Research in Veterinary Science 152 (2022) 207–211

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Evaluation of the ACTH stimulation test using a low dose of a depot formulation in healthy dogs and in dogs with untreated Cushing's syndrome

Carlos Melián [a,b], Beatriz Blanco [c], Pedro J. Ginel [c], Laura Pérez-López [b,*]

[a] Department of Animal Pathology, Faculty of Veterinary Medicine, University of Las Palmas de Gran Canaria, 35413 Arucas, Las Palmas, Spain
[b] University Institute of Biomedical and Health Research, Paseo Blas Cabrera Felipe s/n, 35016 Las Palmas de Gran Canaria, Spain
[c] Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Córdoba, 14005 Córdoba, Spain

ARTICLE INFO

Keywords:
Adrenocorticotropic hormone
Cushing's syndrome
Hypercortisolism
Adrenal gland

ABSTRACT

The sensitivity of the adrenocorticotropic hormone (ACTH) stimulation test to detect Cushing's Syndrome (CS) using a depot formulation needs to be evaluated. The aims of this study were to propose a reference interval (RI) for cortisol values 1-hour after administration of a low-dose of depot ACTH in healthy dogs, and to evaluate the sensitivity of this test to detect CS, differentiating among types of CS based on ultrasound findings.

Forty-one healthy dogs (20 males, 21 females) were prospectively included. Additionally, 90 dogs with CS (31 males, 59 females) were retrospectively included. Dogs with CS were ultrasonographically classified as follows: 44 dogs with symmetrical adrenomegaly consistent with pituitary-dependent hypercortisolism (PDH), 8 dogs with unilateral adrenomegaly and atrophy of the contralateral adrenal gland or unilateral or bilateral adrenomegaly with malignancy features consistent with adrenal-dependent hypercortisolism (ADH), 34 dogs with equivocal adrenal asymmetry (EAA) and 4 dogs with normal adrenal thickness.

In healthy dogs, lower and upper limit of the 95% RI for 1-hour post-ACTH cortisol concentration and their 90% confidence intervals, were 4.4 (2.7–5.8) μg/dl and 18.4 (16.5–20.0) μg/dl, respectively. Post-ACTH cortisol concentration was above the RI in 90.0% (ci95%, 76.1–100) of dogs with CS. An elevated post-ACTH cortisol concentration was detected in 95.5% (ci95%, 76.1–100) of dogs with PDH, 62.5% (ci95%, 46.1–78.9) of dogs with ADH and 88.2% (ci95%, 69.1–100) of dogs with EAA. The sensitivity of the ACTH stimulation test using a low-dose of depot ACTH in high in dogs with CS.

1. Introduction

The adrenocorticotropic hormone (ACTH) stimulation test has been widely used to confirm the diagnosis of Cushing's syndrome (CS) and to monitor trilostane and mitotane treatment in dogs with CS (Bennaim et al., 2019). Cosyntropin (beta-corticotropin) is a synthetic polypeptide form of ACTH. Cosyntropin is also known as tetracosactrin or tetracosactide, and it is identical to the hormonally active N-terminal 24 residues of both human and canine corticotropin (Papich, 2016).

In dogs, a dose of 250 μg per dog (IM or IV) was initially recommended to perform the ACTH stimulation test, as it is used in humans (Frank et al., 2000; Behrend et al., 2013). However, later studies showed that lower doses of ACTH (ie. 1 or 5 μg/kg) are sufficient to produce a maximal stimulation of the adrenal cortex (Kerl et al., 1999; Frank et al., 2000; Aldridge et al., 2016) and currently a dose of 5 μg/kg is generally recommended (Bennaim et al., 2019).

Cosyntropin availability differs between countries and it has been limited at certain time points. A depot formulation of synthetic ACTH is commercially available in some countries and it is used both for diagnostic and treatment purposes in humans. This depot formulation should be administered IM and it has an inorganic zinc complex that adsorbs the active substance and permits its lengthy release (Nuvacthen depot package insert, 2018). Recent studies have shown that depot formulation of tetracosactide may represent an alternative to the non-adsorbed

Abbreviations: ACTH, adrenocorticotropic hormone; CS, Cushing's syndrome; UCCR, Urinary corticoid creatinine ratio; DVTDR, Dorsoventral thickness difference ratio; LDV, Dorsoventral thickness of the larger gland; SDV, Dorsoventral thickness of the smaller gland; ADH, Adrenal-dependent hypercortisolism; PDH, Pituitary dependent hypercortisolism; EAA, Equivocal adrenal asymmetry.

