Original Article
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Evaluation of effects of olfactory and auditory stimulation on separation anxiety by salivary cortisol measurement in dogs

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Separation anxiety (SA) is a serious behavioral problem in dogs. In this study, salivary cortisol was studied to determine if the owner's odor or voice could reduce SA in dogs. Twenty-eight dogs with SA were divided into three groups: group 1 (control), group 2 (with owner's clothes during the separation period; SP) and group 3 (a recording of the owner's voice was played during SP). The dog's saliva was collected after the owner and their dog were in the experimental room for 5 min (PRE). The dog was then separated from the owner for 20 min and saliva collected four times at intervals of 5 min (SP1−4). Finally, the owner was allowed back into the room to calm the dog for 5 min, after which saliva was collected (POST). Evaluation of salivary cortisol concentrations by ELISA revealed that the ratios of SP1 concentration to PRE or POST concentrations were significantly higher in group 1 than in group 2 or 3. Additionally, the concentrations of SP1−PRE and SP1−POST among groups differed significantly. These findings indicate that the owner's odor or voice may be helpful to managing stress in dogs with SA.

Keywords: cortisol, dog, physiology, saliva, separation anxiety disorder

Introduction

Separation anxiety (SA) is defined as any problematic behavior or group of behaviors that occurs exclusively in companion dogs in the owner's physical or virtual absence [18]. Problematic behaviors that occur during the owner's absence are common and make up a significant proportion of the caseloads of behavioral specialists [1]. SA disorder is distressing for the owner as well as the dogs [17,20]. Dogs with SA exhibit their stress via undesirable behaviors, such as excessive vocalization, urination or defecation in inappropriate places, self-harming behavior and/or general destruction [18].

SA accounts for approximately 10 to 20% of the cases referred to dog behaviorists [17], but in older dogs, this proportion may rise to 50% [10]. From 1991 to 2001, 1644 dogs were taken to the behavior clinic at Cornell University, 14.4% of which were diagnosed with SA [2]. In addition, SA is the third most common problem at referral practices in three countries (Canada, USA, and Australia) [10]. These problematic behaviors have the potential to lead owners to abandon or abuse their dogs. To prevent such tragic situations, appropriate diagnostic methods and practical solutions must be developed.

Many studies have been conducted in an attempt to better diagnose and treat this problem [1,12,19,21,22]. The first step is to evaluate the extent to which the dogs are stressed due to the absence of their owners and confirm whether various practical solutions can reduce stress.

Recently, there have been many discussions regarding the methods used to evaluate acute or chronic stress in dogs, and non-invasive methods of measuring stress have been received increased attention because they increase animal welfare while providing more reliable results [3,4]. In particular, use of salivary cortisol to evaluate stress has attracted much attention and is considered very useful [7,11,13,14] because it is relatively easy to take saliva samples and use them to measure stress levels while inducing a minimum amount of physiological changes in the subjects [11,16]. Salivary cortisol is a better measure of adrenal cortical function and a better physiological indicator of stress than plasma cortisol because it is a direct reflection of the biologically active portion of the total cortisol level [9]. Additionally, the concentrations found in saliva were between 5% and 10% of those in found the plasma [23].

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Therefore, although it is a very easy and non-invasive method, salivary cortisol can be a good indicator of acute stress response because it responds very quickly, even when no physiological changes occur in the blood [5,8,14].

Although there are many successful management strategies for SA, some dogs require long-term treatment [15], and in most cases, drug therapy will be needed to address the dogs' intense anxiety [17]. As a result, some owners may abandon their dogs rather than seeking treatment.

Therefore, this experiment was conducted to reduce stress in dogs in the absence of their owners by using the owners' odors or voices. To accomplish this, stress levels were assessed by measuring salivary cortisol levels before, during, and after the dogs were separated from their owners in an unfamiliar environment. The results of this study may determine whether olfactory or auditory stimulation originating from a dog's owner can relieve the stress induced by SA in the dogs.

