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Measurement of cortisol in dog hair: a noninvasive tool for the diagnosis of hypercortisolism

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Background – The clinical signs of hyperadrenocorticism (hypercortisolism) in dogs are known to be caused by chronic overexposure to glucocorticoids. The quantification of cortisol in serum, saliva or urine reflects the cortisol concentration at the time of sample collection, but in suspected hyperadrenocorticism it may be preferable to examine a long-term parameter of cortisol production.

Hypothesis/Objectives – There is a need for a noninvasive method to monitor the long-term production of cortisol in dogs. It seems possible that measuring cortisol levels in hair could represent such a method.

Animals – Hair was collected from 12 dogs with hyperadrenocorticism and from 10 healthy control dogs.

Methods – Immunoreactive cortisol, cortisone and corticosterone concentrations were determined by enzyme immunoassay. High-performance liquid chromatography was performed to test the validity of the cortisol assay.

Results – Levels of immunoreactive cortisol, cortisone and corticosterone were significantly higher in dogs with hyperadrenocorticism than in control dogs. The difference was most pronounced for the cortisol level.

Conclusions and clinical importance – The determination of cortisol in hair offers the advantage that sampling is easier and less invasive than taking blood, urine, faeces or saliva. Measuring cortisol in hair may represent a valuable tool for the diagnosis of hyperadrenocorticism in dogs.

Introduction

Hyperadrenocorticism (HAC) is a complex of physical and biochemical changes resulting from chronic overexposure to glucocorticoids, especially to cortisol. Clinical signs in the dog include polyuria, polydipsia, polyphagia, a pot-bellied appearance and typical skin and hair changes, such as bilateral alopecia.[1] The most frequent cause of the disease is a tumour of the pituitary gland, which secretes adrenocorticotrophic hormone (ACTH) and stimulates the adrenal glands to produce glucocorticoids. The standard approach for diagnosing HAC in dogs involves measuring the activity of the hypothalamic–pituitary–adrenal axis by testing the urinary corticoid-to-creatinine ratio or by an invasive method such as the ACTH stimulation test and/or the low-dose dexamethasone test.[1]

Glucocorticoid concentrations may be measured in a variety of biological samples, including plasma, saliva, urine and hair.[2–6] Compared with taking other types of samples, hair sampling is noninvasive and painless. In addition, the analysis of cortisol in hair provides long-term information about glucocorticoid production rather than about the concentrations at the time of sample collection. In human medicine, hair cortisol analysis has proved to be a useful tool in the diagnosis of chronic pain, stress, drug exposure and hyperadrenocorticism.[5,7–10]

The aim of this study was to investigate whether dogs known to have HAC have higher levels of cortisol in hair. If so, the determination of cortisol in hair samples would represent a useful and noninvasive tool for the preliminary diagnosis of HAC (hypercortisolism) in dogs.

Materials and methods

Animals

Two groups of dogs were evaluated in the study. Dogs (n = 12) with hyperadrenocorticism were obtained from the Clinic for Small Animals at the University of Veterinary Medicine, Vienna. The ages of the animals ranged from 9 to 14 years (median 11 years) and their weights ranged from 2.8 to 40 kg (median 9.25 kg). There were eight females (four entire and four spayed) and four males (entire). Breeds included dachshund (n = 2), Staffordshire terrier (n = 1), Maltese dog (n = 1), Newfoundland (n = 1), poodle (n = 2), doberman (n = 1) and four mixed breed dogs (n = 4). All dogs showed clinical signs of HAC (polyuria, polydipsia, polyphagia or dermatological problems). Each was subjected to a thorough physical examination, and blood samples were taken for haematology and serum biochemical analysis. The diagnosis of HAC was confirmed by screening procedures (urinary corticoid-to-creatinine ratio, ACTH stimulation test and low-dose dexamethasone test) using previously described tests.[1,11] In addition, the adrenal glands were examined ultrasonographically. Only animals with positive results in at least three tests were included in the HAC group.