* Corresponding author.
E-mail address: laura.perezlopez@ulpgc.es (L. Pérez-López).

https://doi.org/10.1016/j.rvsc.2022.08.010
Received 28 September 2021; Received in revised form 19 May 2022; Accepted 8 August 2022
Available online 12 August 2022

synthetic ACTH products in dogs (Ginel et al., 2012; Sieber-Ruckstuh et al., 2015).

Depot tetracosactide (250 μg/kg IM) and cosyntropin (5 μg/kg IV or IM) produced similar cortisol responses at 60 min after administration in healthy dogs (Behrend et al., 2006). The use of tetracosactide depot at dosages of 5 μg/kg or below in dogs with CS have been evaluated in a few studies (Ginel et al., 2012; Sieber-Ruckstuh et al., 2015). However, studies including a larger number of animals and reporting diagnostic performance of the test are still needed.

The aims of this study were: First, to propose reference intervals for cortisol values 1-hour after administration of a low-dose of depot ACTH in healthy dogs. Secondly, to evaluate the sensitivity of this test to detect CS. And finally, to evaluate the sensitivity of this test to detect CS in dogs with different types of CS based on ultrasonographic classification.

2. Material and methods

2.1. Animals and study design

The study was performed at the Veterinary Teaching Hospital of the University of Córdoba, and at Veterinary Teaching Hospital of the University of Las de Gran Canaria and Veterinary Clinic Atántico. study comprised two parts: a prospective cross-sectional study performed from January 1st to December 31st 2017 where healthy dogs were included; and a retrospective study that included dogs with confirmed and untreated CS. Forty-one healthy dogs were recruited from the Veterinary Teaching Hospital of the University of Córdoba, and dogs with CS were recruited from Las Palmas de Gran Canaria (57 from Veterinary Clinic Atántico and 33 from Veterinary Teaching Hospital of the University of Las de Gran Canaria.

Healthy dogs: Dogs were considered to be healthy on the basis of a normal physical examination and routine blood tests results (haematology and biochemistry). All dogs were seronegative for Leishmania and Ehrlichia organisms. Dogs were included if they had not received any medication within the three-month period prior to the study, except for prophylactic parasiticidal therapies. An informed consent was obtained from the owners, and the Committee of Animal Ethics of the University of Cordoba approved all study procedures with reference number 86/2016. All dogs underwent an exogenous ACTH stimulation test using a synthetic tetracosactide formulation with a concentration of 1000 μg/mL. In each test, serum cortisol concentration was measured before and 1-hour after intramuscular administration of 5 μg/kg or at least 0.1 mL of ACTH (Sieber-Ruckstuh et al., 2015).

Dogs with untreated CS: In dogs with CS, data including age, breed, sex, weight, and ultrasound adrenal gland measurements at the time of diagnosis were collected from medical records. CS was suspected if dogs presented clinical signs (polyphagia, polyuria/polydipsia, panting, alopecia or distended abdomen), together with biochemical abnormalities consistent with CS (e.g. increased alkaline phosphatase activity, hyperlipidemia, decreased blood urea nitrogen) (Schofield et al., 2020). Non-adrenal diseases were excluded, except for uncomplicated diabetes mellitus and urinary stones and/or urinary tract infections that can appear concurrently to CS. By contrast, dogs with suspected CS and moderate or severe concurrent diseases were not included. The diagnosis of CS was confirmed if 1-hour after ACTH administration cortisol concentration was >18 μg/dL, or if cortisol was ≥1.4 μg/dL 4 or 8 h after the administration of a low dose of dexamethasone (Bennaim et al., 2019). However, when dogs were considered highly suspicious of CS but ACTH stimulation test and LDDST showed test results within the reference ranges, an elevated urinary corticoid creatinine ratio (UCCR) >80 μg/g was used to confirm the diagnosis (Galac et al., 2009; Behrend et al., 2013; Galeandro et al., 2014). In the case of dogs with concurrent diabetes, confirmation of CS with hormonal tests was not performed until stabilization of diabetes.