Materials and Methods

Subjects

A total of 28 healthy dogs (two intact males, eight neutered males, 15 intact females, and three neutered females) were included in this study. All dogs were housed indoors and privately owned. The mean weight was 4.74 ± 0.41 kg, and the mean age was 4.47 ± 0.56 years, ranging from 1 to 12 years of age. The mean length of time since adoption was 3.63 ± 0.51 years. Five Maltese, five toy Poodles, nine Pomeranians, three Spitzs, three Shih Tzus, one American Cocker Spaniel, one Coton de Tulear, and one mixed breed were used. Nine dogs were adopted from other private owners at under 6 months old, 15 dogs were adopted from a pet shop within 6 months of age, three dogs were readopted in adulthood after being abandoned or from shelters, and one dog was bought from a certified kennel club at under 6 months of age. All dogs were selected on the basis of the results of a simple SA questionnaire [18]: How does your dog behave when you are absent? If they exhibited at least one behavior such as excessive vocalization, destructive behavior toward themselves or the environment, and/or house soiling, the dogs were assumed to have SA. The experiment was immediately stopped and the dog was excluded from experimental analysis if the subject showed immoderate excitement or aggressiveness. This study was approved by the Institutional Animal Care and Use Committees in Seoul National University.

Testing facility

The experiment was conducted in an empty room (3.5 m × 3.7 m) at Seoul National University. In the room, one chair for the owner was situated at 0.9 m from the door, and a blanket (0.45 m × 1.45 m) was placed by the chair. A camera was installed at a position by the door (110 IS; Canon, Japan) and all experiments were recorded. The floor of the room was of a non-slip texture.

Preparing for test

The subjects were divided into three groups according to weight, age and breed. The characteristics of each group are presented in Table 1. Group 1 (n = 10) was a control group to assess salivary cortisol levels of dogs separated from the owners without any treatment. Group 2 (n = 9) was an 'olfactory group' to determine the effects of the owner's odor on SA. For this test, T-shirts were sent to the owners at least one week before the appointed test day and the owners were asked to wear the

Table 1. Information regarding dogs in the three groups. There were no significant differences among groups (Kruskal-Wallis test)

	Group 1	Group 2	Group 3
N	10	9	9
Weight (kg)	4.46 ± 0.85[a]	4.69 ± 0.53[a]	5.09 ± 0.77[a]
Age (yr)	3.85 ± 0.97[b]	5.67 ± 0.87[b]	3.96 ± 1.06[b]
Duration of ownership (yr)	2.50 ± 0.39[c]	5.00 ± 0.96[c]	3.56 ± 1.12[c]
Breeds	Maltese (2), toy Poodle (2), Pomeranian (3), Spitz (2), Shih Tzu (1)	Maltese (2), toy Poodle (1), Pomeranian (2), Spitz (1), Shih Tzu (1), Coton de Tulear (1), mixed breed (1)	Maltese (1), toy Poodle (2), Pomeranian(4), Shih Tzu (1), American Cocker Spaniel (1)
Sex			
Male			
Intact/neutered	0/5	1/2	1/1
Female			
Intact/neutered	5/0	5/1	5/2

Figures in columns marked with the same letters are not significantly different ($p > 0.05$).

t-shirts without cleaning or washing them until the day of the test, then bring the shirt on test day. During the experiment, the t-shirt was placed on the blanket during the separation period. Group 3 (n = 9) was the 'auditory group,' which was evaluated to confirm the effects of the owner's voice on a dog's SA. Prior to test day, the owners were asked to read a story approximately 10 min long to their dogs before going to bed every night for at least one week. In addition, they were instructed to record their voice while reading the story at least once and send it to the experimenter. Voice recorded files were edited to be of uniform length and volume. At the time of testing, the recorded voice was played twice during the separation period.

