Domestic Animal Endocrinology, Volume 46, Pages 20-25, (2014)

Saliva collection by using filter paper for measuring cortisol levels in dogs

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Abstract

Four experiments were conducted to evaluate the accuracy and reliability of noninvasive evaluation of cortisol in saliva of dogs. In experiment 1, we measured the cortisol concentration in the filter paper on which 250-μL cortisol solutions had been quantitatively pipetted and in filter papers dipped in cortisol solution. In experiment 2, we collected the blood and saliva of dogs 3 times at 30-min intervals and compared the cortisol concentrations to examine whether the dynamics of cortisol in the blood and saliva are similar. The results of experiments 1 and 2 showed that the cortisol concentration can be quantitatively measured with this method and that the dynamics of cortisol concentration in the plasma and saliva collected by using filter paper are not different ($P = 0.14$ for experiment 1 and $P = 0.51$ for experiment 2). In experiment 3, to investigate the factors related to inducing stress in dogs by using the filter-paper method of collecting saliva, we compared the cortisol concentrations at 0 and 30 min after collecting the saliva of pet dogs. The dog owners completed a survey on their dogs, providing basic information and reporting the collection of their dog's saliva. We found that the cortisol concentrations increased significantly in dogs whose owners spent >2 min collecting saliva ($P = 0.005$), suggesting that prompt collection of saliva is necessary for accurate assessment of cortisol without induction of a stress response. In addition, the cortisol concentrations increased significantly in dogs whose teeth were not regularly brushed ($P = 0.04$), suggesting that regular teeth brushing mitigates the effect of the collection process on cortisol concentrations in the saliva, with minimal stress to the dogs. In experiment 4, we measured cortisol concentrations in pet dogs accustomed to having their teeth brushed by their owners, before and after interaction with their owners, to assess whether brushing induces stress in dogs. We detected that the cortisol concentrations significantly decreased after human–dog interaction ($P = 0.008$), suggesting that this method does not induce stress in dogs. Our study indicates that the method of saliva collection by using filter paper is effective in measuring the cortisol concentrations to evaluate stress, although certain steps are required to enhance accuracy.

Keywords: Filter paper; Saliva cortisol; Stress; Human–dog interaction; Teeth brushing

1. Introduction

Recently, studies have focused on the welfare of domestic, companion, and experimental animals [1]. Dogs are used not only as companions but also as working animals (eg, guide dogs, police dogs, or laboratory animals for research). Dogs are also used in medical or educational facilities, in animal-assisted activities, animal-assisted education, or animal-assisted therapy. It has been recognized that stress deteriorates performance of working and research dogs [2], as well as their welfare [3], [4], [5].

Periodic evaluation of stress in dogs is important to monitor their welfare, and a simple, accurate method to evaluate the stress experienced by these dogs is necessary for this evaluation. Cortisol is recognized as a major stress marker [6], [7]. Blood is collected to measure plasma cortisol, but the collection of blood itself can be a stressor [8], [9], [10]. Therefore, measuring cortisol in saliva is often used as a noninvasive method [3]. However, cotton-based applicators, which are generally used for saliva collection, are problematic for accurately measuring cortisol concentration, because the ingredients in cotton interfere with measuring the saliva components [11]. An alternative to using cotton swabs is to use filter paper; filter paper absorbs saliva sufficiently, and no ingredients in the filter paper have been shown to interfere with cortisol measurement. In this study, we evaluated the accuracy and reliability of methods of collecting saliva from a dog's mouth by using filter paper to monitor the cortisol level.

2. Materials and methods

2.1. Ethical note

All procedures were conducted according to ethical guidelines of Tokyo University of Agriculture and Technology, and animal use was approved by the University Animal Care and Use Committee.

2.2. Experiment 1

To evaluate the accuracy of our method, we performed cortisol measurements by using a fluid with known cortisol concentration.