Dogs (n = 10) in the control group were 6–10 years of age (median 8.5 years), weighed 6–40 kg (median 31 kg) and comprised two

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females and eight males. All were sexually intact. Breeds included Labrador retriever (n = 2), dachshund (n = 2), Magyar Viszla (n = 1), English springer spaniel (n = 1), German shepherd (n = 1) and three mixed breed dogs (n = 3). The dogs were owned by colleagues and were considered healthy on the basis of medical history and physical examination.

Hair sampling

The region of the vena cephalica was shaved as though for blood sampling. Hair was collected before blood sampling. Samples were labelled and stored at room temperature until analysis.

Extraction of hormones

Hair was cut into 1–3 mm fragments, washed with 7 mL hexane to remove apolar lipids from the surface and dried. Fifty milligrams was mixed in a glass vial with 5 mL methanol, and samples were extracted at room temperature overnight (14 h). After centrifugation (1500 ***g***, 5 min), the supernatant was decanted and the organic solvent evaporated at 60°C under a stream of nitrogen. Extracts were redissolved in 500 μL assay buffer as previously described,[12] and 10 μL was used for enzyme immunoassay (EIA).

Enzyme immunoassay

The EIAs were in-house assays and were performed for cortisol and corticosterone and for cortisone, as previously described.[13,14] In brief, antibodies were raised in rabbits against cortisol-3-carboxymethyloxime (CMO), corticosterone-3-CMO and cortisone-21-hemisuccinate linked to bovine serum albumin. For producing the labels, the steroids were linked to biotin (EZ-Link Biotin PEO-Amine ((+))-Biotinyl-3, 6-dioxaoctanediamine; Pierce - Fisher Scientific Inc., Rockford, IL, USA) and the products purified by high-performance liquid chromatography (HPLC). To fix the anti-steroid antibodies to microtitre plates, the plates were first coated with 50 μg Protein A (Sigma P-7837; Sigma-Aldrich Handels GmbH, Vienna, Austria). Biotinylated steroid was detected by streptavidin–horseradish peroxidase (Roche Diagnostics GmbH, Mannheim, Germany) using 3,3′,5,5′-tetramethylbenzidine.

Possible influences on the hormone concentrations in canine hair were investigated by analysing the concentration of androstenedione, as previously described.[12]

To test the validity of the cortisol assay, HPLC was performed on extracts of hair samples of control dogs. The extracts were purified with C18 Sep-Pak Cartridges (Waters Corp., Milford, MA, USA). After priming the cartridge, the extract was diluted in 5 mL distilled water and extracted. The column was rinsed with 5 mL distilled water and elution performed with 5 mL methanol. After evaporation, the extract was redissolved in 100 μL of 30% methanol. For chromatography, a Novapak C18 column (3.9 mm × 150 mm; Waters Corp.) was used. For HPLC, a linear methanol gradient from 30 to 60% was applied with a flow rate of 1 mL/min. Three fractions were collected per minute, and the concentration of immune-reactive cortisol was measured.

Statistical analysis

According to the Kolmogorov–Smirnov test the data were not distributed normally, so a box plot was used to depict the glucocorticoid concentrations in the two groups. To compare the results of the assays between groups, a Mann–Whitney rank sum test was performed using the SigmaStat 3.1 software (Systat Software Inc., San Jose, CA, USA). Mean differences were considered significant when $P < 0.05$.

To test the linearity of the assay in extracts of hair samples, we performed a serial dilution of the extracts from the hair of five animals and measured the cortisol concentrations. Dilution had no significant influence on the results expressed as nanograms per gram of hair. The intra-assay coefficient of variation for the cortisol assay was 3.5%.

The Spearman rank order correlation test was used to test the correlation of the post-ACTH cortisol concentration in the blood compared with the hair cortisol concentration.

Results

Levels of all three glucocorticoids (cortisol, cortisone and corticosterone) differed significantly between the two groups. In the HAC group, the median immunoreactive cortisol concentration was 5.6 ng/g, compared with 1.50 ng/g in the control group (Figure 1). Hair cortisol concentrations were thus higher in dogs with known hypercortisolism ($P < 0.006$). The median immunoreactive corticosterone content in all 22 samples was 19.8 ng/g. Dogs with HAC had higher values (25.4 ng/g) than healthy dogs (8.4 ng/g). The positive correlation between high corticosterone concentrations and disease signs was statistically significant ($P < 0.015$). The median concentration of immunoreactive cortisone in the samples was 52.5 ng/g; again, dogs with HAC had higher values (65.4 ng/g) than healthy dogs (33.3 ng/g), and the values were positively correlated ($P < 0.003$). In contrast, no significant differences in androstenedione levels were found between the two groups (Figure 1).

