Research in Veterinary Science 117 (2018) 74–80

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Catestatin, vasostatin, cortisol, and visual analog scale scoring for stress assessment in healthy dogs

T. Srithunyarat[a,b,*], R. Hagman[a], O.V. Höglund[a], M. Stridsberg[c], J. Hanson[a], A.S. Lagerstedt[a], A. Pettersson[a]

[a] Department of Clinical Sciences, Swedish University of Agricultural Sciences, Box 7054, SE 75007, Uppsala, Sweden
[b] Department of Surgery and Theriogenology, Faculty of Veterinary Medicine, Khon Kaen University, 40002 Khon Kaen, Thailand
[c] Department of Medical Sciences, Uppsala University, SE 75185, Uppsala, Sweden

ARTICLE INFO

Keywords:
Biomarker
Canine
Chromogranin A
Psychological stress
Saliva

ABSTRACT

The neuroendocrine glycoprotein chromogranin A is a useful biomarker for stress in humans. Chromogranin A epitopes catestatin and vasostatin can be measured in dogs using radioimmunoassays. The objective of this study was to evaluate catestatin and vasostatin as canine stress biomarkers in a clinical setting. Blood and saliva were collected from 33 healthy dogs that were familiar with sampling procedures and the animal hospital environment (control group) and 30 healthy dogs that were unacquainted (stress group). During sampling, stress behavior was scored by the same observer using visual analog scale (VAS). Plasma was analyzed for catestatin and vasostatin, serum for cortisol, and saliva for catestatin. Differences between groups were analyzed using two-sample *t*-tests and $P < 0.05$ was considered significant. Stress behavior VAS score in the control group was significantly lower than in the stress group during blood ($P = 0.002$) and saliva ($P = 0.0009$) sampling. Serum cortisol and saliva catestatin concentrations in the stress group were higher than the control group ($P = 0.003$ and $P < 0.0001$, respectively). Serum cortisol concentrations were correlated with those of saliva ($r = 0.34$, $P = 0.04$) and plasma catestatin ($r = 0.29$, $P = 0.03$). Plasma catestatin and vasostatin did not differ significantly between groups. In conclusion, concentrations of saliva catestatin, and serum cortisol, and stress behavior VAS scores were significantly higher in the stress group. The results indicate that saliva catestatin may be useful as a biomarker for acute psychological stress in dogs.

1. Introduction

Fear and psychological anxiety, which are often experienced by animals when brought to a veterinary clinical practice, induce a stress reaction. This phenomenon is commonly known as the “white coat effect” and has been shown to lead to a fight or flight reaction through activation of the sympatho-adrenal-medullary (SAM) axis and the hypothalamic-pituitary-adrenal (HPA) axis (Marino et al., 2011; Höglund et al., 2012; Tennant, 2013). Although stress is essential for coping with acute changes in the body's homeostasis, the stress response i.e. secretion of catecholamines, chromogranin A (CgA), cortisol, and changes in physiological and behavior parameters (Moberg and Mench, 2000; Hekman et al., 2014) particularly if prolonged, can be detrimental and contribute to disease development in animals as well as in humans (Roizen, 1988; Moberg and Mench, 2000; Sapolsky et al., 2000; Hekman et al., 2014). Stress can be evaluated using both subjective and objective parameters such as behavior and measurement of cortisol and catecholamines in body fluids (Nakane et al., 1998; Akiyoshi et al., 2005; Hekman et al., 2014). However, all currently available methods have shortcomings and new assessment methods are needed.

Chromogranin A (CgA) has shown promise as a biomarker for evaluating stress in humans and a few studies have been done in pigs and dogs (Nakane et al., 1998; Akiyoshi et al., 2005; Lee et al., 2006; Toda et al., 2007; Escribano et al., 2013; Srithunyarat et al., 2017b). The glycoprotein CgA belongs to the Granin family and is stored in chromaffin granules and coreleased with catecholamines and neuroendocrine hormones from the adrenal medulla and sympathetic nerve endings when SAM is activated (Blaschko et al., 1967; O'Connor and Bernstein, 1984). Chromogranin A has a longer half-life, is more stable and easier to handle than catecholamines, and could, in the absence of

Abbreviations: CgA, Chromogranin A; CST, Catestatin; HPA, Hypothalamic-pituitary-adrenal axis; RPM, revolutions per minute; SAM, Sympatho-adrenal-medullary axis; VAS, Visual analog scale; VS, Vasostatin

* Corresponding author.
E-mail addresses: thanikul@kku.ac.th (T. Srithunyarat), ragnvi.hagman@slu.se (R. Hagman), odd.hoglund@slu.se (O.V. Höglund), mats.stridsberg@medsci.uu.se (M. Stridsberg), jeanette.hanson@slu.se (J. Hanson), anne-sofie.lagerstedt@slu.se (A.S. Lagerstedt), ann.pettersson@slu.se (A. Pettersson).