Experimental procedures and data collection

The day before test day, the experimental room was cleaned to remove any possible stray odors. Before the test, the owner was asked to walk with the dog slowly for 10 min to allow the dog to urinate or defecate and relax. To prevent dilution of the saliva, the dog was denied food and water from 2 h before the test until the test ended. The total time of the experiment was 30 min. The experimenter was in the testing room throughout the experimental period to monitor the subject's condition and state. To rule out the response of the dog to the experimenter, the experimenter did not engage in eye contact or physical movement during the experimental period. First, the owner and the dog were introduced into the testing room and given time to adapt to their new surroundings. The owner sat in the chair, while the dog was allowed to move freely around the room. Five minutes later, the owner left the room after the first saliva sample was collected (pre-separation period; PRE). During the 20 min separation period (SP), saliva samples were collected four times at intervals of 5 min (SP1–4). During SP, the dog moved freely around the room and was not allowed contact with the experimenter, except during collection of saliva. After 20 min SP, the owner reentered the room and was given 5 min to calm the dog. The last saliva sample was collected after this 5 min relaxation period (post-separation period; POST).

Saliva was collected by keeping a Salivabio infant swab (Salimetrics, USA) in the dog's mouth for 1 min, after which the swab was stored in a swab storage tube (Salimetrics) and refrigerated. Within one hour, the refrigerated saliva tubes were centrifuged at 2,952 × g for 15 min, then stored at −70°C. Frozen saliva samples were thawed at room temperature for 10 min, then centrifuged at 2,952 × g for 15 min. The absorbance of each sample was measured using an expanded range high sensitivity salivary cortisol EIA kit (Salimetrics), and the salivary cortisol concentrations were calculated as μg/dL.

Statistical analysis

The calculated salivary cortisol levels in the samples were analyzed using the SPSS software (ver. 21.0; SPSS, USA). The alpha value was set at 0.05 in all cases, and all analyses involved two-tailed tests. Differences between two groups at same period were analyzed by the Mann-Whitney *U* test, and three groups were analyzed by the Kruskal-Wallis test. The concentrations from different periods within the group were analyzed by the Wilcoxon signed-ranked test, and concentrations from all periods were analyzed by the Friedman test.

Results

Subjects analysis

There were no significant differences among groups in weight, age, or time since adoption (Table 1).

Cortisol concentration and statistical results

In group 1, the salivary cortisol concentration at PRE was 0.59 ± 0.13 μg/dL, while that at POST was 0.35 ± 0.09 μg/dL, which was significantly different ($p < 0.01$). The SP1 cortisol concentration was 0.84 ± 0.16 μg/dL, which was significantly higher than that of PRE ($p < 0.01$) or POST ($p < 0.01$). During SP, concentrations decreased by 0.64 ± 0.17 μg/dL, 0.52 ± 0.12 μg/dL and 0.42 ± 0.09 μg/dL, respectively. Changes along periods were significantly different ($p = 0.000$). The cortisol at SP1 was 1.68 ± 0.27 times greater than that at PRE and 2.99 ± 0.50 times greater than at POST. Furthermore, the cortisol level at SP1 increased to 0.25 ± 0.06 μg/dL, which was higher than that at PRE and 0.49 ± 0.11 μg/dL more than that at POST.

In group 2, the PRE cortisol concentration was 0.47 ± 0.09 μg/dL, while that at POST was significantly lower, at 0.29 ± 0.06 μg/dL ($p < 0.05$). The SP1 concentration was 0.52 ± 0.09 μg/dL, which represented a non-significant increase from the PRE concentration ($p > 0.05$), but was significantly higher than the concentration at POST ($p < 0.05$). During SP, concentrations were 0.52 ± 0.11 μg/dL, 0.47 ± 0.10 μg/dL and 0.41 ± 0.11 μg/dL, respectively. Changes along the periods were significantly different ($p < 0.005$). The cortisol level at SP1 was 1.17 ± 0.11 times greater than that at PRE and 2.06 ± 0.41 times greater than at that at POST. Furthermore, the cortisol level at SP1 increased by 0.05 ± 0.03 μg/dL relative to that at PRE and 0.23 ± 0.04 μg/dL relative to that at POST.