2.2.1. Subjects

We used filter papers with a diameter of 55 mm (Ashless Quantitative Filter Paper Grade No. 4A; Advantec, Tokyo, Japan) for the experiments and uniformly pipetted 250 μL of cortisol in various concentrations (1, 5, 10, 50, and 100 ng/mL) onto the center of the filter paper (pipette group) or dipped the filter papers directly into the cortisol solutions (dip group). The procedures were repeated 5 times for the 10, 50, and 100 ng/mL cortisol concentrations, and 10 times for 1 and 5 ng/mL cortisol concentrations.

2.2.2. Extraction

Cortisol was extracted from the filter papers with ether. To do this, we folded each filter paper, pushed it into and to the bottom of a glass tube (13 × 100 mm), poured 2 mL of diethyl ether (Wako Pure Chemicals, Osaka, Japan) into each tube, and vortexed each tube for 3 min. After vortexing, the ether was transferred into glass tubes (12 × 75 mm) and evaporated to dryness at 60°C. Next, 0.5 mL of ether was added to the tube to rinse cortisol that was attached to the tube's inner wall to the bottom; the ether was then evaporated again. After cooling, we poured 250 μL of phosphate buffer that contained 1% BSA (Sigma-Aldrich Co, LLC, Tokyo, Japan) into the tube and mixed for another 3 min. For the radioimmunoassay, aliquots of 15 μL of the sample were transferred to the assay tubes and diluted with 85 μL of phosphate buffer and 1% BSA.

2.2.3. Radioimmunoassay

The cortisol concentrations were measured with the double-antibody radioimmunoassay method with ^{125}I labeled radioligands (MP Biomedicals, LLC, Solon, OH, USA), as described previously [12]. The antiserum against cortisol (anti-cortisol-3-[*O*-carboxymethyl] oximino, BSA; HAC-AA71-02RBP) was provided by The Biosignal Research Center (Institute for Molecular and Cellular Regulation, University of Gunma, Gunma, Japan). The cortisol standard (H4001-Hydrocortisone; Sigma-Aldrich Co, LLC) was used for the assay. The intra- and interassay CVs determined in our preliminary study were <10% and 15%, respectively.

2.3. Experiment 2

2.3.1. Subjects

Four intact male beagles housed at Tokyo University of Agriculture and Technology were used. The median age of the dogs was 7.5 y (range, 3–8 y), and the median weight was 12.5 kg (range, 11.2–13.8 kg).

2.3.2. Blood and saliva collection

One milliliter of blood was collected into an EDTA-containing tube and centrifuged at 800 ×*g* for 30 min at 4°C. The plasma samples were pipetted off and stored at −20°C until cortisol extraction. After skin secretions, we immediately collected saliva by inserting a filter paper into the dog's mouth (under its tongue and within the cheek pouch) to thoroughly wet the paper. Plastic gloves were worn throughout the procedure to avoid transferring steroids from human skin secretions. The filter papers were stored in plastic bags at 4°C until cortisol extraction. The cortisol extraction was performed within 2 wk of the sample collection. Blood and saliva were collected 3 times (0, 30, and 60 min). After the first sampling (0 min), we took the subjects for a walk for approximately 10 min. To mitigate the influences of location change, food contamination and pH change [13], [14], and exercise [15], [16], the samplings were performed from 4:00 PM to 6:00 PM, which was >1 h after a meal and exercise, in the kennels in which the dogs were usually housed.

2.3.3. Extraction and radioimmunoassay

To extract plasma cortisol, the plasma samples were diluted with 1% BSA up to 400 μL, and 2.0 mL of ether were added to each tube and mixed for 3 min. After mixing, the tubes were immersed in ethanol that contained dry ice, and the ether was transferred into glass tubes and evaporated to dryness at 60°C. Then, 0.5 mL of ether was added to the tube to rinse the cortisol still attached to the inner wall to the bottom of the tube; the ether was evaporated again. After cooling, 400 μL of phosphate buffer that contained 1% BSA (Sigma-Aldrich Co, LLC) were poured into the tube and mixed for another 3 min. Extraction from the filter papers and radioimmunoassay procedures were described in Experiment 1. We did not correct cortisol concentration for recovery rate, because our study was to determine the accuracy and reliability of the method.