After determining reference intervals for 1-hour post-ACTH cortisol concentration in healthy dogs, the post-ACTH cortisol concentration of dogs with untreated CS was used to evaluate the sensitivity of ACTH stimulation test to detect CS. In addition, dogs with CS were classified into four groups according to the shape and thickness of the adrenal glands on ultrasound examination as previously described (Melián et al., 2021). Thus, dogs were considered that they presented pituitary dependent hypercortisolism (PDH) if they showed symmetrical adrenomegaly without signs of malignancy. Symmetrical adrenal enlargement was considered if the dorsoventral thickness difference ratio (DVTDR) was <20%, DVTDR was defined as the difference between the maximal dorsoventral thickness of the larger gland (LDV) and the maximum dorsoventral thickness of the smaller gland (SDV) with respect to the mean: DVTDR = [2(LDV- SDV)/(LDV + SDV)] x 100 as described by Benchekroun et al., 2010. Dogs were considered to present a functional adrenal tumor leading to adrenal-dependent hypercortisolism (ADH) if they showed unilateral adrenomegaly and atrophy of the contralateral adrenal gland, as well as dogs that had unilateral or bilateral adrenomegaly with ultrasound features consistent with malignancy (ill-defined margins, loss of normal shape, invasion of adjacent structures, and/or suspected metastasis). Adrenal atrophy was considered when the maximum thickness of the smaller adrenal gland was below the lower limit of the reference intervals proposed by Melián et al., 2021. Dogs were considered as having equivocal adrenal asymmetry (EAA) if they showed unilateral or bilateral adrenomegaly with a DVTDR ≥20% (Benchekroun et al., 2010), no signs of adrenal gland atrophy in the smaller gland and no features consistent with malignancy. Finally, dogs were considered of having normal adrenal thickness if they presented adrenal glands of normal shape and size, following the previously proposed reference ranges for different groups of body weight (Melián et al., 2021).

Once dogs with CS were sonographically classified as PDH, ADH, EAA or normal, the sensitivity of the ACTH stimulation test to detect CS using a depot synthetic ACTH was determined for each group.

2.2. Ultrasound evaluation

The ultrasound evaluations were performed by the same person. Exams were done with a LOGIQ P5 Ultrasound System (GE Healthcare, Madrid, Spain) with a linear transducer (3–11 MHz), with a Z.one Pro (Mindray, Madrid, Spain) with the curve array transducer (3–9 MHz) and with a Z.one Ultra (Mindray, Madrid, Spain) with a tightly-curve transducer (4–9 MHz) and a linear array (5–14 MHz). All ultrasound evaluations were performed by one author, and all the measurements were reviewed by two authors.

At the ultrasound examinations, the dogs were placed in dorsal or lateral recumbency. Images were taken from a subcostal angle (as opposed to intercostal) whenever possible. Maximum dorsoventral thickness of either cranial or caudal pole for the left and right adrenal gland were recorded.

2.3. Analytical procedures

Blood samples were obtained in EDTA tubes for hemogram, and in serum separator tubes for biochemistry and cortisol. Biochemistry included alkaline phosphatase, alanine aminotransferase, total proteins, cholesterol, and triglycerides measured by spectrophotometry.

The adrenocorticotropic hormone (ACTH) (Nuvacthén Depot®, 1000 μg/mL, Sigma Tau, Spain) stimulation test was performed by the determination of serum cortisol before and 1 h after intramuscular (IM) administration of 5 μg/kg or at least 0.1 mL of ACTH. The low-dose dexamethasone (sodium phosphate) suppression test (0.01 mg/kg) IV or IM for assessment of serum cortisol consisted in cortisol determination before and 4 and 8 h after injection of dexamethasone (Peterson, 2007; Bennaim et al., 2019).

Serum basal cortisol and 1-hour post-ACTH cortisol were measured by chemiluminescence immunoassay (Immulite 2000®, Siemens Medical Solutions, Madrid) in healthy dogs, and by chemiluminescence

immunoassay (Immulite 1000®, Siemens Medical Solutions, Madrid) in dogs with CS. Both creatinine and cortisol in urine were measured by chemiluminescence immunoassay (Beckman Coulter Au680, Barcelona for creatinine and Immulite 2000®, Siemens Medical Solutions, Madrid for cortisol).

2.4. Statistical analysis

The normality of the parameters was assessed with the Shapiro-Wilk test and Q-Q plots. Non-normally distributed data are shown as median and interquartile range (25-75th percentile). Differences between variables were determined with the Mann Whitney *U* test Test. Statistical significance was set at $p < 0.05$ and statistical analyses were performed with IBM SPSS Statistics 25 software.