In group 3, the PRE cortisol concentration was 0.56 ± 0.11 μg/dL, which was significantly higher than the value of 0.37 ± 0.09 μg/dL ($p < 0.05$) observed at POST. The concentration at SP1 was 0.57 ± 0.12 μg/dL, which was significantly higher than that at POST ($p < 0.01$), but not significantly different than that at PRE ($p > 0.05$). During SP, concentrations were 0.52 ± 0.41 μg/dL, 0.50 ± 0.13 μg/dL, and 0.50 ± 0.14 μg/dL. Hormonal changes along periods were significantly different ($p < 0.005$). The cortisol level at SP1 was 1.10 ± 0.18 times greater than that at PRE and 1.62 ± 0.14 times greater than that at POST. Furthermore, the cortisol level at SP1 was 0.01 ± 0.09 μg/dL higher than that at PRE and 0.19 ± 0.06 μg/dL higher than that at POST.

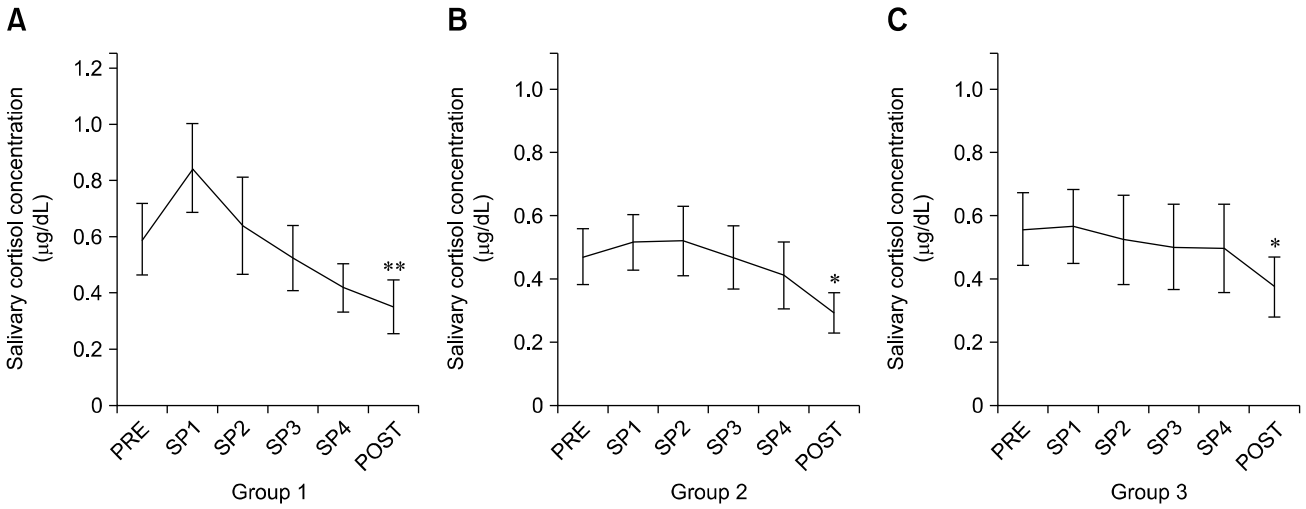

Fig. 1. Hormonal results of the three groups (mean ± SE). (A) Variation in salivary cortisol level in group 1. Changes along periods were significantly different ($p = 0.000$). (B) Variation in salivary cortisol level in group 2. Changes along the periods were significantly different ($p < 0.005$). (C) Variation in salivary cortisol level in group 3. Hormonal changes along periods were significantly different ($p < 0.005$). There were no significant differences among groups at corresponding sampling times ($p > 0.05$). *$p < 0.05$, **$p < 0.01$ in comparison of pre-separation period (PRE) and post-separation period (POST). SP, separation period.

