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Evaluation of Hair Cortisol in the Diagnosis of Hypercortisolism in Dogs

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Background: Measurement of hair cortisol is a noninvasive technique used for several purposes in humans and in animals.

Objectives: To measure hair cortisol concentrations (HCC) in dogs with spontaneous hypercortisolism (HC) and determine whether it can represent a useful diagnostic test for this syndrome.

Animals: Twenty-two dogs with spontaneous HC before treatment, 28 sick control dogs (SCD), and 40 healthy dogs.

Methods: In this prospective, observational clinical study, the HCC was measured by an RIA assay after extraction in HC dogs, in dogs with other chronic diseases, and in healthy dogs. The diagnostic accuracy of HCC was evaluated by subjecting data from dogs with HC and dogs with other chronic diseases to receiver operating characteristic (ROC) curve analysis.

Results: Median (range) cortisol concentration in dogs with HC was 4.53 pg/mg (0.32–74.62 pg/mg) and was significantly higher than in SCD (1.49 pg/mg, 0.13–14.19 pg/mg) and healthy dogs (1.28 pg/mg, 0.34–5.38 pg/mg). Within the 3 groups, there was a large overlap of HCC. The area under the ROC curve was 0.80 (95% CI: 0.67–0.92). A cut-off value of HCC of 1.93 pg/mg revealed 91% sensitivity and 61% specificity to diagnose HC.

Conclusions and Clinical Importance: Hair cortisol concentrations are higher in dogs with HC compared to SCD and healthy dogs. It is a noninvasive technique that should be further investigated as a possible diagnostic procedure for the diagnosis of HC in dogs.

Key words: Cushing's syndrome; Diagnostic test; Hyperadrenocorticism; Hypothalamic-pituitary-adrenal-axis; Steroid hormones.

The use of noninvasive techniques to assess the hypothalamic-pituitary-adrenal axis activity has recently aroused great interest in humans. Measurements of cortisol in serum, urine, and saliva reflect systemic cortisol concentrations at the time of sample collection or shortly before collection but cannot assess past cortisol levels.[1] Hair cortisol concentrations (HCC) are assumed to reflect integrated cortisol secretion over a period of several months in people, and might provide a sensitive marker for stress-associated endocrine changes.[2] In rhesus monkey HCC increases in response to major life stressors.[3] There is a significant correlation between HCC and mean salivary cortisol concentrations provided to support the validity of HCC as an index of long-term cortisol secretion. There is a validated method to measure cortisol concentrations in canine hair.[4]

Although the mechanism of cortisol incorporation into the hair is not fully understood, measurement of HCC permits evaluation of chronic stress in both humans and animals.

Cushing's syndrome (CS), also known as hypercortisolism (HC), consists of signs caused by prolonged exposure to increased glucocorticoid levels. There is higher HCC in CS patients compared to healthy controls.[1] The identification of markedly elevated HCC in Cushing's patients was confirmed by the results of a more recent study.[2]

Abbreviations:

ADH	adrenal-dependent hypercortisolism
CS	Cushing's Syndrome
HC	hypercortisolism
HCC	hair cortisol concentrations
PDH	pituitary-dependent hypercortisolism
SCD	sick control dogs

A diagnosis of HC in dogs is currently based on clinical signs, laboratory findings, and the results of the low-dose dexamethasone suppression test (LDDS test), the ACTH stimulation test (ACTH test), and an increase in the urine cortisol-to-creatinine ratio or various combinations.[5] However, such tests are time consuming and invasive, and some owners find it difficult to collect the urine sample. The development of a noninvasive method requiring sample material that is easy to collect and store would be an advantage in the diagnosis of HC in dogs.

The aim of the study was to measure HCC in dogs with HC and determine whether it can represent a useful diagnostic test for this syndrome. For this purpose, the HCC in healthy dogs, in dogs with HC, and in a group of sick control dogs were compared.

Materials and Methods

Approval for this study was given by the Scientific-Ethics Committee, University of Bologna, Italy.