2.4. Experiment 3

2.4.1. Subjects

Twenty-one dogs comprising 15 females (3 intact, 12 spayed) and 6 males (1 intact, 5 neutered) were used. The median of age of the dogs was 4.0 y (range, 0.5–14 y), and median weight was 6.7 kg (range, 2.2–31.5 kg). Seventeen breeds were represented. The purpose and experimental design were explained to the owners, and owners' consents were obtained before any procedure began.

2.4.2. Saliva collection

We explained to the owners how to collect saliva by using filter paper, and the owners collected their dogs' saliva. Saliva was collected twice (0 min and 30 min). To mitigate the influence of location change, saliva collection was performed where each dog usually stayed from 2:00 PM to 7:00 PM. In the interval between saliva collections, the owners attended to their dogs as usual, although they were instructed not to give a stimulus, such as food and exercise. Other experimental conditions were the same as in experiment 2.

2.4.3. Questionnaire

Owners were asked to fill out a questionnaire about their dogs and the saliva collection. The questionnaire items were as follows: the basic information on their dogs (breed, sex, age, weight, spayed/castrated or intact, and the history of present illness), the time required to collect the dog's saliva at 0 min, and whether they regularly brushed the dog's teeth.

2.4.4. Extraction and radioimmunoassay

Saliva cortisol was extracted from the filter papers and cortisol concentrations were measured with radioimmunoassay. Extraction and radioimmunoassay procedures were described in Experiment 1.

2.5. Experiment 4

2.5.1. Subjects

A total of 7 dogs (3 spayed female and 4 neutered males) were used in this experiment. The median age of the dogs was 6 y (range, 1–11 y) and median weight was 6.7 kg (range, 4.2–30.0 kg). Five breeds were represented. All subjects were pets and did not have a current illness. The dog's teeth were brushed by its owners more than twice a week for >1 y. The purpose and experimental design was explained to owners, and owners' consent was obtained before any procedure began.

2.5.2. Saliva collection and questionnaire

We explained to the owners the method of collecting saliva by using filter paper, and the owners collected their dogs' saliva as described in Experiment 3. Saliva was collected before and 30 min after the human–dog interaction. Experimental conditions were the same as in experiment 2. After saliva collection, owners were asked to fill out a questionnaire about the basic information on their dogs and the time required to collect the dogs' saliva. The filter papers were stored at 4°C until further analysis.

2.5.3. Human–dog interaction

After the first saliva collection, the dogs interacted with their owners for 30 min. During the interaction, the owners stroked, petted, played with, and spoke to their dogs. No restrictions were imposed on the type of interaction.

2.5.4. Extraction and radioimmunoassay

Saliva cortisol was extracted from the filter papers, and the cortisol concentrations were measured by radioimmunoassay. Extraction and radioimmunoassay procedures were described in Experiment 1.

2.6. Statistical analysis

In experiment 1, the difference in the detected cortisol concentrations between the pipette and dip groups was tested with 2-way ANOVA without repeated measures. In experiment 2, the generalized liner mixed model (GLMM) was used to evaluate the similarity in the behavior of cortisol concentrations in saliva and serum. In this analysis, cortisol concentration was set as a dependent factor. Time of collection, collection type (saliva or blood), and interaction between time of collection and collection type were set as independent factors. Identification of the dog was set as a random effect. If the model detected a significant effect of the interaction, the behavior of the cortisol concentration in saliva and blood was different. In experiment 3, a paired t test was conducted to determine whether there was a difference between cortisol concentrations collected at 0 min and that collected at 30 min. The influences of sex, breed, age, castration/spay, and history of illness on cortisol concentrations were tested with ANOVA with repeated measures. Fisher exact test was performed to analyze the relationship between tooth-brushing ritual and the time required to collect saliva at 0 min. Dogs >15 kg were grouped as large-breed dogs (n = 7), and the other dogs (n = 14; <15 kg) were grouped as midsized to small-breed dogs. Dogs <1 y were grouped as young dogs (n = 4), dogs between 1 and 7 y were grouped as adult dogs (n = 12), and dogs >7 y were grouped as senior dogs (n = 5). In experiment

