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Short Communication

A novel method for assessing chronic cortisol concentrations in dogs using the nail as a source

Z. Mack, H.B. Fokidis*

Department of Biology, Rollins College, Winter Park, FL 37289, USA

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ABSTRACT

Cortisol, a glucocorticoid secreted in response to stress, is used to assess adrenal function and mental health in clinical settings. Current methods assess cortisol sources that reflect short-term secretion that can vary with current stress state. Here, we present a novel method for the extraction and quantification of cortisol from the dog nail using solid phase extraction coupled to enzyme-linked immunosorbent assay. Validation experiments demonstrated accuracy ($r = 0.836$, $P < 0.001$) precision (15.1% coefficients of variation), and repeatability (14.4% coefficients of variation) with this method. Furthermore, nail cortisol concentrations were positively correlated to an established hair cortisol method ($r = 0.736$, $P < 0.001$). Nail cortisol concentrations did not differ with dog sex, breed, age, or weights; however, sample size limitations may preclude statistical significance. Nail cortisol may provide information on cortisol secretion integrated over the time corresponding to nail growth and may be useful as a tool for diagnosing stress and adrenal disorders in dogs.

1. Introduction

Cortisol is an adrenal glucocorticoid that plays a central role in the vertebrate stress response where acutely it rapidly liberates glucose from energy stores, suppresses inflammation, and promotes immune cell proliferation, but the latter can be reversed with chronic exposure [1,2]. During an adaptive acute response, basal blood cortisol increases rapidly (ie, 3–5 min) during stress, but then returns to baseline after it subsides [1,3]. Chronic stress, often stemming from disease, elevates cortisol above the normal baseline concentrations [4–8]. Cortisol measurements are used to assess animal welfare and diagnose adrenal dysfunction, yet current methods measure transient concentrations reflecting an animal's current state and not long-term secretory patterns related to disease or chronic stress.

Blood sampling involves animal restraint which can be stressful, leading to overestimated baseline concentrations [9]. Similarly, salivary sampling measures the 5% to 10% of "free" cortisol that is not bound to carrier proteins within the blood [10,11] and is also impacted by acute stress [12], and even frequency of tooth brushing [11]. The urinary cortisol-to-creatinine ratio provides information on cortisol patterns within the previous 24 h [13,14], but it is not specific to diagnosing hyperadrenocorticism in canines, as both polyuria and polydipsia may lead to similar results [15,16]. Cortisol measurements from blood, saliva, and urine can fluctuate daily [17] and are influenced by any stress an animal is experiencing [18], such as the unfamiliar environment of a veterinary clinic.

Many keratinized tissues contain glucocorticoids including feathers [19,20], baleen plates [21], turtle claws [22], and hair [23]. In young adults, hair cortisol concentrations were aligned with major life stress events [24]. Similarly, dogs diagnosed with hyperadrenocorticism have greater hair cortisol concentrations compared with healthy dogs [23]. Hair testing measures cortisol secretion during

* Corresponding author. Tel.: +1-407-646-2452; fax: +1-407-646-2494.
E-mail address: hfokidis@rollins.edu (H.B. Fokidis).

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hair follicle growth, but it may be affected by body area sampled [23], fur care products [23], hair color [25], and possible local hair follicle synthesis of cortisol [26,27]. These issues complicate an analysis of long-term cortisol secretion based on hair sampling.

Mammalian nail contains a keratinized epithelium with a vascularized inner corium, or "quick" [28] where cortisol passively accumulates in the nail from the bloodstream. Nails in college-aged adults during "academic stress" had greater cortisol concentrations than during a less stressful period [29]. Human nail methodologies can analyze very small quantities of cortisol with a high degree of steroid recovery [30]. Analyzing nail cortisol has several practical applications that may make it preferable to established methods. Dog nails are routinely clipped without effecting outward appearance, a concern with hair sampling, particularly for short-haired breeds [23]. Nail sampling is less invasive than blood or saliva collection and is likely not influenced by current "stressful" conditions. We present a novel method for the isolation and quantification of cortisol from the nail of the domestic dog and compare these with concentrations isolated from hair samples and between individuals of varying age, size, and sex.

2. Materials and methods

Employees at Loch Haven Veterinary Clinic in Winter Park, Florida, collected hair and nail samples from 23 dogs during the course of normal care (from June to August 2015) and after written permission from owners was obtained. The institutional animal care and use committee at Rollins College was consulted, but as researchers did not directly handle dogs and samples were obtained as a byproduct of routine animal care, no specific approval was required. Nails were clipped below the quick from all toes, and these were pooled to provide a sufficient sample. Hair samples were only collected from the underbelly surface coat of 19 of 23 dogs to facilitate comparison with the nail. All samples were collected on the same date and time, and additional information provided by the owners included sex (n = 11 males, 12 females), age (means ± standard error [SE]: 6.93 ± 0.78 yr, range: 1–16 yr), weight (means ± SE: 21.4 ± 3.50 kg, range: 5.4-74.8 kg), and breed (n = 13 pure bred, 10 mixed). The health status of all the dogs in this study was unknown because of confidentiality, and all dogs were reported as being spayed or neutered.