https://doi.org/10.1016/j.rvsc.2017.11.015
Received 26 April 2017; Received in revised form 11 October 2017; Accepted 20 November 2017

neuroendocrine tumors and gastrointestinal disease, therefore be an alternative marker for evaluating the SAM response (Blaschko et al., 1967; Crout, 1968; Derbyshire and Smith, 1984; O'Connor et al., 1989; Escribano et al., 2014). That CgA can be actively secreted into saliva has been shown to occur in rats, horses, pigs, and humans (Kanno et al., 1999; Sato et al., 2002; Saruta et al., 2005). Because saliva sampling is a noninvasive method, it may be preferable for stress monitoring purposes as the sampling itself, in humans, is less likely to induce a stress response than sampling of other body fluids or blood (Vincent and Michell, 1992; Nakane et al., 1998; D'Amico et al., 2014). In dogs, the CgA epitopes catestatin (CST) and vasostatin (VS) can be measured using radioimmunoassay in both blood and saliva and have been shown to be unaffected by age, gender, breed, and time of day (Srithunyarat et al., 2017b). However, no studies comparing CST, VS, cortisol, and stress score using stress behavior visual analog scale (VAS) between healthy dogs under nonstressful and stressful condition have previously been presented.

The aim of this study was to evaluate the potential of CST and VS as canine psychological stress biomarkers in a clinical setting. This study investigated concentrations of CST, VS, and cortisol, and stress behavior VAS score in healthy dogs where one group was accustomed to the sampling procedures and environment and the other was unaccustomed.

2. Materials and methods

2.1. Study design and ethical approval

This study comprises data and samples collected during two separate earlier studies (Srithunyarat et al., 2016; Srithunyarat et al., 2017b). These studies were approved by the Uppsala Ethical Committee (C301/12) and Khon Kaen University (KKU) Ethical Legislation (AEKKU 26/2557). All owners were informed and gave their consent prior to participation of their dog. No sedative drugs were used during or prior to the sampling procedures and owners were present throughout the clinical parts of the investigation.

2.2. Dogs

All dogs were healthy, and aged between one to eight years old. The average (mean ± SD) age, body weight, and body condition scores are illustrated in Table 1.

2.2.1. Stress group

Thirty privately-owned healthy female dogs, of ten different breeds, Chihuahua, Thai Ridgeback, Thai Bangkaew, Pomeranian, Shih Tzu, Maltese, Siberian Husky, Labrador Retriever, Poodle, and Mixed Breed, admitted for elective ovariohysterectomy at KKU Veterinary Teaching Hospital, were included in the study. All sampling and assessments were made prior to premedication and surgery as previously described (Srithunyarat et al., 2016). All dogs were regarded as healthy based on history, a complete physical examination (including assessment of the mental status, general attitude, appetite, mucus membrane appearance, capillary refill time, rectal temperature, body weight, body condition score, hydration status, hair and skin condition, heart and respiratory rate and sounds by auscultation, abdominal organs, musculoskeletal system and lymph nodes by palpation, and mouth, ear, and eye examination), and blood screening tests (hematology including hematocrit, hemoglobin, red blood cell, white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, and platelet counts and blood biochemistry including creatinine, alanine aminotransferase, and total protein and parasite blood smears for *Dirofilaria immitis, Babesia cania, Hepatozoon canis, Ehrlichia canis, Trypanosoma evansi,* and *Anaplasma platys*). Dogs were unaccustomed to the sampling procedures and the animal hospital environment. Samples were collected between 8:30 a.m. to 11:30 a.m. Food was withheld for at least 6 h prior to sampling.

Table 1
Age, body weight and body condition score in 33 control and 30 stress dogs.

Parameters	Control group (n = 33)	Stress group (n = 30)
Age (months)	47 ± 25	28 ± 26
Body weight (kg)	35.8 ± 10.1	11.6 ± 7.0
Body condition score (of 9)	5 ± 1	5 ± 1

Data presented as mean ± SD.
Dogs in the control group were familiar with sampling procedures and the animal hospital environment whereas dogs in the stress group were unfamiliar with these events.

2.2.2. Control group

Thirty-three privately-owned healthy dogs that routinely donate blood at the University Animal Hospital (UDS), Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, were included as previously described (Srithunyarat et al., 2017b). Twenty-four were males and nine females, of fourteen different breeds (Boxer, Bernese Mountain, Collie, Dalmatian, Flat Coated Retriever, German Shepherd Dog, Golden Retriever, Great Dane, Greyhound, Labrador Retriever, Leonberger, Shorthaired Pointer, White Shepherd, and Mixed Breed). These dogs were included as the control group as they were well accustomed to the sampling procedures and the animal hospital surrounding, and considered to express little or no signs of stress. Sixteen of these dogs visited the animal hospital on several occasions for blood donation, and in these dogs only one randomly chosen sampling occasion was included in this study. All dogs were healthy based on a complete physical examination and blood screening (hematology including hematocrit, hemoglobin, red blood cell, white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, and platelet counts, and biochemistry including creatinine, alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, and albumin), blood type (DEA 1.1), and the presence of vector borne diseases including *Anaplasma phagocytophilum, Anaplasma platys, Borrelia burgdorferi, Ehrlichia canis, Ehrlichia ewingii, and Dirofilaria immitis* (Snap 4DX test, IDEXX Laboratories, Maine, USA). Samples were collected between 8:00 a.m. to 1:30 p.m. Food had not been withheld prior to sampling.