As the cortisol assay revealed the greatest differences between the two groups, the validity of the test was assessed by HPLC. As shown (Figure 2), two immunoreactive peaks were eluted from the column, one with the same elution pattern as cortisol, while the other eluted with the same retention time as cortisone.

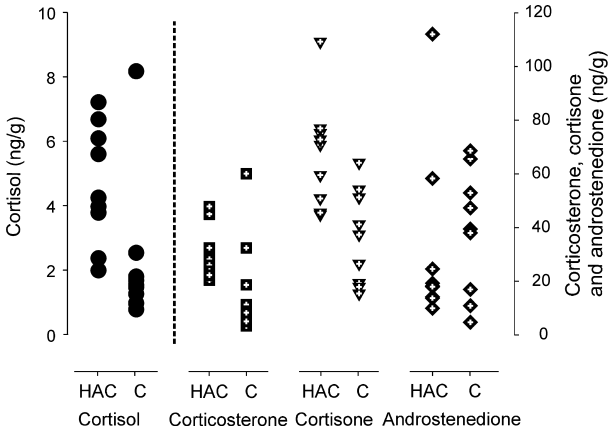

Figure 1. Immunoreactive cortisol, corticosterone, cortisone and androstendione levels in hair in dogs with hypercortisolism (HAC; n = 12) and in healthy dogs (C; n = 10).

The cortisol–cortisone correlation was significant ($P \leq 0.001$, Spearman rank order correlation). There was no significant association between the post-ACTH blood cortisol concentration and the hair cortisol concentration (Figure 3).

Discussion

Several studies have shown that cortisol can be detected in hair.[3–5,10,15,16] Skin from humans[17,18] and dogs[19–22] is able to produce cortisol. The source of the hormone is not clear, and various possibilities have been considered in the literature (for example see Skobowiat *et al.*).[19] The concentration of cortisol determined in dog hair has previously been shown to correlate with that measured in faeces[4] and in saliva.[6] We are not aware of any data relating to the diagnosis of hypercortisolism (HAC) based on cortisol determination in the hair of dogs. This is surprising, because hair cortisol concentrations are expected to change more gradually than concentrations in blood plasma and thus hair would seem to represent an ideal type of sample for analysing the average concentrations of glucocorticoid metabolites in dogs over time.

In humans, there is a positive association between increased adrenal activity and elevated concentrations of glucocorticoids in the hair, with concentrations of cortisol in the hair being higher in humans with HAC.[9,23] This finding is consistent with the present results in dogs, although in contrast to the situation in humans we generally found higher concentrations of cortisone than of cortisol in dog hair. The difference between the species could be a result of different activities of the 11β-hydroxysteroid dehydrogenase. Alternatively, it is possible that the two compounds are taken up differently into the hair because of their different affinities to the corticoid-binding protein or simply because of the lower polarity of cortisone, which means that it can be better absorbed by the hair. The finding that androstenedione concentrations did not differ between the two study groups shows that the higher cortisone values are not due to different fat concentrations in the skin or to increased activity of the sebaceous glands, because androstenedione is fairly lipophilic.

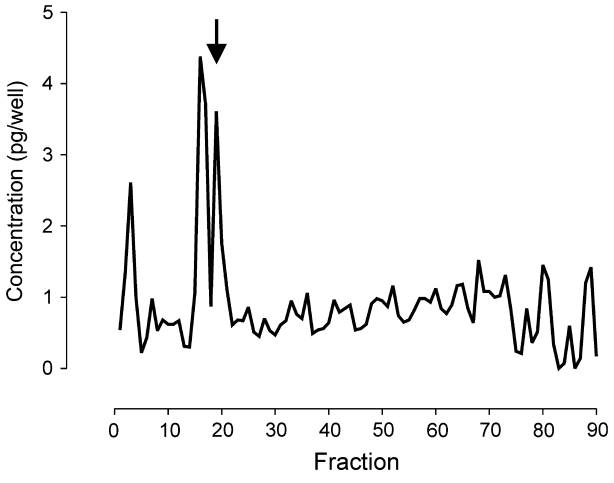

Figure 2. High-performance liquid chromatography elution pattern of cortisol-immunoreactive substances. The arrow represents the elution position of the cortisol standard.