Nail samples were ground to a powder using a handheld attrition mill (Glas-Col LLC, Atlanta, GA, USA), which maximized endogenous cortisol recovery among multiple methods tested [30]. Hair samples (30–50 mg of hair) were first cut into fragments (1–4 mm), as described in [25]. Samples were then stored at −20°C until extraction. Prepared nail and hair samples were suspended in 84% methanol (MeOH) in a 19:1 ratio (volume-to-sample mass) and left overnight at 4°C. Samples were then centrifuged at 3,000 × *g* for 10 min at 4°C and the supernatant collected and diluted with 10 mL of deionized water. Cortisol was extracted using a solid phase extraction (SPE) method [31] with unendcapped carbon-bonded silica C18 filter column cartridges (Agilent Technologies, Santa Clara, CA, USA) on a vacuum manifold at a constant flow rate of about 2 drops per second. Columns were first primed with 3 mL of 100% ethanol (EtOH), then equilibrated with 10-mL deionized water before loading the diluted 10-mL sample. Next, 10 mL of 40% MeOH was used to remove fats (eg, triglycerides, cholesterols, and fatty acids) from the sample that could interfere with the assay. Columns were then run dry, and cortisol was eluted using 5 mL of 90% MeOH. Samples were dried in a speed vacuum concentrator (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) at 60°C for 4 h. All SPE extractions included a solvent blank as a negative control. Dried extracts were stored at −20°C until assayed.

Cortisol concentration was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Arbor Assays Inc, Ann Arbor, MI, USA). The manufacturer's reported assay specifications are sensitivity = 17.3 pg/mL, limit of detection = 45.4 pg/mL, intra-assay precision = 8.8%, inter-assay precision = 8.1%, cross-reactivities, dexamethasone = 18.8%, prednisolone = 7.8%, corticosterone = 1.2%, cortisone = 1.2%, and all other tested steroids <0.1%. Dried extracts were reconstituted using 2 μL of absolute EtOH and 60 μL of the kit assay buffer. The assay was performed as per manufacturer's instructions. Final concentrations were calculated using raw absorbance data interpolated from the standard curve using GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla, CA, USA). All subsequent statistical analyses were conducted using SigmaPlot 13 (SYSTAT Software Inc, San Jose, CA, USA).

To determine if substances within the nail matrix bias cortisol measurement, parallelism between the assay standard curve and a serial dilution of a pooled nail sample (from 1:2–1:64 with assay buffer) was tested using linear regression of log-transformed data. Whether loading more sample (from 1.5–63.0 mg) increased recovery of endogenous cortisol was also tested using logistic regression analysis. Accuracy was defined as exogenous cortisol recovery from pooled nail extracts spiked with cortisol at a range of concentrations (from 2–113 ng/mL) that lie within the linear range of the standard curve and assessed by Pearson's correlation between cortisol added and recovered. As a negative control, a subset of samples was pretreated with dextran-coated charcoal (DCC; 2:1 sample by mass) overnight to eliminate endogenous cortisol. All samples and standards were run in duplicate, and every 96-well microplates (n = 6) contained samples from the same pool run in triplicate. Assay precision and repeatability are presented as percentages for inter-assay (ie, between microplates) and intra-assay (ie, within microplates) coefficients of variation (%CVs), respectively. Sensitivity (ie, minimal detection limit) was defined as a difference of 2 standard deviations between the means of the blank standard (n = 12) and the pooled nail sample (based on n = 18 replicates across 3 microplates).

Hair and nail samples from the same individuals were assayed on the same microplates to allow comparison of cortisol concentrations using Pearson's correlations. Multiple linear regression analysis was used to investigate variation in nail cortisol concentration with age, sex, weight, and pure vs mixed breeds added as predictor variables. Two sample t-tests were used to validate negative controls and assay blanks. All data were first tested for normality and equal variance and transformed if necessary before analysis.

For all statistical tests, the alpha level for significance was $P < 0.05$, and data are shown as means ±SE.