2.3. Sampling protocol

Blood and saliva samples were collected prior to evaluation of stress behaviors in all dogs included in the study. The sampling and scoring procedures were performed using the same methods and techniques in both dog groups. All scoring procedures were performed by one person only. All samples were collected within an hour after arrival at the respective hospitals.

2.3.1. Blood and saliva collection

Blood was collected from the distal cephalic vein using butterfly needles (BD Vacutainer, Becton-Dickson, Plymouth, United Kingdom) into lithium heparin tubes and clot activator tubes (BD Vacutainer, Becton-Dickson, Plymouth, UK) and centrifuged at 3300 rpm for 5 min. The obtained heparinized plasma and serum samples were freeze stored in cryotubes (Low Temperature Freezer Vials, VWR, Stockholm, Sweden). For practical reasons, in order not to interfere with the clinical work conducted at the different animal hospitals, the order in which blood and saliva samples were collected was randomized with an interval between saliva and blood sampling of < 10 min. Blood samples from the control dogs were collected by two veterinary nurses and from stress group dogs by one veterinarian (TS).

Saliva samples were collected using a swab (SalivaBio, Salimetrics, PA, USA) placed in the oral cavity for 60–90 s by the same veterinarian (TS) for all dogs. The swabs were centrifuged at 3000 rpm for 15 min and the saliva deposited was freeze stored.

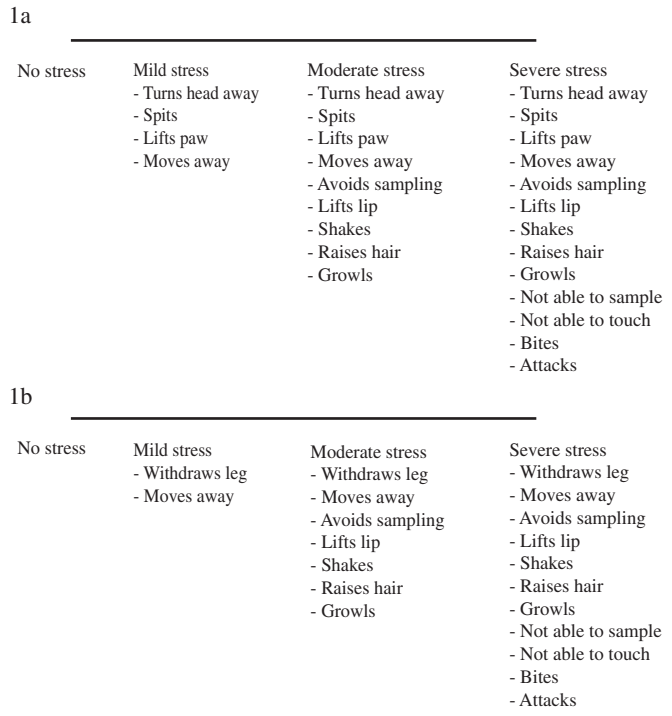

Fig. 1. Stress behavior visual analog scale (VAS) scoring used during blood and saliva sampling.
1a Criteria used during saliva sampling.
1b Criteria used during blood sampling.
These criteria (Srithunyarat et al., 2017b) were modified from Norling et al. (2012).

In the control group, samples were directly, after collection, stored and kept at − 70 °C until analysis. Samples from the stress group were initially directly frozen at − 20 °C after collection. After completed sampling of the stress group in Thailand (March to June 2015), samples were transported by a private transportation company (Temperature control, World Courier, Bangkok, Thailand) with temperature monitored to remain below − 20 °C, to SLU, Uppsala, Sweden, within 48 h. The samples were then freeze stored at − 70 °C until analysis. At no point were any samples thawed prior to analysis.

2.3.2. Visual analog scale (VAS)

Stress behavior VAS during each blood and saliva sampling occasion was assessed using a plain 100-mm line. The pre-established subjective criteria and VAS used in this study to determine stress behaviors during blood and saliva sampling have previously been described and are illustrated in Fig. 1 (Norling et al., 2012; Srithunyarat et al., 2016; Srithunyarat et al., 2017b). All stress behavior VAS scores were determined by one observer (TS).

2.4. Analysis of catestatin and vasostatin

All samples were analyzed for CST and VS in duplicate using competitive radioimmunoassay at the Clinical Chemistry Laboratory, Uppsala University Hospital, Uppsala, Sweden as previously described (Stridsberg et al., 2014). For each CST and each VS analysis, 300 µL saliva and 100 µL plasma were required. Saliva sample volumes obtained were insufficient for analysis of both CST and VS, which is why only CST was measured in saliva. Samples from the two groups were analyzed in two different batches. All analysis was performed by the same experienced laboratory technician. At no point were any samples thawed prior to analysis. The limit of detection was 0.01 nmol/L for plasma CST and VS and 0.04 nmol/L for saliva CST. The overall coefficient of variance (CV) in this study was < 10%.