4, a *t* test was used to determine whether there was a difference between the cortisol concentrations before and after the interaction. With the exception of GLMM, statistical analyses were performed with GraphPad Prism (Graph Pad Software, Inc, San Diego, CA, USA). We conducted GLMM by using R (http://www.r-project.org/). The difference was considered significant if the *P* value was <0.05.

3. Results

3.1. Experiment 1

We measured cortisol of known concentrations and applied the cortisol solution onto filter paper by pipette or dipped the paper into the solution. We detected the mean ± SEM cortisol concentrations of 79.58 ± 4.02 ng/mL for 100 ng of solution, 39.15 ± 0.83 ng/mL for 50 ng of solution, 8.13 ± 0.33 ng/mL for 10 of ng solution, 4.43 ± 0.16 ng/mL for 5 ng of solution, 0.97 ± 0.04 ng/mL for 1 ng of solution in the pipette group and 86.84 ± 5.71 ng/mL, 43.28 ± 2.49 ng/mL, 7.45 ± 0.27 ng/mL, 4.54 ± 0.13 ng/mL, 0.88 ± 0.03 ng/mL in the dip group (Fig. 1). The mean recovery percentage of the pipette group was 85.0 ± 2.9 (n = 5) and that of the dip group was 85.3 ± 2.3 (n = 5). No significant difference was found in cortisol concentrations between the pipette and dip groups ($F = 3.85$, $P = 0.14$).

3.2. Experiment 2

Mean cortisol concentrations in serum were 4.08 ± 1.34 ng/mL at 0 min, 3.71 ± 0.63 ng/mL at 30 min, and 1.57 ± 0.27 ng/mL at 60 min (Fig. 2). Mean cortisol concentrations in saliva were 1.28 ± 0.43 ng/mL, 1.50 ± 0.44 ng/mL, and 0.31 ± 004 ng/mL, respectively. Cortisol concentrations in serum was always significantly higher than that in saliva ($F = 12.45$, $P = 0.003$; Fig. 2). Time of collection also had a significant effect on cortisol concentrations ($F = 3.71$, $P = 0.047$). The interaction between time of collection and collection type was not significant ($F = 0.70$, $P = 0.51$).

3.3. Experiment 3

The mean cortisol concentrations at 0 and 30 min in dogs whose owners spent <2 min collecting saliva (n = 11) were 1.22 ± 0.09 ng/mL and 1.14 ± 0.11 ng/mL, respectively. No significant difference was found between the cortisol concentrations at 0 and 30 min ($P = 0.36$). Conversely, in dogs whose owners spent >2 min collecting saliva (n = 10), the mean cortisol concentrations at

0 and 30 min were 0.99 ± 0.13 ng/mL and 1.26 ± 0.16 ng/mL, respectively. A significant difference was found between the cortisol concentrations at 0 and 30 min (P = 0.005; Fig. 3).

In dogs whose teeth were regularly brushed (n = 12), the cortisol concentrations at 0 and 30 min were 1.16 ± 0.11 ng/mL and 1.14 ± 0.10 ng/mL, respectively, with no significant difference between them (P = 0.81; Fig. 4). Conversely, the cortisol concentrations at 0 and 30 min in dogs whose teeth were not regularly brushed (n = 9) were 1.05 ± 0.11 ng/mL and 1.28 ± 0.18 ng/mL, respectively; a significant difference was found between them (P = 0.042). To assess the relationship between tooth-brushing ritual and collection time, we performed a Fisher exact test. No significant relationship was found between these 2 factors (P = 0.67).