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Healthy Dogs

Forty clinically healthy dogs, consisting of 14 females (12 spayed) and 26 males (7 neutered), were used. They ranged from 7 to 15 years in age (median, 10 years). Breeds included Epagneul Breton (n = 1), Bracco Italiano (1), Dalmatian (1), Siberian Husky (1), Maremma Sheepdog (1), Pointer (1), Rottweiler (1), Italian short-haired Segugio (1), English Setter (1), Wirehaired Pointing Griffon (1), and mixed-breed dogs (30). The dogs were considered healthy based on the medical history and results of the physical examination.

Dogs with HC

Twenty-two dogs with HC were included in the study. They consisted of 12 females (8 spayed) and 10 males (1 castrated), which ranged in age from 6 to 16 years (median, 12 years). Breeds included Boxer (n = 2), Dachshund (2), Miniature Schnauzer (2), Bolognese (1), Wire haired Dachshund (1), Coton de Toulear (1), Maltese (1), Italian Segugio (1), Yorkshire Terrier (1), and mixed-breed dogs (10). Hematologic, urinary, and biochemical analyses, ACTH stimulation test, and ultrasonographic examination of the adrenal glands were carried out in all dogs. Low-dose dexamethasone suppression test was performed in 16 dogs and endogenous ACTH was measured in 11 dogs. Dogs were included in the study when clinical and laboratory findings were consistent with HC, the LDDS test, ACTH test, or both were positive for HC, treatment with trilostane or adrenalectomy resulted in an adequate response, and no other treatments had been administered. A diagnosis of pituitary-dependent hypercortisolism (PDH) or adrenal-dependent hypercortisolism (ADH) was based on the ultrasonographic appearance of the adrenal glands, the results of the LDDS test, and the concentrations of endogenous ACTH. Pituitary-dependent HC was diagnosed in 17 dogs and ADH in 5 dogs. All dogs with HC were consecutively enrolled at the time of diagnosis and before treatment.

Sick Control Dogs (SCD)

Twenty-eight sick dogs were included in the study. The Sick Control Dogs (SCD) older than 6 years were consecutively admitted when illness was first diagnosed. They consisted of 15 females (8 spayed) and 13 males (3 castrated), which ranged in age from 6 to 15 years (median 10 years). Breeds included Siberian Husky (n = 3), Border Collie (2), Medium Schnauzer (2), Miniature Poodle (2), Airedale Terrier (1), Bearded Collie (1), Boxer (1), Cairn Terrier (1), English Setter (1), Terranova (1), Volpino Italiano (1), and mixed-breed dogs (12). The diseases of the dogs included diabetes mellitus (6), nonclassified neoplasia (3), heart failure (3), hypothyroidism (2), diabetic ketoacidosis (2), pyometra (2), chronic hepatopathy (2), chronic kidney disease (1), prostatic carcinoma (1), pancreatitis (1), dirofilaria immittis infection (1), insulinoma (1), thyroid neoplasia (1), liver neoplasia (1), osteosarcoma (1), mammary carcinoma (1), protein losing enteropathy (1), hypoglycemic syndrome (1), and prosencephalic syndrome (1). Four dogs had more than 1 disease at the same time. Dogs that had received glucocorticoids within the previous 60 days were not enrolled.

Sample Collection and Endocrine Tests

Hair samples were collected from the xiphoid region by shaving with an electronic shaver to the level of skin for each dog. The shaving area was about 9–10 cm^2.

Hair samples were identified, labeled, and stored at room temperature until analysis. Blood samples for the determination of endogenous ACTH concentrations were collected from the jugular vein or cephalic vein into EDTA-coated plastic tubes placed on ice. The samples were immediately centrifuged at 4°C, 500 × *g* for 8 minutes, and plasma was immediately transferred to plastic tubes and stored at −80°C until analysis. ACTH stimulation test and LDDS test were performed by injecting intravenous tetracosactide esacetate[a] and dexamethasone,[b] respectively, as previously described.[5]

Extraction from Hair and Hormone Determinations

The extraction of the cortisol from the hair was performed as described by Accorsi et al.[4] Hair was first minced into 1–3 mm length fragments and 60 mg of trimmed hair was placed in a glass vial. Methanol (concentration ≥99.9%) was added and the vials were incubated at +50°C by gentle shaking for 18 hours.