2.5. Cortisol analysis

Serum samples were analyzed in duplicate for cortisol using solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000, Siemens, Erlangen, Germany) at the Clinical Chemistry Laboratory, UDS, SLU, Uppsala, Sweden. Samples from the two groups were analyzed in two different batches. All cortisol analysis was performed by the same experienced laboratory technician. At no point were any samples thawed prior to analysis. The detection limit for cortisol analysis was 10 nmol/L. The overall CV was < 5%.

2.6. Statistical analysis

Prior to analysis, residuals were checked for normality and homoscedasticity. Plasma vasostatin concentrations were skewed, and after transformation to natural log, data reached normality. Comparison of blood and saliva sampling stress behavior VAS, plasma and saliva CST, plasma VS, and serum cortisol between the control and stress groups were made using independent *t*-tests (Olsson, 2011). Correlations of all variables were analyzed using Proc corr and all analyses were performed using SAS program version 9.4 (SAS Institute, 2014). *P*-values < 0.05 were considered statistically significant for all tests used in the study.

3. Results

3.1. Stress behavior visual analog scale

Mean ± SD stress behavior VAS scores in the stress group were significantly higher than the control group (19 ± 20 vs 38 ± 22 mm, $P < 0.0001$ for blood sampling and 22 ± 19 vs 42 ± 24 mm, $P = 0.0002$ for saliva sampling) (Fig. 2). Blood and saliva sampling stress behavior VAS scores were significantly correlated ($r = 0.86$, $P < 0.0001$).

3.2. Serum cortisol

Mean ± SD serum cortisol concentrations in the control and stress groups were 65 ± 28 and 175 ± 79 nmol/L, respectively. Serum cortisol concentration in the stress group was significantly higher than in the control group ($P < 0.0001$) (Fig. 2). Serum cortisol was significantly correlated with saliva CST ($r = 0.34$, $P = 0.04$) and with plasma CST ($r = 0.29$, $P = 0.03$).

3.3. Plasma catestatin and vasostatin

Mean ± SD plasma CST concentrations in the control and stress groups were 0.76 ± 0.10 and 0.76 ± 0.17 nmol/L, respectively. Mean ± SD plasma VS concentrations in the control and stress groups were 0.42 ± 0.39 and 1.13 ± 2.16 nmol/L, respectively. Both plasma CST and VS did not significantly differ between stress and control group (Fig. 2).

3.4. Saliva catestatin

In some dog, saliva volume were insufficient for analyzing. Mean ± SD saliva CST concentrations in the control (from 27 samples) were 0.64 ± 0.24 and 1.17 ± 0.48 nmol/L in stress groups (from 12 samples). Saliva CST concentration in the stress group was significantly higher than in the control group ($P = 0.007$) (Fig. 2). Saliva CST was also significantly correlated with saliva sampling stress behavior VAS score ($r = 0.47$, $P = 0.003$).