3. Results

A serially diluted nail sample modeled using linear regression ($R^2 = 0.994$, $F_{1, 5} = 691.07$, $P \leq 0.001$) had the same slope as the standard curve ($P = 0.672$; Fig. 1A) indicating parallel displacement of the cortisol antibody. Increased sample loading in the assay predictably increased extracted cortisol concentrations ($R^2 = 0.909$, $F_{1, 4} = 30.01$, $P < 0.001$, n = 5; Fig. 1B). Recovery of known cortisol concentrations from nail extracts ranged from 88% to 97% and was correlated to the amount of cortisol that was added to the sample pool (r = 0.836, $P \leq 0.001$, n = 7; Fig. 2). Pretreatment of a sample pool with DCC, removed cortisol (0.02 + 0.013 ng/mg) to a concentration comparable with the solvent blank (0.03 ± 0.004 ng/g; t = 0.371, $P = 0.407$). The nail cortisol assay demonstrated a high degree of precision and reliability with intra-assay and inter-assay variation at 12.4% and 15.1%, respectively (n = 6 plates). Sensitivity of the assay was determined at 38 pg/mL. Nail cortisol concentrations were not significantly correlated to hair cortisol concentrations (r = 0.366, $P = 0.15$, n = 19). However, a significant positive correlation between nail and hair cortisol concentrations was observed (r = 0.736, $P \leq 0.001$, n = 14; Fig. 3) after removal of all outliers (n = 5 hair samples) that were ≥2 standard deviations from the mean. Nail cortisol concentrations neither significantly influenced by dog breed, sex, age, or weight nor by any of the interactions of these variables (Table 1).

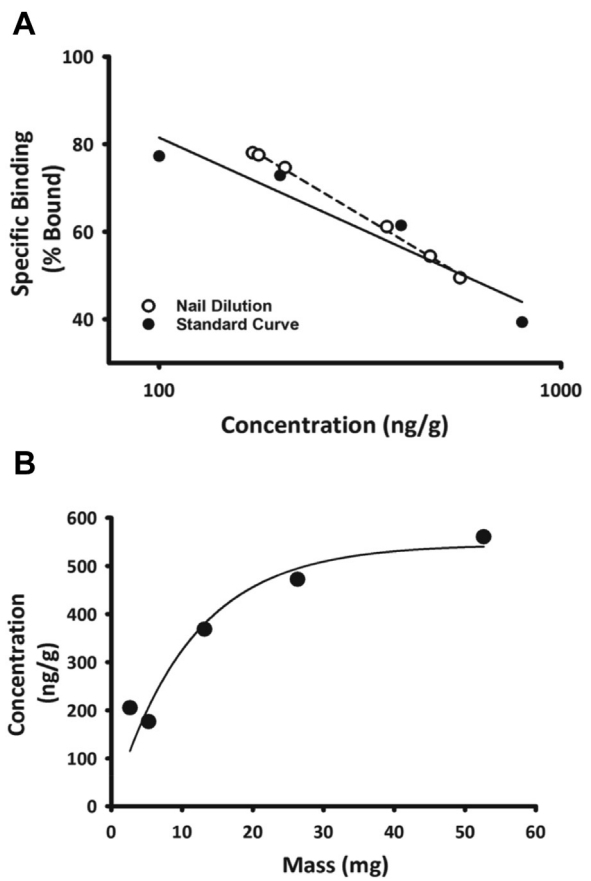

Fig. 1. (A) The percentage of dog nail cortisol specifically bound to the assay antibody as a function of cortisol concentrations of a sample pool. Samples within the linear range were parallel to the standard curve suggesting no substances were present in the nail matrix that could interfere with the assay. (B) Endogenous dog nail cortisol concentrations recovered from varying sample masses derived from a pooled sample.

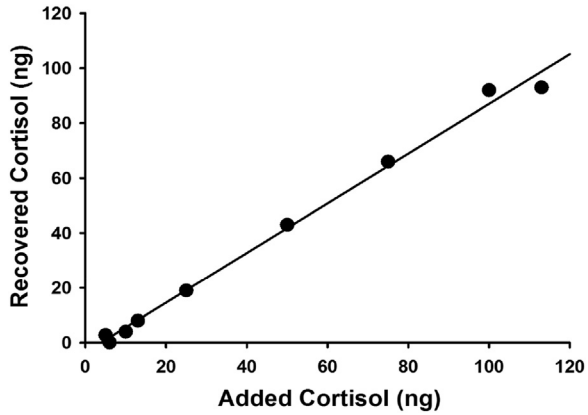

Fig. 2. Recovery of exogenous cortisol from dog nail extracts, demonstrating a significant relationship between the concentration recovered from nail samples that were spiked with varying amounts of cortisol.