The vials' contents were then filtered to separate the liquid phase. The latter was evaporated to dryness under an air-stream suction hood at 37°C and dry residue was then dissolved into PBS 0.05 M, pH 7.5. Cortisol concentrations were determined by RIA based on ^{3}H-steroid by competitive adsorption.[6] Analysis was performed in duplicate. Parameters for the analysis validation were as follows: sensitivity 0.26 pg/mg, intra-assay variability 6.8%, inter-assay variability 9.3%, specificity (%): cortisol 100, corticosterone 9.5, 11α-hydrossi-progesterone 8.3, cortisone 5.3, 11α-desossicortisol 5.0, progesterone 0.6, desossicorticosterone 0.5, 20α-dihydrocortisone 0.4, testosterone 0.3, aldosterone 0.1, dehydroepiandrosterone, 5α-pregnenolone, 17β-estradiolo, cholesterol <.0001. All of the concentrations were expressed in pg/mg of hair shaft. Serum cortisol and plasma ACTH concentrations were determined with kits[c,d] that have been validated previously for use in dogs.[7,8]

Statistical Analysis

Results were analyzed by nonparametric statistics.[e,f] Median and ranges are given. Kruskal–Wallis test followed by Dunn's Multiple Comparison Test was performed to compare data from dogs with HC, SCD, and healthy dogs. Categorical data were compared by Fisher's exact test or χ^2-test depending on the number of cases in each group. Mann–Whitney *U*-test was performed to determine whether dogs with PDH or ADH differed significantly in terms of HCC. The correlations between HCC and serum cortisol concentrations before and after administration of ACTH were determined using Spearman's test. A receiving operating characteristic (ROC) curve was used to determine the area under the curve (AUC) and select the optimum HCC cut-off values to diagnose or exclude a state of HC. Ninety-five percent confidence intervals were calculated for ROC curves. A P value <.05 was considered significant.

Results

Age, sex, neutered status, and breed distribution were not significantly different between groups. Median body weight in dogs with HC (10.2 kg, range 3.1–45.7 kg) was significantly ($P = .03$) lower compared with SCD (23.5 kg, range 5.0–55.6 kg). Hair cortisol concentration was 4.53 pg/mg (0.32–74.62 pg/mg), 1.49 pg/mg (0.13–14.19 pg/mg), and 1.28 pg/mg (0.34–5.38 pg/mg) in dogs with HC, SCD, and healthy dogs, respectively. Hair cortisol concentrations in dogs with HC were significantly higher ($P < .001$) compared with SCD and healthy dogs. There was a great deal of overlap in HCC between groups (Fig 1). No significant

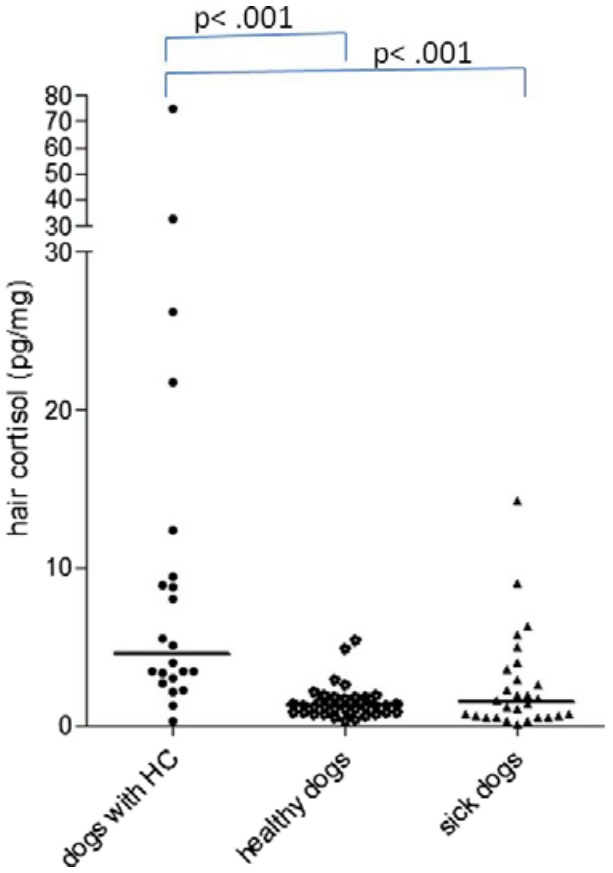

Fig 1. Hair cortisol concentrations (HCC) in dogs with hypercortisolism (HC) (n = 22), healthy dogs (n = 40), and sick control dogs (SCD) (n = 28). Dogs with HC have significantly higher HCC than healthy dogs and SCD.