4. Discussion

The healthy dogs in this study that were unaccustomed to the

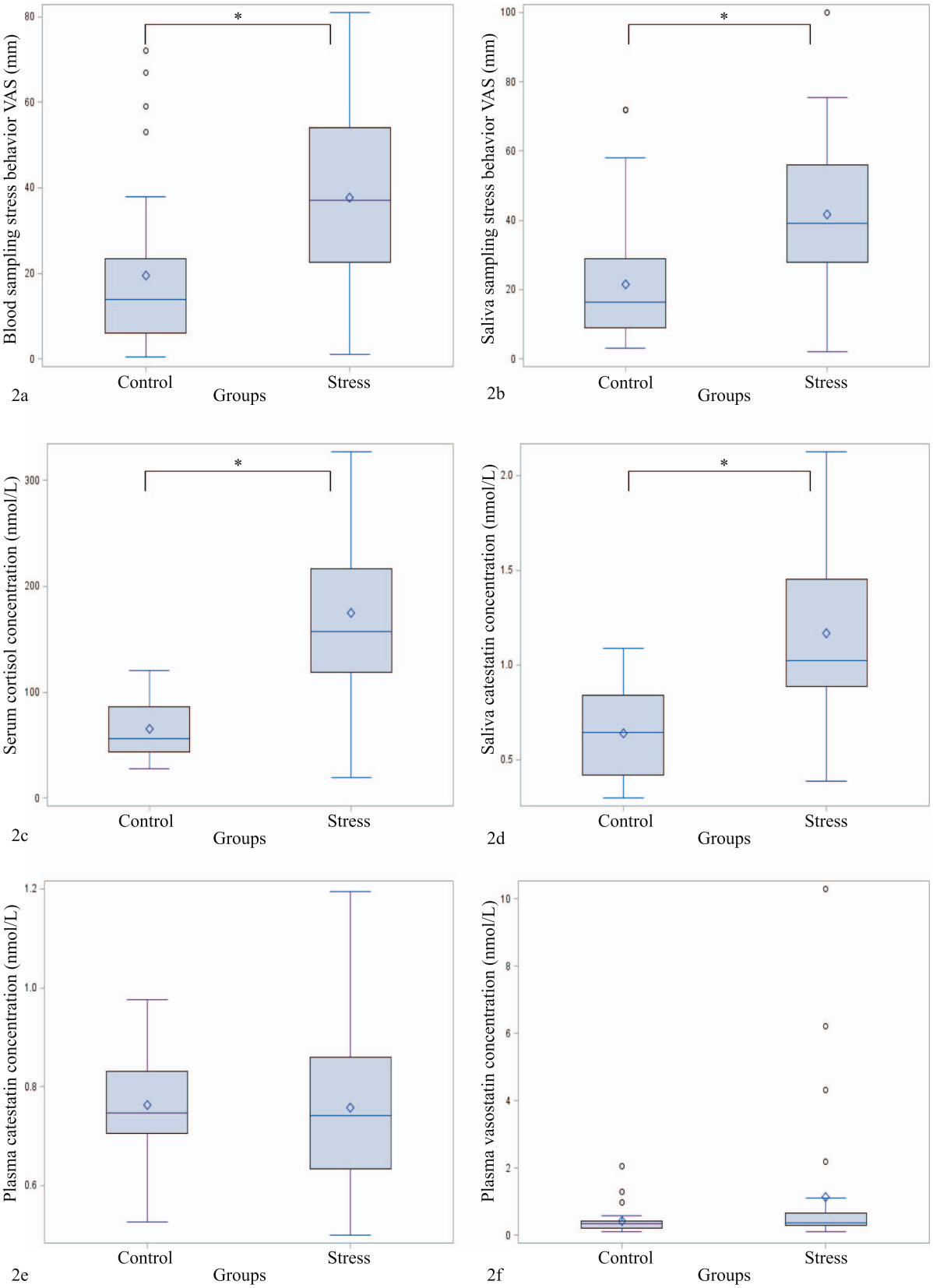

Fig. 2. Stress visual analog scale (VAS), serum cortisol, catestatin, and vasostatin concentration in 33 control and 30 stress dogs.
2a Blood sampling stress behavior VAS.
2b Saliva sampling stress behavior VAS.
2c Serum cortisol concentration.
2d Saliva catestatin concentration.
2e Plasma catestatin concentration.
2f Plasma vasostatin concentration.
*Significant difference between the groups ($P < 0.05$).

animal hospital environment (stress group) had higher stress behavior scores and serum cortisol concentrations compared to dogs that were familiar with the handling and sampling procedures and the animal hospital environment (control group). Importantly, the dogs in the stress group also had significantly higher saliva CST concentrations. These results indicate that saliva CST may have a potential as a possible biomarker for acute psychological stress in dogs.

Although dogs may perceive varying degrees of stress when exposed to new situations such as an animal hospital environment, stress behavior VAS scores during blood and saliva sampling were significantly higher in the stress group than the control group, which suggested an increased stress experienced by dogs that were not accustomed to sampling procedures and the animal hospital setting. Behavioral changes are common signs of stress in animals and can be used for evaluation of stress levels. The VAS score used in this study was based on avoidance behavior (Srithunyarat et al., 2017b). The dogs in the control group used for blood donation were all well accustomed to the sampling procedures and the animal hospital environment. They did not seem to associate the procedures with discomfort or fear as demonstrated by the overall low avoidance behavior recorded by the stress behavior VAS scores. Dogs in the stress group, however, exhibited significantly more avoidance behaviors than control dogs, as demonstrated by the stress behavior VAS scores. Assessments of behavioral changes should ideally be performed by a single trained observer using the same criteria to eliminate interobserver variability, as was done in this study. It would have been preferable if the observer had been blinded, but this was not possible for practical reasons. The significantly increase in stress behavior VAS scores in the stress group observed here, most likely reflected the dogs' experience of acute psychological stress. This is further supported by the significantly higher serum cortisol concentrations found in the stress group than the control group.

Cortisol concentrations in body fluids, most often in serum, have traditionally been used for stress and pain evaluations in both humans and animals (Tennant and Hermann, 2002; Mastrocinque et al., 2012; Michelsen et al., 2012; Hekman et al., 2014; Höglund et al., 2015). During acute stress, stimulation of the HPA axis occurs, which in turn induces hypersecretion of cortisol resulting in increased circulating concentrations. Although circadian variation, gender, age, and episodic secretion may affect serum cortisol concentrations in dogs (Kemppainen and Sartin, 1984; Giannetto et al., 2014), in this present study, we found that the serum cortisol concentration was almost three times higher in the dogs in the stress group than the control group. The significant increase in serum cortisol seen in the stress group further supports the notion that these dogs in fact were experiencing acute psychological stress. In addition, the presence of low serum cortisol concentrations confirmed that dogs in the control group were unstressed and that the stress behavior VAS used in this study was useful to reflect levels of experienced stress.