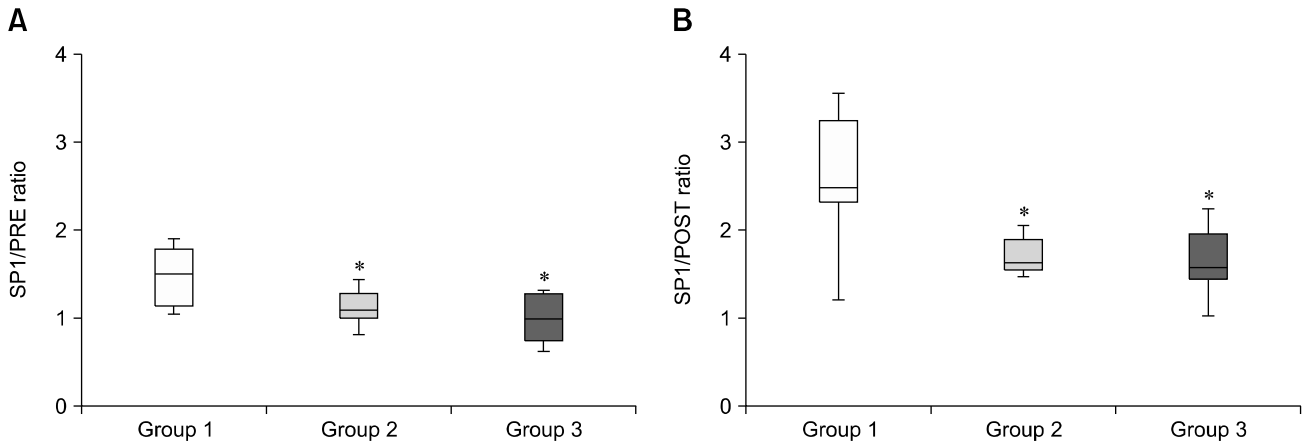

Fig. 2. Cortisol concentration ratio of SP1 at different time points (mean ± SE). (A) At SP1 to that at PRE. (B) At SP1 to that at POST. *$p < 0.05$ in comparison with group 1.

Overall variations in each group with time are shown in Fig. 1. There were no significant differences among groups at corresponding sampling times ($p > 0.05$). However, the ratio of the concentration at SP1 to that at PRE (SP1/PRE) was significantly different among groups ($p < 0.05$) (panel A in Fig. 2). Comparison of group 1 and 2 revealed significant differences ($p < 0.05$). In addition, there were significant differences between group 1 and 3 ($p < 0.05$). Likewise, the ratio of the concentration of SP1 to that of POST (SP1/POST) differed significantly among groups ($p < 0.01$) (panel B in Fig. 2). Specifically, there were significant differences between group 1 and 2 ($p < 0.05$) and between group 1 and 3 ($p < 0.05$).

In addition, when comparing the differences in concentrations between PRE and SP1 among groups (SP1−PRE), these levels were significantly different ($p < 0.05$) (panel A in Fig. 3). In the same manner, the differences in concentrations between POST and SP1 among groups (SP1−POST), there were significant differences ($p < 0.05$) (panel B in Fig. 3).

Discussion

This study was conducted to evaluate the effects of olfactory or auditory stimulation by owners on SA in dogs based on salivary cortisol concentrations. Measurement of the salivary cortisol concentration is an effective method of assessing stress levels in dogs that is also non-invasive and induces minimum