The 95% reference interval (RI) for cortisol concentration 1-hour after administration of a depot formulation of ACTH was determined in healthy dogs. Outliers were identified using the Reed method. The RI was obtained using a robust method as described by Horn (Horn et al., 1998) The 90% confidence intervals for the extremes of the RI were obtained by means of the bootstrap, (Efron, 1979) the number of iterations was 10,000 and size of the sample was 41. The RI and the confidence interval were determined according to the American Society for Veterinary Clinical Pathology Reference Intervals guidelines, (Friedrichs et al., 2012) and they were obtained through the MedCalc 18.11.6 software.

3. Results

3.1. Healthy dogs

A total of 41 healthy dogs were included in the study, 20 male and 21 female dogs. Median age was 8.5 (6–11) years. Breeds were represented as follows: mixed-breed dogs (19), Spanish greyhound (10), Beagle (3), Cocker Spaniel (2), Miniature Schnauzer (2), Poodle (1), French Bulldog (1), Dachshund (1), Labrador Retriever (1) and German Shorthaired Pointer (1).

Biochemical profile and cortisol values of healthy dogs are represented in Table 1. Lower and upper limit of the 95% reference interval for 1-hour post-ACTH cortisol concentration and their 90% confidence intervals were 4.4 (2.7–5.8) ug/dl and 18.4 (16.5–20.0) ug/dl respectively.

Table 1
Hematocrit, biochemical profile, serum cortisol and urine cortisol-creatinine ratio determinations in healthy dogs and dogs with CS. Data are given as median and IQR (25th, 75th percentile) Basal and post-ACTH cortisol were measured in 41 healthy dogs and 90 dogs with CS.

Variable (unit)	Healthy	CS	p
	N = 41	N = 90	
Hematocrit (%)	53 (49–56)	48 (43–53)	0.001
Total proteins (g/dL)	7.0 (6.6–7.6)	6.5 (6.0–7.5)	0.037
Cholesterol (mg/dL)	237 (210–251)	367 (290–470)	<0.005
Triglycerides (mg/dL)	102 (71–132)	196 (119–355)	<0.005
ALT (U/L)	41 (31–52)	223 (123–374)	<0.005
ALKP (U/L)	42 (25–84)	592 (292–1258)	<0.005
Basal Cortisol (μg/dL)	3.2 (1.8–4.9)	6.5 (3.9–9.8)	<0.005
1-hour post ACTH Cortisol (μg/dL)	11.0 (9.5–13.7)	29.5 (22.0–36.8)	<0.005
UCCR	34 (24–46)	244 (174–632)	<0.005

Hematocrit, biochemical profile and UCCR were determined in 29 healthy dogs. These parameters were not recorded in all dogs with CS: hematocrit was recorded in 54 dogs, total proteins in 50 dogs, cholesterol in 46, triglycerides in 30 dogs, ALT in 55 dogs, ALKP in 57 dog and UCCR in 9 dogs.
*p values <0.05 reflect a significant difference between healthy and CS dogs.
ALT = alanine aminotransferase, ALKP = alkaline phosphatase, UCCR = urine cortisol creatinine ratio, 1-hour Cortisol = cortisol 1 h after a low dose of a depot ACTH administration.

3.2. Dogs with hyperadrenocorticism

A total of 90 dogs with CS were included, 31 males and 59 females. Median age was 11.0 (9.8–12.0) years. Breeds were represented as follows: Yorkshire Terriers (32), mixed-breed dogs (24), Poodle (7), Chihuahua (6), French Bulldog (6), Maltese (4), Fox terrier (2), Shih Tzu (2), Shar-pei (1), Boxer (1), Giant Schnauzer (1), Beagle (1), German Shorthaired Pointer (1), Cocker Spaniel (1) and Pinscher (1).