Plasma CST and VS concentrations did not differ significantly between the control and stress groups. This is in contrast to the findings in a previous study in dogs where acute stress, brought about by insulin induced hypoglycemia under experimental conditions, lead to increased levels of circulating CgA, catecholamines, and cortisol (Akiyoshi et al., 2005). Possibly the magnitude of psychological stress associated with the hospital visit in the present study may not have been strong enough to cause changes in plasma CST and VS concentrations compared to the stress induced by hypoglycemia. The CST and VS concentrations in the present study overlapped with the reference ranges for healthy dogs (Srithunyarat et al., 2017b). A similar overlap was seen in studies investigating plasma CST and VS as potential biomarkers for pain-induced stress in dogs undergoing ovariohysterectomy (Srithunyarat et al., 2016; Srithunyarat et al., 2017a; Srithunyarat et al., 2017b). The results of the present study indicate that neither plasma CST nor VS are suitable as biomarkers for monitoring this degree of acute stress in dogs in a clinical setting.

In this study, we found that saliva CST concentrations significantly increased in response to stress, and that the levels were two times higher in dogs in the stress group than dogs in the control group. The mean saliva CST concentration in the stress group was higher than the reference range reported previously for healthy dogs accustomed to the sampling procedures (Srithunyarat et al., 2017b). Although saliva CST correlated significantly with serum cortisol and saliva sampling stress VAS scores, the correlations were weak. In dogs and humans, cortisol passively diffuses from blood to saliva. Saliva and plasma cortisol are therefore closely correlated (Vincent and Michell, 1992; Den et al., 2007). There is evidence in some mammals that CgA can be produced and actively secreted from saliva glands; however, secretion patterns vary between different species and studies in dogs are lacking (Kanno et al., 1999; Sato et al., 2002; Saruta et al., 2005). In our study, saliva CST did not correlate significantly with plasma CST. However, blood and saliva samples could not be collected simultaneously which may have affected the results. Although further studies are needed, our results support the notion that saliva and plasma CST may be secreted through different routes during SAM activation even in dogs (Kanno et al., 1999).

Saliva CST may be found to be a useful indicator for acute psychological stress in dogs. However, the stress reaction, per se, leads to reduced saliva production which results in difficulties obtaining sufficient saliva volumes in stressed dogs, at least with the current methods (Srithunyarat et al., 2016). Pharmacological induction of saliva secretion is not an option because it may affect emotions and secretion of neuroendocrine peptides. Although saliva sampling techniques need to be improved to obtain sufficient volumes or a method developed for CST saliva analysis that requires very low sample volumes, saliva CST shows promise as a future clinically valuable objective biomarker for stress in dogs.

There were limitations in this study that should be considered. In accordance with the routines for blood donation at UDS and surgery at KKU, food was not withheld from blood donor dogs while dogs scheduled for elective surgery had a minimum period of six hours of fasting before surgery which may have affected the results. However, we have previously shown, when evaluating plasma CST and VS concentrations over time in healthy dogs, that the concentrations do not vary significantly in relation to time span between feeding and sampling (Srithunyarat et al., 2017b). In addition, saliva CgA concentrations have been shown to be unaffected by snack eating in humans (Toda et al., 2004) and thus is less likely to be affected by this difference. All sampling was performed according to the same standardized techniques and the stress behavior VAS score was assessed by the same observer in both dog groups, however, it would have been preferable if the observer had been blinded. The samples from each group were acquired from two different locations and analyzed in different batches. Evaluation of stress assessments should ideally be performed in dogs with a more homogenous population i.e. similar location, sample collection order, age, breed, body weight as well as investigation of at least two samples within the same individual. Other confounding factors such as storage and transportation of samples may affect the results. However, we have previously shown that both CST and VS concentrations are unaffected by age, gender, breed, body weight, and time of day (Srithunyarat et al., 2017b). No circadian variation in salivary CgA concentrations could be demonstrated in Beagle dogs in an earlier study (Kanai et al., 2008). A study on CgA levels in porcine saliva, using a time-resolved immunoflourometric assay with antibodies directed to CgA 359–379, which includes the CST epitope (CgA 361–372), showed porcine CgA to be very stable, allowing for storage at 4 °C for 2 days and at − 20 °C for up to 1 year. Further, CgA levels were unaffected by repeated freeze-thawing cycles (O'Connor et al., 1989; Escribano et al., 2014). All samples included in this study were directly frozen at − 20 °C (stress group) or − 70 °C (control group) and remained frozen until analysis at the same respective laboratory by the same experienced technician. Although measurements of CST and VS concentrations reflect both the intact CgA molecule and the respective bioactive

peptides, information is limited concerning CST and VS secretion and biodegradation in dogs which is a limitation. Even taking into consideration the previously described limitations, plasma CST and VS concentrations in this study did not differ significantly from the previously described reference ranges (Srithunyarat et al., 2017b) illustrating the limited value of these CgA epitopes for evaluating moderate psychological stress in a clinical environment. Further studies with more standardized protocols are warranted to investigate the potential of saliva CST as a psychological biomarker in dogs.

5. Conclusion

Psychological stress increased stress behavior VAS and concentrations of serum cortisol and saliva CST in dogs unaccustomed to the sampling procedures and animal hospital environment. Our findings indicate that saliva CST may be useful as objective biomarker for stress in healthy dogs in a clinical setting.