4. Discussion

Cortisol plays an integral role in the stress response and chronically high concentrations can be associated with disease and compromised health in dogs. Here, we developed a noninvasive cortisol measurement based on its extraction from the dog nail. This method demonstrates that the dog nail contains a quantifiable amount of cortisol that can be extracted and quantified with reliability, accuracy, and precision. Concentrations obtained with this method are comparable with circulating levels in dogs [32–34] and were correlated to concentrations obtained

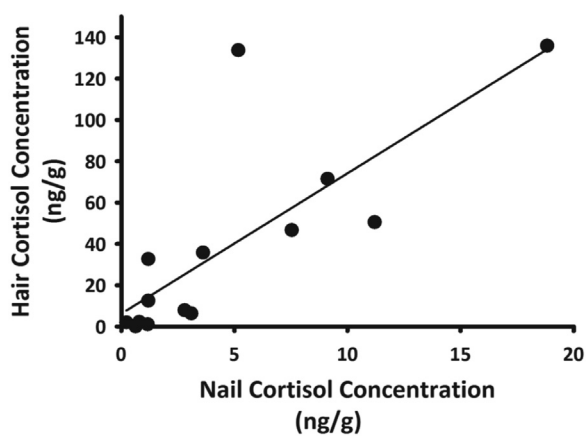

Fig. 3. Correlation between cortisol concentrations obtained from nail and hair samples collected from the same individual dogs.

Table 1
Results of multiple linear regressions between cortisol extracted from dog nails and breed, sex, age, and dog weight.

Predictor variables	R	t	*P*
Constant	3.70	0.35	0.735
Breed	−4.59	−0.78	0.452
Sex	0.10	0.60	0.562
Age	19.31	2.14	0.056
Weight	−10.49	−1.85	0.091
Breed*sex	−74.64	−0.28	0.789
Breed*age	33.45	0.29	0.782
Breed*weight	103.15	1.01	0.340
Sex*age	3.22	0.24	0.814
Sex*weight	−1.71	−0.58	0.575
Age*weight	−0.61	−0.89	0.394
Breed*sex*age	−8.25	−0.73	0.478
Sex*age*weight	1.32	1.10	0.296
Breed*sex*age*weight	0.32	0.60	0.562

The overall regression model was not statistically significant ($R^2 = 0.585$, df = 15, F = 1.429, $P = 0.288$) and * indicates interactions between predictor variables.

using an established hair cortisol extraction. Thus, nail cortisol may be a useful veterinary biomarker for assessing physical and mental dog health as related to stress.

A serial dilution of pooled nail extract ran parallel to the assay standard curve demonstrating that cortisol successfully bound to the antibody without substances in the nail matrix interfering with the steroid–antibody interaction. Furthermore, cortisol concentrations increased with the amount of nail sample extracted, as previously documented for corticosterone in turtle claws [22]. However, this relationship was curvilinear, and recovery did not increase with sample load beyond 50 mg of nail, suggesting this is the maximal sample amount to be extracted. The strong correlation between steroid added and recovered and complete steroid removal from the matrix with DCC pretreatment [35], collectively suggest that nail cortisol values reflected actual embedded cortisol, as opposed to spurious results, antibody cross-reactivity or nonspecific binding.

Nail cortisol concentrations were correlated to the well-established hair cortisol marker after statistical outliers were removed from the hair data. Outliers were present only for the hair data highlighting the variability of this marker which has been the subject of substantial review [36–38]. Hair cortisol concentrations differ between coat colors suggesting that melanin pigments may influence levels [25], as can chemicals products, such as shampoo [23]. Interestingly, the absolute cortisol concentrations in nail were less than in hair, and this may be explained by a slower rate of diffusion from circulation into the nail compared with within the hair follicle. The correlation between nail and hair cortisol suggests that the extracted steroid originated from circulation and not local synthesis within the nail, although this cannot be entirely excluded. Hair cortisol has been used to diagnose hypercortisolism [23,39] and assess interactions with humans in dogs [40], and similar studies can be done using nail cortisol.

Nail cortisol did not differ between males vs females, pure vs mixed breeds, nor dogs of varying ages nor body size. Near-significant trends were apparent but may have been limited by lower statistical power associated with samples size (power = 0.675 <desired 0.80). Previous research has reported differences in circulating cortisol between spayed and intact females [41]. In this study, all dogs were spayed or neutered; thus, the effect of this procedure on nail cortisol is unknown. Plasma cortisol in dogs diagnosed with hyperadrenocorticism was elevated in older individuals [42]. The health status of the dogs in this study was unknown because of confidentiality, and the majority was <10 yr old, and this may have confounded an accurate interpretation of age-related steroid patterns. The dogs in this study were household pets that presumably have been exposed to different environments during the nail growth period (eg, presence of multiple animals or children, different diets, outdoor vs indoor housing) which can lead to individual variation in nail cortisol secretion.

This pilot study has validated SPE and ELISA for the quantification of dog nail cortisol, and future research should specifically test the effects of chronic stress and examine alignment with circulating cortisol. Furthermore, studies of dogs diagnosed with hypoadrenocorticism or hyperadrenocorticism would validate this biomarker in the clinical setting.

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