No significant difference was found between the cortisol concentrations at 0 and 30 min in males (n = 6; P = 0.32), females (n = 15; P = 0.52), large breeds (n = 7; P = 0.33), midsized and small breeds (n = 14; P = 0.43), young (n = 4; P = 0.15), adults (n = 12; P = 0.84), seniors (n = 5; P = 0.13), castrated dogs (n = 5; P = 0.32), spayed (n = 12; P = 0.56) and not spayed (n = 3; P = 0.80), dogs with a history of illness (n = 4; P = 0.52), and dogs without a history of illness (n = 17; P = 0.12). There was only 1 non-castrated dog in this experiment, and his cortisol concentrations at 0 and 30 min were 1.02 ng/mL and 1.00 ng/mL, respectively (Table 1). No significant influence was found for sex (F = 0.25, P = 0.62), weight (F = 0.68, P = 0.42), age (F = 0.38, P = 0.69), castration/spay (F = 0.33, P = 0.81), or history of illness (F = 0.001, P = 0.97) on cortisol concentrations.

3.4. Experiment 4

All saliva was collected in <2 min before human–dog interaction. Figure 5 shows the cortisol concentrations before and after interaction, which were 1.66 ± 0.34 ng/mL and 1.04 ± 0.28 ng/mL, respectively. The cortisol concentrations significantly decreased after human–dog interaction (P = 0.008).

4. Discussion

In the present study, we evaluated the accuracy and reliability of using filter paper as a method by which to collect and measure cortisol concentrations in dogs. We showed that we could quantitatively measure cortisol concentrations in saliva obtained with this method. We did not detect significant differences between the cortisol dynamics in plasma and saliva, suggesting that cortisol concentration in saliva behaves in a fashion similar to that in serum. The cortisol concentrations at 30 min were higher than those at 0 min when an owner spent >2 min collecting the dog's saliva or when an owner did not regularly brush the dog's teeth. In dogs whose owners regularly brushed their teeth and spent <2 min collecting its saliva, the cortisol concentrations decreased after human–dog interaction. These results indicate that the method of collecting saliva by using filter paper can be used to measure cortisol concentrations and to evaluate stress levels in dogs without creating additional stress in the dogs when the dog is accustomed to having its teeth brushed and the saliva is collected promptly.

There are many methods by which to collect saliva [17], [18], [19], [20]. In humans, the passive-drool method is recommended, and various kits have been developed for this [19], [21], [22]. With this method, the subject collects his or her own saliva by using a straw. To apply these kits to dogs, a human must collect the dog's saliva; however, there is a risk of injuring the dog's mouth or the dog accidentally ingesting the straw. Thus, the cotton-based collection method has generally been used in dogs [18]. It has been reported that the ingredients in cotton interfere with the accurate measurement of concentrations of saliva cortisol by using radioimmunoassay or enzyme immunoassay [11], [23], [24]. With the use of filter paper as outlined in the present study, the dog's owner puts the filter paper into the dog's mouth to collect the saliva. The merits of this method are a) the influences of the cotton ingredients are avoided, and b) the cost of performing the method is low.

In dogs whose teeth were not brushed by their owners, the cortisol concentrations significantly increased after saliva collection. The method of collecting saliva by using filter paper requires the owner to touch the dog's mouth. Dogs without a ritual of teeth brushing were assumed not to be accustomed to being touched in their mouths; therefore, this was a stressor for the dogs. Conversely, in dogs accustomed to having their teeth brushed by their owners, the cortisol concentration did not significantly increase. These results suggest that it is necessary to consider whether a dog's teeth were regularly brushed when this method is applied to evaluate cortisol concentration.

A protracted amount of time to collect saliva might be stressful to the dog and elicit an increase in cortisol concentration. In experiment 3, we tested the influence of the method of collecting saliva by using filter paper in the cortisol concentration. In dogs whose owners spent >2 min collecting saliva, the cortisol concentrations significantly increased from 0 to 30 min; however, the absolute value of cortisol concentration at 0 min in the dogs with collection times

>2 min was lower than those with the collection times >2 min. This might lead to a seeming increase in cortisol concentration.