differences in HCC between SCD and healthy dogs were observed. Hair cortisol concentrations in dogs with PDH (5.13 pg/mg, 0.32–74.62 pg/mg) were not significantly different (P = .87) from dogs with adrenal-dependent HC (3.94 pg/mg, 2.10–21.76 pg/mg). No correlations were found between HCC and basal serum cortisol concentrations (P = .85) nor between HCC and post-ACTH serum cortisol values (P = .46). Figure 2 shows the ROC curve for the HCC. The area under the ROC curve was 0.80 (95% CI: 0.67–0.92). A cut-off value of HCC 1.93 pg/mg revealed 91% sensitivity (95% CI: 70.8–98.9) and 61% specificity (95% CI: 40.9–78.5) with a positive likelihood ratio and a negative likelihood ratio of 2.55 and of 0.14 to diagnose HC.

Discussion

The present study indicates that HCC is significantly higher in dogs with HC compared to SCD and healthy control dogs. This findings support the hypothesis that increased HCC reflect excessive endogenous cortisol secretion and are in accordance with studies performed in humans where the CS patients could be distinguished from healthy controls based on their HCC.[1,2]

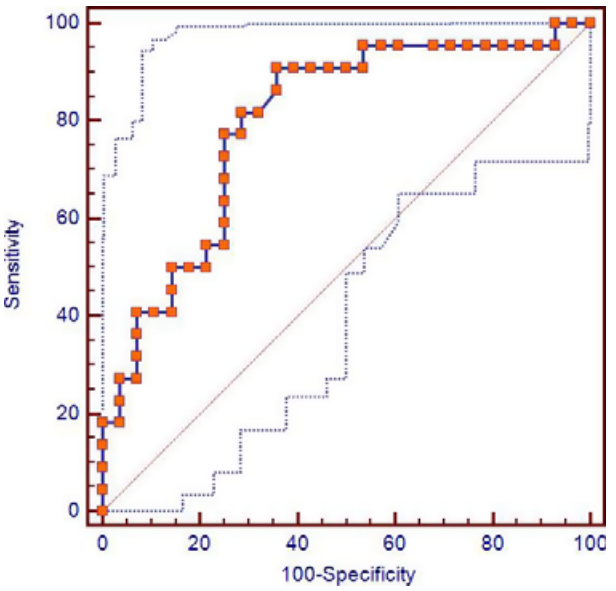

Fig 2. Receiver operating characteristics (ROC) curve for the hair cortisol concentrations when used to distinguish between dogs with hypercortisolism and sick control dogs. The line with the squares is the ROC curve and the dashed gray lines represent the 95% confidence interval. The solid central line represent the null hypothesis (area under the ROC curve = 0.5).

HCC moved parallel to the clinical course of disease and HCC levels decrease after treatment in humans.[1,2] In our study, we measured HCC only at the time of diagnosis of HC; therefore, further evaluations are needed to investigate whether the treatment of HC produces a decrease in HCC in dogs. Hair samples were collected from the xiphoid region for a practical reason and for better compliance of the owners. Abdominal ultrasonography is commonly performed in the diagnostic work-up of dogs with HC and to perform such procedure the abdominal region is usually shaved; furthermore, hair that is shaved in the xiphoid region does not produce any evident aesthetic damage to the dog.

Hair growth patterns in humans vary across different regions of the scalp, which could potentially influence the HCC.[9] It is unknown if in dogs the hair growth and HCC levels are different if measured in other regions of the body but further studies are needed to investigate such hypothesis.