In 82 out 90 dogs the diagnosis of CS was confirmed with an ACTH stimulation test. The remaining eight dogs showed a post- ACTH cortisol concentration within the reference range. and the diagnosis was confirmed with either a LDDST or an UCCR or both (Table 2). Only in three dogs the diagnosis was confirmed only with UCCR (values: 192, 320 and 844). These three dogs had a clinical presentation highly suspicious of CS, biochemical alterations consistent with CS and non-adrenal diseases were rule out. In one of these three dogs the UCCR was measured in several occasions and the UCCR was persistently high. In other dog the UCCR was measured once and the ultrasound examination showed adrenomegaly in the right adrenal gland with a maximum thickness of 12.7 mm whereas the left adrenal gland had decreased size consistent with atrophy with a maximum thickness of 2.5 mm in the sagittal plane. In the last dog, clinical signs were suggestive of CS and the dog presented bilateral adrenomegaly and high-normal post-ACTH cortisol concentration and, after ruling out non-adrenal disease, the diagnosis was confirmed with UCCR = 844. In this dog, the result of the ACTH stimulation test performed 10 days after initiation of trilostane treatment was consistent with CS, and in the long-term the dog showed good clinical response and normalization of the post-ACTH cortisol concentration.

Seventy six out of 90 dogs with CS (84.4%) had a minimum of one month of follow-up. These dogs had a significant reduction in basal (6.2 (3.7–9.4) vs 3.2 (2.1–5.2) μg/dL; $p < 0.005$) and post-ACTH cortisol (29.4 (21.7–35.2) vs 7.9 (4.9–11.6) μg/dL; $p < 0.005$) after trilostane treatment. Furthermore, all dogs that were treated with trilostane and had follow-up ($n = 76$), showed a partial to complete clinical response after trilostane treatment.

Among the 14 dogs with CS without follow-up record or with less than one month of follow-up, 11 dogs did not had follow-up because they returned to the referring veterinarian for treatment and monitoring. The remaining three dogs died or were euthanized shortly after diagnosis from complications including neurological signs due to suspected pituitary macroadenoma, pancreatitis or poor quality of life due to severe spondylarthrosis.

Cortisol concentration at 0 and 1-hour after administration of a depot tetracosactide were significantly higher in dogs with CS compared to healthy dogs ($p < 0.0005$) (Table 1) (Fig. 1). The overall sensitivity of cortisol concentration to detect CS 1-hour after IM administration of a low dose of a depot formulation of tetracosactide was 90.0% (81/90).

The 90 dogs with CS were ultrasonographically classified as follows: 44 dogs with PDH (48.9%), 8 dogs with ADH (8.9%), 34 dogs with EAA (37.8%) and 4 dogs with normal adrenal thickness (4.4%). Sensitivity of cortisol concentration to detect CS 1-hour after administration of a low dose of a depot formulation of tetracosactide in each category of CS is shown in Table 3.

4. Discussion

This study demonstrates that 1-hour cortisol concentration after intramuscular injection of a low dose of a depot formulation of ACTH is a useful test to confirm the diagnosis of canine CS, yielding an overall sensitivity of 90.0%, which is similar to the sensitivity reported in previous studies using other forms of ACTH (ie, cosyntropin) to perform the ACTH stimulation test (57–95%) (Behrend et al., 2013).

The maximum stimulation effect using a low dose of cosyntropin in dogs occurs 60–90 min after ACTH injection (Kerl et al., 1999); meanwhile the maximum stimulation effect produced by the depot

Table 2
Hormone function tests with positive results in dogs diagnosed with CS.

	ACTH stimulation test	LDDST	UCCR	ACTH stimulation test + LDDST	ACTH stimulation test + UCCR	LDDST + UCCR
Dogs (n = 90)	77	2	3	2	3	3