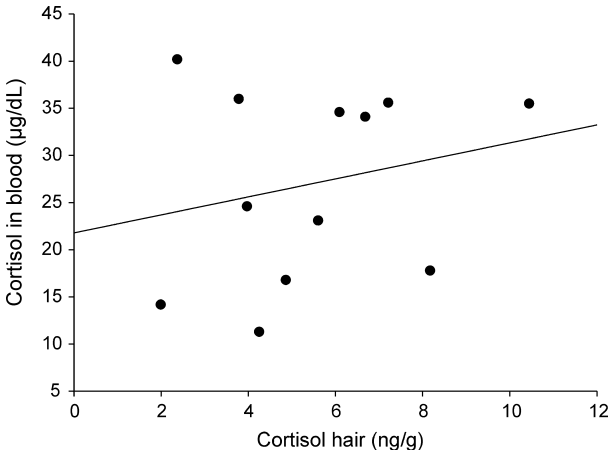

Figure 3. Comparison of individual levels of immunoreactive cortisol in hair and blood in dogs with hypercortisolism ($n = 12$).

Hair treatment seems to be associated with lower concentrations of cortisol in hair in humans,[24] so it will be important to investigate whether washing, special shampoos and lotions or frequent swimming may influence the validity of the test.[25] It would also be important to investigate whether there are differences between pigmented and nonpigmented hair,[19] between different body areas (in this study only hair from the region of the vena cephalica was used) or between animals with different patterns of hair growth[9] or body mass indexes.[26,27]

It should be noted that the HAC group and the control group differed in median age and, more importantly, in size and body weight, although the body mass index was not analysed. In general, HAC is more common in small and medium-sized dogs and tends to appear fairly late in life,[11] so dogs in the HAC group are on average older and smaller than dogs in the control group. The difference between the two groups has no bearing on the results of the study, which was primarily undertaken to assess the usefulness of measuring cortisol concentrations in hair. Nevertheless, further studies with a better matched control group would be beneficial. In contrast to previous studies,[26,27] no positive correlation between hair cortisol and body weight was found in dogs (data not shown).

In humans, hair grows at an average rate of one centimetre per month, so differences in hormone content along the length of the hair fibres can be used to give an indication of variations in cortisol exposure over the past weeks or months. Cortisol may be measured in all segments of the hair shaft in humans.[9] In dogs, the hair follicle has a lifelong capacity for producing hair growth. Hormonal changes are known to affect the hair cycle; high levels of glucocorticoids reduce the rate of hair growth.[28] In addition, seasonal and breed variation may lead to different patterns of hair growth and moult. For these reasons, it is not clear whether it would be possible to monitor the development of hypercortisolism in dogs by examining successive samples along hair fibres. Additional work would be required to address this aspect.

In guinea-pigs it was shown that systemically administered radioactive cortisol was deposited in the hair only in small amounts, whereas the immunoreactive glucocorticoid concentration in hair was fairly high;

therefore, the authors suggested a local production of glucocorticoids in hair follicles.[29] In dogs with pituitary-dependent HAC, the ACTH production may cause an increased glucocorticoid production by the skin, and the source of the glucocorticoids measured in the hair of the dogs remains unknown. Hyperadrenocorticism is analogous to a permanent stress response and thus the cortisol value is high in saliva, urine, faeces, blood and hair. In dogs with suspected pituitary-dependent HAC, the measurement of cortisol in hair offers numerous benefits. It is a noninvasive and painless method. Hair can be collected easily at any time of the day or year and can be stored and posted at room temperature. It should be noted that the validation experiment shows that the cortisol assay cross-reacts with other metabolites, so the results should be interpreted as cortisol-immunoreactive substances rather than necessarily cortisol itself. Nevertheless, the correlation between the measured hormone levels and the clinical tests shows the usefulness of the method for an initial diagnosis.