The AUC measures the probability of a correct diagnostic classification, and the value of 0.80 found in the present study indicates a moderately discriminative power. The sensitivity at HCC 1.93 pg/mg was 91% (95% CI: 70.8–98.9%). Accordingly, the specificity at HCC 1.93 pg/mg was only 61% (95% CI: 40.9–78.5%). In light of these findings, to overcome low specificity, HCC seems to be a noninvasive technique that could be used in dogs with a high suspicion of HC.

The analysis of cortisol in hair in human medicine constitutes a highly promising method for the

retrospective assessment of integrated cortisol secretion over extended periods of time[9] and segmental analysis of the hair can provide historical information of the patient for some months before the time of sampling.[1] One study performed in healthy dogs was unable to find any significant differences in the HCC between the proximal and the distal hair sections.[10]

An important point in this respect concerns the question of whether local cortisol production in the hair follicle also adds to HCC. In guinea pigs, a study showed that only very limited amounts of systemically administered radioactive cortisol were deposited in the hair, while at the same time large amounts of unlabeled glucocorticoids could be found.[11] This strongly suggests local production in hair follicles. Therefore, it is still unclear to what extent, for example, local skin irritation alters glucocorticoid levels in hair and further studies on this topic are needed. Indeed in humans, the hair follicle (in vitro) itself is effective in producing cortisol following CRH stimulation, and thus is equivalent to the hypothalamic-pituitary-adrenal axis. However, evidence from studies showing a close correspondence between HCC and conditions with well-defined changes in classical hypothalamic-pituitary-adrenal axis components supports the notion that HCC sensitively reflects systemic cortisol levels and, in turn, may only be marginally influenced by local cortisol production.[12]

Hypercortisolism is reported to have predominance of hairless and atrophic telogen follicles[13] and this aspect could potentially influence the HCC. Further studies aimed to evaluate the influence of the hair cycle on HCC are recommended.

The present study had a number of limitations, such as the lack of evaluation of the color of the hair sampled. One study found a relationship between hair color and HCC in healthy dogs; eumelanin (black) hairs are lower in cortisol than pheomelanin (yellow) hairs.[10] We do not know whether the color of hair sampled was homogeneous in the 3 groups of dogs tested here. However, as animals were randomly selected and no breed within each group was overrepresented, it is unlikely that a specific hair color would have been significantly more present in 1 group. Moreover, it is not known if the color of the hair, like in healthy dogs, also influences the HCC in sick dogs and in dogs with HC. Furthermore, the mild influence of the hair color, unlikely would change the clinical interpretation of the HCC in dogs with HC. Another limitation is that we did not evaluate the washing procedures before sample collection. Frequent washing procedures may partially influence HCC in humans,[9] but is unknown if a similar condition is present in dogs. However, the substantial lower frequency of hair washing procedure in dogs compared with humans makes improbable that this aspect could significantly influence the HCC in dogs.

As with other screening tests for HC, HCC cannot differentiate dogs with PDH from dogs with ADH. Other tests, such endogenous plasma ACTH determination, the LDDS test, high-dose dexamethasone suppression test, and diagnostic imaging are helpful in making this distinction.[5]

In conclusion, this is the 1st study to document that hair cortisol levels are high in dogs with HC. The main advantage of this method is that hair sampling is an easy procedure. Hair cortisol concentrations were significantly higher in HC dogs compared with healthy dogs and SCD, but a large overlap of results was observed. Because of the lack of specificity, this test could be considered a noninvasive procedure only in dogs with a high suspicion of HC.

Footnotes

[a] Synacthen, Novartis, Origgio, Italy
[b] Dexadreson, dexamethasone phosphate, 2 mg/mL; Intervet, Peschiera Borromeo, Italy
[c] Immulite cortisol, Diagnostic Product Corporation, Los Angeles, CA
[d] Immulite ACTH, Diagnostic Product Corporation
[e] GraphPad Prism 5, GraphPad Software Inc, San Diego, CA
[f] MedCalc 10.2.0.0, Mariakerke, Belgium

Acknowledgments

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Conflict of Interest: Authors disclose no conflict of interest.

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