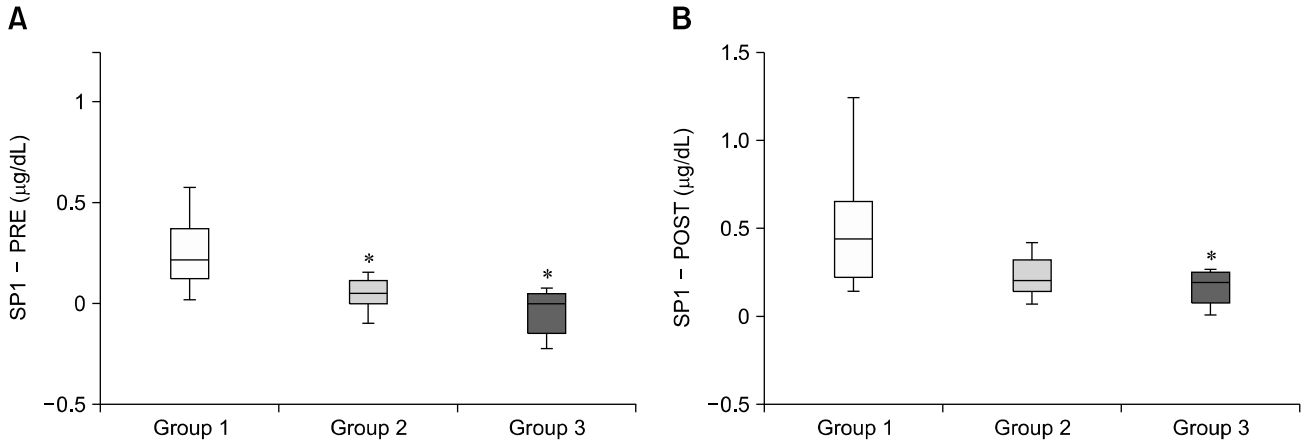

Fig. 3. The differences in cortisol concentration between time points (mean ± SE). (A) Between PRE and SP1. (B) Between SP1 and POST. *$p < 0.05$ in comparison with group 1.

physiological changes in the animals [3,6,13].

It should be noted that the sample size was very small and included a variety of individual histories, as well as different breeds, sexes, and ages. The dogs also displayed various degrees of SA. Therefore, the results of the examination may not be representative of all dogs with SA. In addition, deviation from the average cortisol level was very large in each dog, indicating that some of the results may not be meaningful or representative of a larger group. Nevertheless, the results revealed clear differences in salivary cortisol concentrations among groups.

The concentrations of PRE were similar in all groups; however, stress levels increased more rapidly in group 1 than in the other groups. During SP, the concentrations in group 2 and 3 were lower than in group 1, although these differences were not statistically significant. Therefore, the ratio of SP1 to PRE (SP1/PRE) or POST (SP1/POST), and the differences in concentration between SP1 and PRE (SP1−PRE) or POST (SP1 −POST) were estimated to identify relative increases in stress levels because of the owner's absence.

The increase in the ratio of SP1 to PRE in groups 2 and 3 was significantly lower than that in group 1. Stress levels increased immediately after the owners left, while during SP these levels decreased to various degrees in all groups. POST levels were the lowest in all the groups, although some of the dogs were extremely excited by their owner's return.

These results suggest that the PRE cortisol concentration can be used to indicate increased stress due to the unfamiliar surroundings, and that POST cortisol concentration may be assumed as a baseline cortisol level because the owners calmed the dogs gently and almost all dogs rapidly relaxed with 5 min. The increase in concentration at SP1 over that at POST (assumed baseline) was statistically different among groups, and the ratio in groups 2 and 3 was lower than that in group 1. In addition, the differences in concentration between PRE and SP1 or POST and SP1 were significantly different among groups.

These results indicated that stress induced by the owner's departure could be reduced physiologically by allowing the dog to sniff the owner's odor or hear the owner's recorded voice. Accordingly, this method may be useful to owners when applied along with practical training and drug therapy as a way of treating dogs with SA. This method is easy to implement and allows for more efficient management of SA when combined with other techniques. Furthermore, it is worthwhile to improve the dogs' welfare; they may otherwise live the majority of their time at home alone with their anxiety.

In conclusion, companion dogs with SA become stressed in the absence of the owners and exhibited various problematic behaviors including excessive barking, destruction, and improper urination and defecation. This study demonstrated that the owner's odor or voice could reduce separation related stress significantly at the physiological level. Overall, the methods employed herein could be a useful and practical management solution for dogs and owners struggling with SA, especially if applied in combination with other training techniques or drug therapies.

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Conflict of Interest

There is no conflict of interest.

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