One of the purposes of our study was to examine whether this method could be a stressor on dogs. In experiment 4, we investigated whether we could monitor stress reduction in dogs after interaction with their owners by collecting saliva by using filter paper for measuring cortisol. The cortisol concentrations significantly decreased after the interaction. It is reported that human–dog interaction decreases cortisol concentration in dogs [25], [26], [27]. If our method of collecting saliva had induced stress on dogs, we would not detect a significant decrease in cortisol concentrations. Our results suggest that the method induced so little stress on dogs, that we detected a reduction in cortisol concentrations after human–dog interaction, when saliva is collected promptly and dogs' teeth were regularly brushed.

In conclusion, the method by which to collect saliva by using filter paper for measuring cortisol concentration is accurate and reliable. Furthermore, the method does not induce stress on the subjects if the dogs are accustomed to their mouths being touched by the teeth-brushing ritual and if the saliva is collected quickly. Our study provides a relatively noninvasive, stress-free method by which to assess cortisol in dogs.

Acknowledgment

We thank the staff of the Veterinary Physiology Laboratory, Tokyo University of Agriculture and Technology, for supporting our experiments. We also thank Ms. Kathy Clutz Oyama for correcting the language of our manuscript.

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Ethics declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Figures

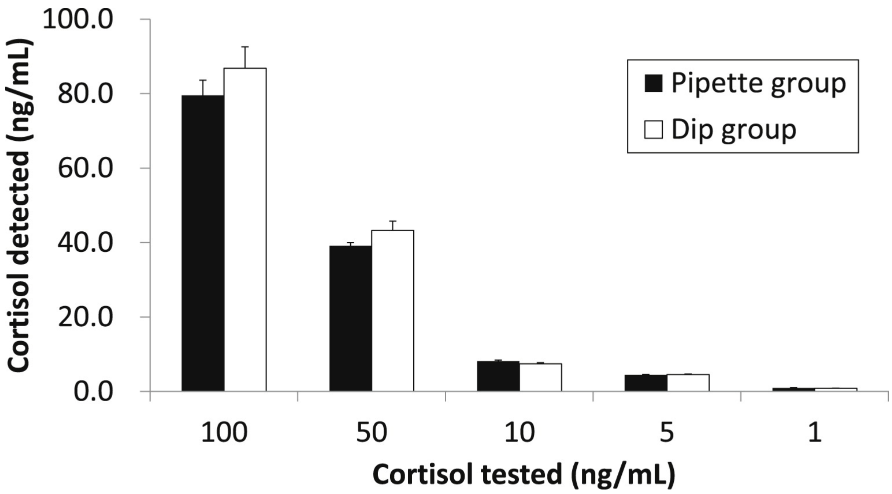

Figure 1 Cortisol concentrations detected in filter papers with cortisol solution. Cortisol solution of known concentration was measured. The solution was pipetted onto filter paper (pipette group) or filter paper was dipped into the cortisol solution (dip group). Error bars represent SEM.