In summary, it has been shown that hair cortisol is elevated in dogs with HAC (hypercortisolism). Measurement of cortisol in hair thus represents a novel method for assessing glucocorticoid overproduction in dogs and should be explored further as a noninvasive method for diagnosing HAC in dogs.

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Résumé

Contexte – Les signes cliniques de l'hyperadrénocorticisme chez le chien sont connus pour être dus à une exposition chronique aux glucocorticoïdes. La quantification du cortisol sérique, salivaire ou urinaire reflète la concentration en cortisol au moment de la collection de l'échantillon mais dans les cas d'hyperadrénocorticisme suspectés il peut être préférable d'examiner un paramètre de production du cortisol au long-cours.

Hypothèses/Objectifs – Il existe un besoin de méthode non-invasive pour suivre la production au long-cours du cortisol chez le chien. Il semble possible que la mesure des taux de cortisol dans les poils puisse être une telle méthode.
Sujets – Les poils ont été collectés sur 12 chiens atteints d'hyperadrénocorticisme et sur 10 chiens sains de contrôle.
Méthodes – Les concentrations de cortisol, cortisone et corticostérone immunoréactifs ont été déterminés par test immuno-enzymatique. Une chromatographie liquide à haute performance a été réalisée pour vérifier la validité de l'analyse du cortisol.
Résultats – Les taux de cortisol, cortisone et corticostérone immunoréactifs étaient significativement plus élevés chez les chiens atteins d'hyperadrénocorticisme que chez les chiens contrôles. La différence était plus prononcée pour les taux de cortisol.
Conclusions et importance clinique – La détermination du cortisol dans les poils, offre l'avantage d'un prélèvement simple et moins invasif que la prise de sang, d'urine, de fèces ou de salive. La mesure du cortisol dans les poils peut s'avérer être un outil valable pour le diagnostic de l'hyperadrénocorticisme chez le chien.

Resumen
Introducción – los signos clínicos de hiperadrenocorticismo (hipercortisolismo) en perros pueden ser causados por la exposición crónica a gluocorticoides. La cuantificación del cortisol en suero, saliva y orina refleja la concentración de cortisol en el momento de la toma de muestras, pero en casos sospechosos de hiperadrenocorticismo puede ser preferible examinar un parámetro a largo plazo de la producción de cortisol.
Hipotesis/Objetivos – se necesita un método no invasivo para controlar la producción de cortisol a largo plazo en perros. Puede ser posible que la evaluación de los niveles de cortisol en pelos represente dicho método.
Animales – se obtuvo pelo de 12 perros con hiperadrenocorticismo y de 10 perros control sanos.
Métodos – se determinaron las concentraciones de cortisol, cortisona y corticosterona inmunoreactivos mediante prueba de enzimoinmunoensayo. Se utilizó cromatografía liquida de alta resolución para validar el ensayo de cortisol
Resultados – los niveles de cortisol, cortisona y corticosterona inmunoreactivos fueron significativamente mayores en perros con hiperadrenocorticismo que en perros control. La diferencia fue mas pronunciada en los niveles de cortisol.
Conclusiones e importancia clínica – la determinación del cortisol en pelos ofrece la ventaja que la toma de muestras es mas fácil y menos invasiva que la toma de sangre, orina, heces o saliva. La evaluación del cortisol en pelos puede representar una herramienta útil para el diagnostico de hiperadrenocorticismo en perros.