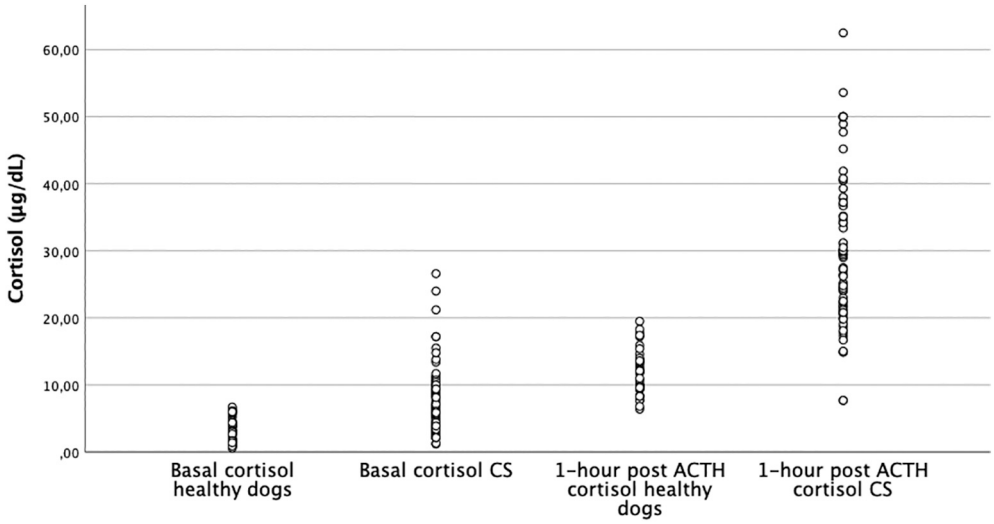

Fig. 1. Basal and 1-hour cortisol concentration in 41 healthy dogs and in 90 dogs with Cushing's syndrome.

Table 3
Median and 25-75th percentil of 1-hour post ACTH cortisol values among different groups of dogs with Cushing's syndrome and sensitivity of the ACTH stimulation test to detect Cushing's syndrome in these groups using a low dose of a depot formulation of tetracosactide (5 µg/ kg or at least 0.1 mL, IM).

	PDH	FAT	EAA	Normal	Total
	N = 44	*N* = 8	*N* = 34	N = 4	*N* = 90
1-hour post ACTH cortisol concentration (µg/dL)	30.0 (24.4–37.7)	21.9 (10.2–34.6)	28.4 (21.8–31.4)	24.8 (21.0–29.3)	29.5 (22.0–36.8)
N dogs with post-ACTH cortisol >18.4 µg/dL.	42	5	30	4	81
Sensitivity (95% confidence interval)	95.5 (76.1–100)%	62.5 (46.1–78.9)%	88.2 (69.5–100)%	100 (78.1–100)%	90.0 (71.3–100)%

formulation of tetracosactide have been studied in healthy dogs and it occurs later (120–180 min post-injection) compared to soluble ACTH formulation (Nuvacthen depot, package insert, 2018). However, similar concentrations of cortisol have been observed in dogs with CS 60 min post stimulation with either cosyntropin or depot formulation of tetracosactide (Ginel et al., 2012; Sieber-Ruckstuh et al., 2015). In other words, measuring cortisol concentration at the time of maximum stimulation after using a depot formulation of ACTH, might not be necessary since similar concentrations of cortisol occur 1-hourour post stimulation with either cosyntropin or depot formulation of tetracosactide in dogs with CS. Nonetheless, specificity still needs to be evaluated in dogs with non-adrenal diseases to assess whether 1-hour, 2-hour or 3-hours post-ACTH cortisol will differentiate between dogs with CS and dogs with other illnesses.

In the present study, upper limit for 1-hour post ACTH cortisol concentration using a low dose of a depot formulation of ACTH was initially assessed in healthy dogs. This upper limit of 18.4 µg/dL was used to calculate the sensitivity of ACTH stimulation test to confirm the diagnosis of CS. However, reference ranges might vary among laboratories and interpretation of results should be based on each laboratory reference range.

It is known that the sensitivity of the ACTH stimulation test could vary depending on the type of CS. False negative results are more frequently observed in dogs with ADH; these dogs could present a subnormal cortisol response as the production of cortisol does not depend on ACTH, showing little or no response to exogenous ACTH in some cases (Peterson, 1982). Previous studies have shown that the sensitivity of the ACTH stimulation test to detect CS in dogs with PDH ranged between 84 and 92% and between 57 and 63% in dogs with ADH (Peterson, 1982; Reusch and Feldman, 1991; Kaplan et al., 1995; Norman et al., 1999; Monroe et al., 2012; Nivy et al., 2018). The results of the present study using a depot ACTH formulation showed similar results with higher sensitivity to detect CS in dogs consistent with PDH than in dogs consistent with ADH (95.5 vs 62.5%). Consequently, the diagnosis of CS should not be excluded on the basis of normal post-ACTH cortisol concentration in dogs with suspected CS and ultrasonographic findings consistent with ADH. In such cases, performing a different diagnostic test (ie, low-dose dexamethasone suppression test and/or urine corticoid to creatinine ratio) is recommended. It should be noted that performing an ultrasound evaluation previously or at the time of the ACTH stimulation test could add valuable information for the interpretation of the ACTH stimulation test results and for the general diagnostic approach of dogs with suspected CS. However, due to the small number of dogs that were considered as ADH in this study, the results of the sensitivity in this group should be interpreted carefully.

