress. Contrarily, the advanced class exercise did not stress the dogs very much. Similar to a report outlining that repeated stress stimuli reduced the blood cortisol levels in humans (Yanagisawa, Yukimura, & Yamada, 1986), serum cortisol levels during the elementary and intermediate classes were high, and serum cortisol levels during the advanced class were low. The result is also consistent with observation of Rooney, Clark, and Casey (2016) that reported reduction of fear and anxiety due to habituation in working dogs. Blood cortisol levels were reported to increase when a pig was restrained by a harness (Marchant-Forde *et al.*, 2012). Since the highest cortisol levels were observed in the dogs in the intermediate class immediately after they wore harnesses, putting on a harness could be a restraint-related stress load in response to which the serum cortisol levels might have increased. The stress levels caused by training loads may depend not only on the degree of difficulty associated with the exercises given to the dogs but also on the ability of the dogs to adapt to the training. Serum cortisol levels have been shown to be sensitive to the stress caused by the training given to dogs.

However, the serum NGF and Mg^{2+} levels were hardly affected with or without training, as shown in Figs. 2 and 3. Apart from during the elementary class, immediately following the trainings, the serum NGF levels were almost the same as the S-state levels. The changes in serum Mg^{2+} levels in response to given training loads almost reflected the behavior of the serum NGF levels. The most characteristic observations with regard to the serum NGF and Mg^{2+} levels during the three classes were that they were the lowest during the elementary class and the highest during the advanced class. This implies that these levels increased as the training stages progressed from the elementary to advanced classes.

NGF has been reported to show various effects on the nervous, immune, and endocrine systems, and blood NGF is affected by both physiological and biological conditions (Alleva & Santucci, 2001; Aloe *et al.*, 1994). In dogs in an elementary class, serum NGF levels tended to increase just after the exercise load and returned to the preliminary level after 1 h. The change suggests that NGF levels might be quickly affected by stress load in less adopted dogs with anxiety. Serum NGF levels have been reported to increase owing to stress resulting from strenuous exercise (Aloe *et al.*, 1986; Aloe *et al.*, 1994; Ando *et al.*, 2016; Dugué *et al.*, 1993; Kawamoto *et al.*, 1996; Matsuda *et al.*, 1991; Schedlowski *et al.*, 1993). Contrarily, NGF levels have been reported to be low in dogs with neurologic disorders (Kordass *et al.*, 2016) and in patients with depression (Wiener *et al.*, 2015). Furthermore, because NGF has been reported to act as an antidepressant, reduced serum NGF levels may lead to depression (McGeary, Gurel, Knopik, Spaulding, & McMichael, 2011; Shi *et al.*, 2010). Therefore, blood NGF levels might reflect the state of mental or emotional strain and anxiety. Since these levels were the highest during the advanced class and the lowest during the elementary class, it was conjectured that the dogs in the advanced class were mentally stable and that those in the elementary class were the most unstable. Blood NGF is involved in maintaining functions of immune systems (Kannan *et al.*, 1991; Kawamoto *et al.*, 1995; Sawada *et al.*, 2000; Susaki *et al.*, 1996). The dogs in the advanced class showed high serum NGF levels, indicating that their physical status might be suitably managed.

Since Mg^{2+} is necessary for internal biological reaction to protect individuals from environmental, physical, and mental stress (Golf, Bender, & Gruttner, 1998; Soldatovic, Matovic, Vujanovic, & Stojanovic, 1998; Zieba, Tata, Dudek, Schlegel-zawadzka, & Nowak, 2000), Mg^{2+} might be applied to biologic reaction of cells and tissues in less adopted dogs. Recently, we have reported that serum Mg^{2+} levels decreased due to stress caused by environmental changes, nervousness owing to situations the animals are unaccustomed to, aging, and physical exercise (Ando *et al.*, 2017). The dogs in the intermediate and advanced classes exhibited high serum Mg^{2+} levels, suggesting their adaptation to the environment and had their training life. In contrast, the dogs in the elementary class showed the lowest serum Mg^{2+} levels, because of their stress caused by environmental changes or the levels of tension and impulsive movements cause by anxiety.