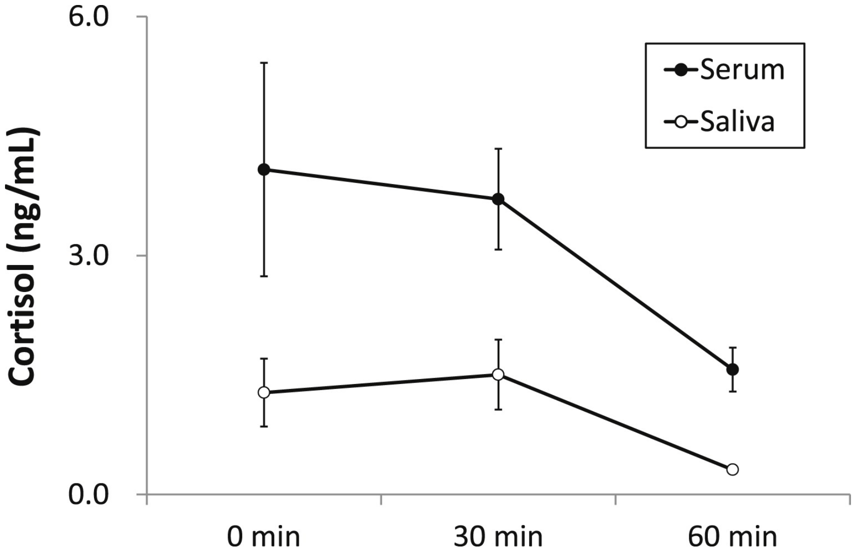

Figure 2 Cortisol concentrations in the serum and saliva. Cortisol concentration was evaluated at 0, 30, and 60 min in the serum and saliva. The cortisol concentrations in the serum were always higher than those in the saliva. No difference in cortisol dynamics was detected between the serum and saliva. Error bars represent SEM.

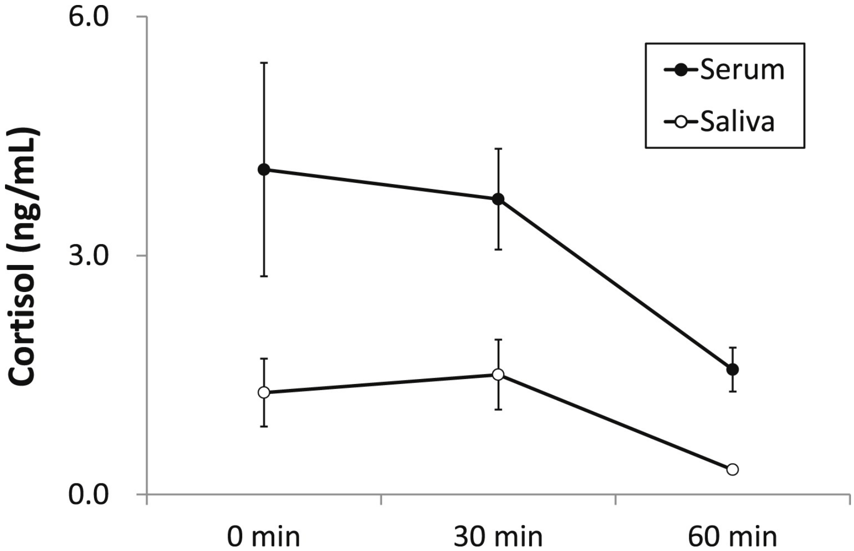

Figure 3 Cortisol concentrations in dogs whose owners spent <2 min or >2 min collecting saliva. No significant difference was observed between cortisol concentrations when the saliva was collected within 2 min at times 0 min. Conversely, the cortisol concentration at 30 min was higher than that at 0 min when the saliva collection required >2 min. Error bars represent SEM. $^{**}P < 0.01$.

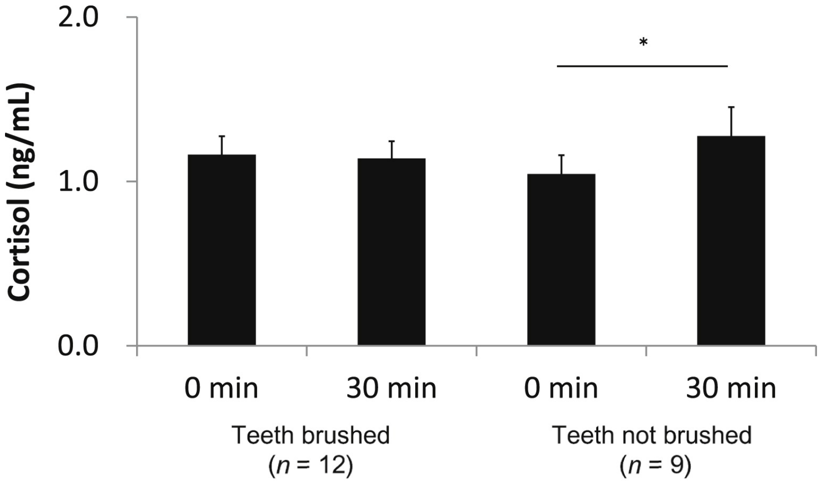

Figure 4 Cortisol concentrations in dogs whose teeth were brushed and not brushed. No significant difference was detected between cortisol concentration in saliva collected at 0 and 30 min when the dogs' teeth were regularly brushed. Conversely, the cortisol concentration in the saliva collected at 30 min was higher than that at 0 min when the dogs' teeth were not regularly brushed. Error bars represent SEM. *$P < 0.05$.