Zusammenfassung
Hintergrund – Die klinischen Symptome des Hyperadrenocorticismus (Hypercortisolismus) bei Hunden werden bekanntlich durch ein Übermaß an Glukokortikoiden verursacht. Die Quantifizierung des Cortisols im Serum, im Speichel oder im Urin reflektiert die Cortisolkonzentraion zum Zeitpunkt der Probenahme, aber bei Verdacht auf Hyperadrenocorticismus könnte es besser sein, einen Langzeitparameter der Cortisolproduktion zu untersuchen.
Hypothese/Ziele – Es besteht der Bedarf einer nicht-invasiven Methode, die Langzeitproduktion von Cortisol bei Hunden zu überwachen. Die Messung von Cortisolwerten im Haar scheint eine derartige Methode darzustellen.
Tiere – Es wurden Haare von 12 Hunden mit Hyperadrenocorticismus und von 10 gesunden Kontrollhunden gesammelt.
Methoden – Konzentrationen des immunreaktiven Cortisols, Cortison und Corticosteron wurden mittels Enzymimmunassay bestimmt. Eine Hochleistungsflüssigkeitschromatographie wurde durchgeführt, um die Validität des Cortisol Assays zu testen.
Ergebnisse – Die Werte des immunreaktiven Cortisols, des Cortisons und des Corticosterons waren signifikant höher bei Hunden mit Hyperadrenocorticismus als bei Kontrollhunden. Der Unterschied war beim Cortisolwert am markantesten.
Schlussfolgerungen und klinische Bedeutung – Die Cortisolbestimmung im Haar bietet den Vorteil der leichteren Probenahme, die auch weniger invasiv als die Entnahme von Blut, Urin, Kot oder Speichel ist. Die Messung von Cortisol im Haar könnte ein wertvolles Hilfsmittel für die Diagnose des Hyperadrenocorticismus bei Hunden sein.

要約

背景 – イヌの副腎皮質機能亢進症(副腎皮質ホルモン過剰症)の臨床症状は糖質コルチコイドに対する慢性的な暴露過剰により生じることが知られている。血清、唾液、あるいは尿中におけるコルチゾールの定量化はサンプル回収時のコルチゾール濃度を反映するが、しかし副腎皮質機能亢進症が疑われる場合はコルチゾール産生の長期間におけるパラメーターの検査が望ましいと思われる。

仮説/目的 – イヌにおけるコルチゾールの長期間の産生をモニターするための非侵襲的な方法が必要とされている。被毛のコルチゾール値を測定することが 1 つの方法として可能と思われる。

供与動物 – 副腎皮質機能亢進症の12頭のイヌおよび10頭の健常なコントロール犬から被毛を回収した。

方法 – 免疫反応性コルチゾール、コルチゾン、ならびにコルチコステロン濃度を酵素免疫測定法で測定した。高性能液体クロマトグラフィーをコルチゾール分析の有効性を検査するために行った。

結果 – 免疫反応性コルチゾール、コルチゾン、ならびにコルチコステロンの値は副腎皮質機能亢進症のイヌでコントロール犬と比較し有意に高かった。その差はコルチゾール値で最も顕著であった。

結論および臨床的な重要性 – 被毛におけるコルチゾールの測定はサンプリングが血液、尿、便あるいは唾液の採取に比べてより容易でより侵襲が少ないという利点がある。被毛のコルチゾール測定はイヌの副腎皮質機能亢進症の診断にとって有益な手段となるかもしれない。

摘要

背景 – 已知犬肾上腺皮质机能亢进（皮质醇增多症）的临床症状，是由慢性糖皮质激素分泌过度所引起。血清、唾液或尿液皮质醇定量反映出样本收集时的皮质醇浓度，但怀疑有肾上腺皮质机能亢进时，可能长期监测皮质醇值更理想。

假设/目的 – 需要找到一种无侵袭性的方法，以长期监测犬皮质醇的产生。测量毛发中皮质醇水平或许可行。

动物 – 收集12只肾上腺皮质机能亢进患犬和10只健康对照犬的毛发。

方法 – 通过酶免疫分析测定免疫反应性皮质醇、可的松和皮质酮浓度。进行高性能液相色谱分析检测皮质醇试验的有效性。

结果 – 肾上腺皮质机能亢进患犬的反应性皮质醇、可的松和皮质酮水平显著高于对照犬。皮质醇水平的差异更显著。

结论和临床价值 – 与采血、尿、粪便和唾液相比，毛发皮质醇检测的优势是采样方便，伤害更小。测量毛发皮质醇可能能作为诊断犬肾上腺皮质机能亢进的一个有效方法。