Authors' contributions

TS designed the study and collected the samples. AP, RH, OVH, and MS gave input on the study design and data collections. MS performed CgA analyses and TS performed statistical analysis. The manuscript was drafted by TS and revised with assistance of AP, RH, OVH, MS, JH, and ASL. All authors read and approved the final manuscript.

Funding

This study was financially supported by the Royal Thai Government scholarship for TS Doctoral studies, SLU internationalization travel grant for travelling expenditures, and Sveland Animals Insurance Company for sample analysis.

Competing interests

All authors declared that have no competing interests.

Consent of publication

Not applicable.

Availability of data and material

All the data supporting the findings is included within the manuscript.

Acknowledgments

The authors would like to thank the Clinical Chemistry Laboratory, UDS, the Department of Clinical Sciences, SLU, Uppsala, Sweden, KKU Veterinary Teaching Hospital, and the Department of Surgery and Theriogenology, Faculty of Veterinary Medicine, KKU, Khon Kaen, Thailand, for allowing the use of laboratory equipment and for great collaboration. Annika Rikberg, Louise Pettersson, Britta Liby, and Kristoffer Dreimanis, all at SLU, and all veterinarians and veterinary assistants at KKU Veterinary Teaching Hospital are acknowledged for their kind help.

References

Akiyoshi, H., Aoki, M., Shimada, T., Noda, K., Kumagai, D., Saleh, N., Sugii, S., Ohashi, F., 2005. Measurement of plasma chromogranin A concentrations for assessment of stress responses in dogs with insulin-induced hypoglycemia. Am. J. Vet. Res. 66, 1830–1835.

Blaschko, H., Comline, R.S., Schneider, F.H., Silver, M., Smith, A.D., 1967. Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. Nature 215, 58–59.

Crout, J.R., 1968. Sampling and analysis of catecholamines and metabolites. Anesthesiology 29, 661–669.

D'Amico, M.A., Ghinassi, B., Izzicupo, P., Manzoli, L., Di Baldassarre, A., 2014. Biological function and clinical relevance of chromogranin A and derived peptides. Endocrine Connections 3, R45–54.

Den, R., Toda, M., Nagasawa, S., Kitamura, K., Morimoto, K., 2007. Circadian rhythm of human salivary chromogranin a. Biomed. Res. 28, 57–60.

Derbyshire, D.R., Smith, G., 1984. Sympathoadrenal responses to anaesthesia and surgery. Br. J. Anaesth. 56, 725–739.

Escribano, D., Soler, L., Gutierrez, A.M., Martinez-Subiela, S., Ceron, J.J., 2013. Measurement of chromogranin A in porcine saliva: validation of a time-resolved immunofluorometric assay and evaluation of its application as a marker of acute stress. Animal 7, 640–647.

Escribano, D., Gutierrez, A.M., Fuentes-Rubio, M., Ceron, J.J., 2014. Saliva chromogranin A in growing pigs: a study of circadian patterns during daytime and stability under different storage conditions. Vet. J. 199, 355–359.

Giannetto, C., Fazio, F., Assenza, A., Alberghina, D., Panzera, M., Piccione, G., 2014. Parallelism of circadian rhythmicity of salivary and serum cortisol concentration in normal dogs. J. Appl. Biomed. 12, 229–233.

Hekman, J.P., Karas, A.Z., Sharp, C.R., 2014. Psychogenic stress in hospitalized dogs: cross species comparisons, implications for health care, and the challenges of evaluation. Animals 4, 331–347.

Höglund, K., Hanas, S., Carnabuci, C., Ljungvall, I., Tidholm, A., Häggström, J., 2012. Blood pressure, heart rate, and urinary catecholamines in healthy dogs subjected to different clinical settings. J. Vet. Intern. Med. 26, 1300–1308.

Höglund, O.V., Hagman, R., Stridsberg, M., 2015. Chromogranin A and cortisol at intraoperative repeated noxious stimuli: surgical stress in a dog model. SAGE Open Med 3 (2050312115576432).

Kanai, K., Hino, M., Hori, Y., Nakao, R., Hoshi, F., Itoh, N., Higuchi, S., 2008. Circadian variations in salivary chromogranin a concentrations during a 24-hour period in dogs. J. Vet. Sci. 9, 421–423.

Kanno, T., Asada, N., Yanase, H., Iwanaga, T., Ozaki, T., Nishikawa, Y., Iguchi, K., Mochizuki, T., Hoshino, M., Yanaihara, N., 1999. Salivary secretion of highly concentrated chromogranin a in response to noradrenaline and acetylcholine in isolated and perfused rat submandibular glands. Exp. Physiol. 84, 1073–1083.

Kemppainen, R.J., Sartin, J.L., 1984. Evidence for episodic but not circadian activity in plasma concentrations of adrenocorticotrophin, cortisol and thyroxine in dogs. J. Endocrinol. 103, 219–226.

Lee, T., Shimizu, T., Iijima, M., Obinata, K., Yamashiro, Y., Nagasawa, S., 2006. Evaluation of psychosomatic stress in children by measuring salivary chromogranin A. Acta Paediatr. 95, 935–939.