From the observations on the serum cortisol, NGF, and Mg^{2+} levels of the dogs in all the three classes, the serum cortisol levels were demonstrated to be sensitive to the stress caused by the given training load, and the serum NGF and Mg^{2+} levels might be a reflection of the stress owing to mental instability, emotional strain, and environmental changes experienced during daily life. Finally, in order to understand the relationship between stress due to environmental changes and stress caused by mental instability or emotional strain, the serum NGF and Mg^{2+} levels of all dogs were plotted in Fig. 4. Although we used the S-state values to measure the serum NGF and Mg^{2+} levels, similar trends were obtained using the values measured after the training ended (3 h).

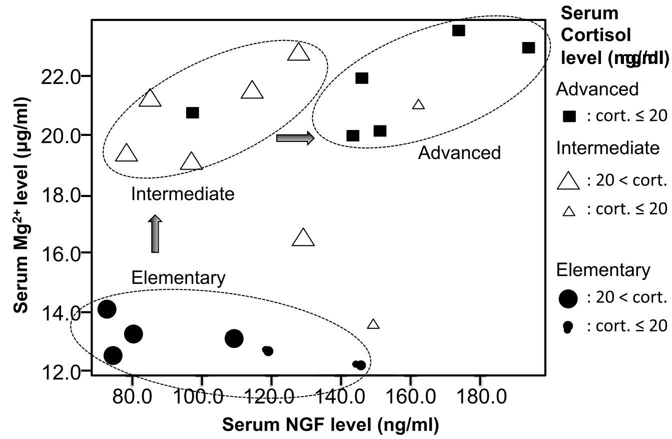

Fig. 4. The state map of each guide dog candidate during the different training phases
Each point represents the serum cortisol, NGF, and Mg^{2+} levels. The serum cortisol levels are divided into two using medians. The serum Mg^{2+} levels are presented on the vertical axis, and the serum NGF levels are presented on the horizontal axis.

It is evident from the map that the dogs in the elementary class were located in the domain in which both the serum NGF and Mg^{2+} levels were low, thereby implying that they were under stress resulting from mental instability and also from being in unfamiliar surroundings. Additionally, the dogs with higher serum cortisol levels appeared to experience stress even in the S-state. In contrast, the dogs in the advanced class were distributed on the map in the domain in which both the serum NGF and Mg^{2+} levels were high, thereby implying that they were mentally stable and familiar with the environment of everyday life; however, the serum cortisol levels of each dog in this class were lower than those of dogs in the other classes, suggesting that the dogs in this class did not feel stress in the S-state compared to the other dogs. Most of the dogs in the intermediate class were distributed in the domain with low serum NGF levels and high serum Mg^{2+} levels. The dogs in the elementary class displayed both low serum NGF and Mg^{2+} levels at the same time while dogs in the intermediate class displayed only increased serum Mg^{2+} levels as the training stages progressed. In other words, the changes that occurred in the dogs that were upgraded from the elementary to the intermediate class took place so that the dogs could adapt to the environmental changes. It is likely that the mental stability levels of the dogs in the intermediate class reach those of the dogs in the advanced class after they get used to the environmental changes. The variations in the serum NGF and Mg^{2+} levels may represent a visualization of the growth process that takes place during the training stages that involves the dogs in the elementary class progressing to the advanced class. Particularly, the fact that several dogs in the intermediate class either belonged to the advanced or elementary classes may be the reason for the differences observed with respect to individual dogs during the training phase.

Conclusion

In this study, the stress status in the training process that candidates of the guide dogs for the blind undergo was investigated by measuring the variations in the serum cortisol, NGF, and Mg^{2+} levels. Data of all the dogs were mapped on a plot consisting of two axes representing the serum NGF and Mg^{2+} levels with high or low cortisol levels. In the map, domains were divided in three groups corresponding to the elementary (high cortisol, low NGF, low Mg^{2+}), intermediate (high cortisol, low NGF, high Mg^{2+}), and advanced (low cortisol, high NGF, high Mg^{2+}) classes. Therefore, it was possible to identify that serum NGF and Mg^{2+} levels along with serum cortisol levels are important factors to comprehend the stress status of a dog.

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Conflict of Interest Statement

None.

Contributions

None.

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