Dogs with PDH usually present symmetrical bilateral adrenomegaly, whereas dogs with ADH commonly present unilateral adrenomegaly and atrophy of the contralateral adrenal gland or they might present unilateral or bilateral adrenomegaly with ultrasound features consistent with malignancy. Nonetheless, ultrasonographic differentiation between PDH and FAT is not always possible as more than a third of dogs with CS present adrenomegaly with EAA (Melián et al., 2021). Some dogs with PDH could present nodular hyperplasia leading to adrenal asymmetry, and dogs with ADH does not always present contralateral

adrenal gland atrophy and some degree of overlap in the ultrasound thickness of hyperplastic, normal, and atrophied adrenal glands have been reported (Hoerauf and Reusch, 1999; Benchekroun et al., 2010). Moreover, dogs with bilateral adrenal tumours, concurrent ADH and PDH, or other concurrent neoplasia (pheochromocytoma) may also be classified as dogs as EAA (Beatrice et al., 2018; Galac and Grinwis, 2018; van Bokhorst et al., 2019). The stimulation ACTH stimulation test showed a good sensitivity (88.2%) to detect CS in dogs with EAA (88.2%) and was similar to the sensitivity observed in dogs with PDH (95.5%).

Unexpectedly, the hematocrit values of dogs with CS were significantly lower compared to healthy dogs. Differences regarding breed or sample size might have affected results of this parameter.

Some limitations are acknowledged in this study. First, the specificity of the ACTH stimulation test using a depot formulation of ACTH could not be evaluated and further studies including dogs with non-adrenal diseases would be needed. In addition, the type of CS was differentiated only by ultrasonography, therefore dogs with EAA could not be classified as ACTH-dependent or ACTH-independent CS. In most dogs, data on the ACTH concentration at the time of diagnosis were not available, and neither were the results of histopathologic examination or high dexamethasone suppression test. Morevover, for the LDDST a dexamethasone sodium phosphate presentation was used, and not the dexamethasone in polyethylene glycol as previously used intramuscularly (Peterson, 2007). In addition, the administered dose was 0.01 mg/kg although this calculation was not based on the dexamethasone content. However, due to the small number of dogs diagnosed with LDDST, we believe that is unlikely that this would influence the results obtained from the study. Finally, other limitation was the number of dogs considered as presenting ADH was small, and further studies assessing the sensitivity of the ACTH stimulation test using a depot formulation of tetracosactide in dogs with ADH could be necessary.

In conclusion, the 1-hour post-ACTH cortisol concentration using a low-dose of a depot ACTH formulation has good sensitivity to detect CS. Further studies are needed to evaluate the specificity of the test and to assess whether the sensitivity of the test is lower in dogs with ultrasonographic findings consistent with ADH compared to dogs with ultrasonographic findings consistent with PDH or EAA.

Declaration of Competing Interest

None.

Acknowledgments

Financial support was received from the Research group BIO307 PAIDI, Junta de Andalucía.

A research communication of this study was presented as a poster for the 2021 ECVIM-CA Congress.

References

Aldridge, C., Behrend, E.N., Kemppainen, R.J., Lee-Fowler, T.M., Martin, L.G., Ward, C. R., Bruyette, D., Pannu, J., Gaillard, P., Lee, H.P., 2016. Comparison of 2 doses for ACTH stimulation testing in dogs suspected of or treated for Hyperadrenocorticism. J. Vet. Intern. Med. 30, 1637–15641.

Beatrice, L., Schär, Boretti F., Sieber-Ruckstuhl, N.S., Mueller, C., Fraune-Kümmerle-Raune, C., Hilbe, M., Grest, P., Reusch, C.E., 2018. Concurrent endocrine neoplasias in dogs and cats. a retrospective study (2004–2014). Vet. Rec. 182, 323.

Behrend, E.N., Kemppainen, R.J., Bruyette, D.S., Busch, K.A., Lee, H.P., 2006. Intramuscular administration of a low dose of ACTH for ACTH stimulation testing in dogs. Journal of American Veterinary Medical Association 229, 528–530.

Behrend, E.N., Kooistra, H.S., Nelson, R., Reusch, C.E., Scott- Moncrieff, J.C., 2013. Diagnosis of spontaneous canine hyperadrenocorticism: 2012 ACVIM consensus statement (small animal). Journal of Veterinar Internal Medicine 27, 1292–1304.