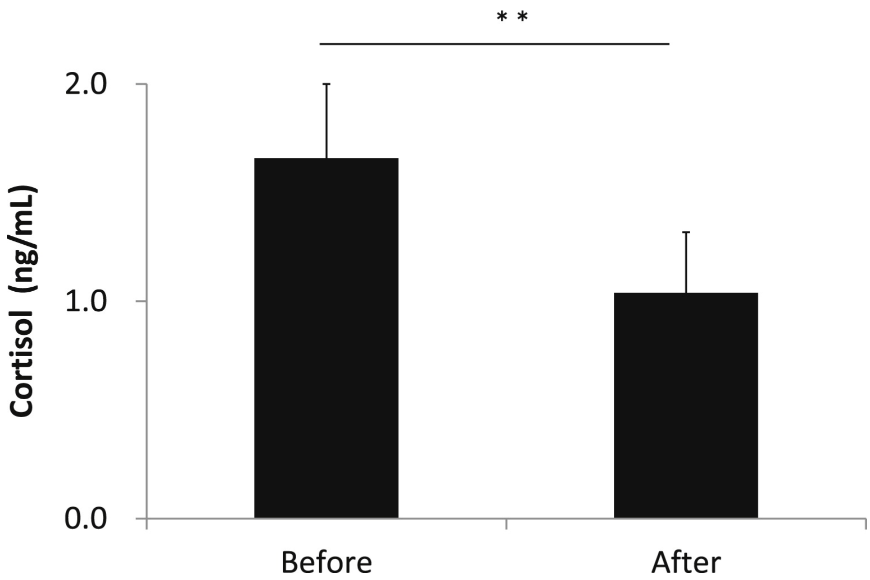

Figure 5 Cortisol concentrations before and after human–dog interaction. The cortisol concentration in dogs' saliva was lower after human–dog interaction than before the interaction. Error bars represent SEM. $^{**}P < 0.01$.

Table 1. Cortisol concentrations and basic information of the dogs in experiment 3.

	No. of dogs	Cortisol (ng/mL)		*P* value[a]	*P* value[b]
		0 min	30 min		
Sex					
Male	6	1.13 ± 0.13	1.31 ± 0.20	0.32	0.62
Female	15	1.11 ± 0.10	1.15 ± 0.11	0.52	
Weight					
Large	7	1.15 ± 0.10	1.25 ± 0.12	0.33	0.42
Mid-sized and small	14	1.04 ± 0.12	1.09 ± 0.16	0.43	
Age					
Young	4	1.12 ± 0.28	1.34 ± 0.27	0.15	0.69
Adult	12	1.08 ± 0.10	1.11 ± 0.12	0.84	
Old	5	1.18 ± 0.16	1.31 ± 0.20	0.13	
Castrated/spayed					
Castrated	5	1.15 ± 0.16	1.37 ± 0.24	0.32	0.81
Not castrated	1	1.02	1	—	
Spayed	12	1.13 ± 0.11	1.19 ± 0.12	0.56	
Not spayed	3	0.99 ± 0.31	1.01 ± 0.28	0.8	
History of illness					
Yes	4	1.20 ± 0.10	1.10 ± 0.17	0.52	0.97
No	17	1.09 ± 0.10	1.22 ± 0.11	0.12	

Data are expressed as means ± SEM.

[a]Analyzed to show the difference between cortisol concentrations at 0 and 30 min.

[b]Analyzed to show the effect of each category.