Marino, C.L., Cober, R.E., Iazbik, M.C., Couto, C.G., 2011. White-coat effect on systemic blood pressure in retired racing Greyhounds. J. Vet. Intern. Med. 25, 861–865.

Mastrocinque, S., Almeida, T.F., Tatarunas, A.C., Imagawa, V.H., Otsuki, D.A., Matera, J.M., Fantoni, D.T., 2012. Comparison of epidural and systemic tramadol for analgesia following ovariohysterectomy. J. Am. Anim. Hosp. Assoc. 48, 310–319.

Michelsen, J., Heller, J., Wills, F., Noble, G.K., 2012. Effect of surgeon experience on postoperative plasma cortisol and C-reactive protein concentrations after ovariohysterectomy in the dog: a randomised trial. Aust. Vet. J. 90, 474–478.

Moberg, G.P., Mench, J.A., 2000. The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Pub, Wallingford, UK; New York, NY, USA, pp. 377.

Nakane, H., Asami, O., Yamada, Y., Harada, T., Matsui, N., Kanno, T., Yanaihara, N., 1998. Salivary chromogranin A as an index of psychosomatic stress response. Biomed. Res. (6), 401–406.

Norling, Y., Wiss, V., Gorjanc, G., Keeling, L., 2012. Body language of dogs responding to different types of stimuli. In: Proceeding of the 46th Congress of the International Society for Applied Ethology 2012. Wageningen Academic, Wageningen, pp. 199.

O'Connor, D.T., Bernstein, K.N., 1984. Radioimmunoassay of chromogranin A in plasma as a measure of exocytotic sympathoadrenal activity in normal subjects and patients with pheochromocytoma. N. Engl. J. Med. 311, 764–770.

O'Connor, D.T., Pandian, M.R., Carlton, E., Cervenka, J.H., Hsiao, R.J., 1989. Rapid radioimmunoassay of circulating chromogranin-a - in vitro stability, exploration of the neuroendocrine character of neoplasia, and assessment of the effects of organ failure. Clin. Chem. 35, 1631–1637.

Olsson, U., 2011. Statistics for Life Science 1, First ed. Studentlitteratur, Lund, pp. 351.

Roizen, M.F., 1988. Should we all have a sympathectomy at birth? Or at least preoperatively? Anesthesiology 68, 482–484.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.

Saruta, J., Tsukinoki, K., Sasaguri, K., Ishii, H., Yasuda, M., Osamura, Y.R., Watanabe, Y., Sato, S., 2005. Expression and localization of chromogranin A gene and protein in human submandibular gland. Cells Tissues Organs 180, 237–244.

SAS Institute, 2014. SAS/STAT User's Guide Version 9.4. SAS Institute, Cary NC.

Sato, F., Kanno, T., Nagasawa, S., Yanaihara, N., Ishida, N., Hasegawa, T., Iwanaga, T., 2002. Immunohistochemical localization of chromogranin A in the acinar cells of equine salivary glands contrasts with rodent glands. Cells Tissues Organs 172, 29–36.

Srithunyarat, T., Höglund, O.V., Hagman, R., Olsson, U., Stridsberg, M., Lagerstedt, A.S., Pettersson, A., 2016. Catestatin, vasostatin, cortisol, temperature, heart rate, respiratory rate, scores of the short form of the Glasgow composite measure pain scale and visual analog scale for stress and pain behavior in dogs before and after ovariohysterectomy. BMC Res Notes 9, 381.

Srithunyarat, T., Hagman, R., Hoglund, O.V., Stridsberg, M., Olsson, U., Hanson, J., Nonthakotr, C., Lagerstedt, A.S., Pettersson, A., 2017a. Catestatin, vasostatin, cortisol, and pain assessments in dogs suffering from traumatic bone fractures. BMC Res

Notes 10, 129.
Srithunyarat, T., Hagman, R., Höglund, O.V., Olsson, U., Stridsberg, M., Jitpean, S., Lagerstedt, A.-S., Pettersson, A., 2017b. Catestatin and vasostatin concentrations in healthy dogs. Acta Vet. Scand. 59 (1).
Stridsberg, M., Pettersson, A., Hagman, R., Westin, C., Höglund, O., 2014. Chromogranins can be measured in samples from cats and dogs. BMC Res Notes 7, 336.
Tennant, F., 2013. The physiologic effects of pain on the endocrine system. Pain Ther 2, 75–86.
Tennant, F., Hermann, L., 2002. Normalization of serum cortisol concentration with opioid treatment of severe chronic pain. Pain Med. 3, 132–134.
Toda, M., Morimoto, K., Nagasawa, S., Kitamura, K., 2004. Effect of snack eating on sensitive salivary stress markers cortisol and chromogranin a. Environ. Health Prev. Med. 9, 27–29.
Toda, M., Kusakabe, S., Nagasawa, S., Kitamura, K., Morimoto, K., 2007. Effect of laughter on salivary endocrinological stress marker chromogranin a. Biomed. Res. 28, 115–118.
Vincent, I.C., Michell, A.R., 1992. Comparison of cortisol concentrations in saliva and plasma of dogs. Res. Vet. Sci. 53, 342–345.