Benchekroun, G., de Fomel-Thibaud, P., Rodríguez-Piñeiro, M.I., Rault, D., Besso, J., Cohen, A., Hernandez, J., Stambouli, F., Gomes, E., Garnier, F., et al., 2010. Ultrasound criteria for differentiating ACTH dependency from ACTH independency in 47 dogs with hyperadrenocorticism and equivocal adrenal asymmetry. J. Vet. Intern. Med. 24, 1077–1085.

Bennaim, M., Shiel, R.E., Mooney, C.T., 2019. Diagnosis of spontaneous hyperadrenocorticism in dogs. Part 2: adrenal function testing and differentiating tests. Vet. J. 252, 105343.

Efron, B., 1979. Bootstrap methods: another look at the jackknife. Ann. Stat. 7, 1–26.

Frank, L.A., DeNovo, R.C., Kraje, J.W., Oliver, J.W., 2000. Cortisol concentrations following stimulation of healthy and adrenopathic dogs with two doses of tetracosactrin. J. Small Anim. Pract. 41, 308–311.

Friedrichs, K., Harr, K., Freeman, K., Szladovits, B., Walton, R.M., Barnhart, K.F., Blanco-Chavez, J., 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet. Clin. Pathol. 41, 441–453.

Galac, S., Grinwis, G., 2018. Concurrent endocrine neoplasia: more common than you thought? Vet. Rec. 182, 320–322.

Galac, S., Buijtels, J.J.C.W.M., Kooistra, H.S., 2009. Urinary corticoid: Creatinine ratios in dogs with pituitary-dependent hypercortisolism during trilostane treatment. J. Vet. Intern. Med. 23, 1214–1219.

Galeandro, L., Sieber-Ruckstuhl, N.S., Riond, B., Hartnack, S., Hofmann-Lehmann, R., Reusch, C.E., Boretti, F.S., 2014. Urinary corticoid concentrations measured by 5 different immuno assays and gas chromatography mass spectrometry in healthy dogs and dogs with hypercortisolism at home and in the hospital. J. Vet. Intern. Med. 28, 1433–1441.

Ginel, P.J., Sileo, M.T., Blanco, B., Garfia, B., Quintvalla, F., 2012. Evaluation of serum concentrations of cortisol and sex hormones of adrenal gland origin after stimulation with two synthetic ACTH preparations in clinically normal dogs. American Journal Veterinary Research 73 (237–24).

Hoerauf, A., Reusch, C.E., 1999. Ultrasonographic characteristics of both adrenal glands in 15 dogs with functional adrenocortical tumors. J. Am. Anim. Hosp. Assoc. 35, 193–199.

Horn, P.S., Pesce, A.J., Copeland, B.E., 1998. A robust approach to reference interval estimation and evaluation. Clin. Chem. 44, 622–631.

Kaplan, A.J., Peterson, M.E., Kemppainen, R.J., 1995. Effects of disease on the results of diagnostic tests for use in detecting hyperadrenocorticism in dogs. Journal of American Veterinary Medical Association 207, 445–451.

Kerl, M.E., Peterson, M.E., Wallace, M.S., Melián, C., Kemppainen, R.J., 1999. Evaluation of a low-dose synthetic adrenocorticotropic hormone stimulation test in clinically normal dogs and dogs with naturally developing hyperadrenocorticism. Journal of Americn Veterinary Medical Associations 214, 1497–1501.

Melián, C., Pérez-López, L., Saavedra, P., Ravelo-García, A.G., Santos, Y., Jaber, J.R., 2021. Ultrasound evaluation of adrenal gland size in clinically healthy dogs and in dogs with hyperadrenocorticism. Vet. Rec. 188, e80.

Monroe, W.E., Panciera, D.L., Zimmerman, K.L., 2012. Concentrations of noncortisol adrenal steroids in response to ACTH in dogs with adrenal-dependent hyperadrenocorticism, pituitary-dependent hyperadrenocorticism, and nonadrenal illness. J. Vet. Intern. Med. 26, 945–952.

Nivy, R., Refsal, K.R., Ariel, E., Kuzi, S., Yas-Natan, E., Mazaki-Tovi, M., 2018. The interpretive contribution of the baseline serum cortisol concentration of the ACTH stimulation test in the diagnosis of pituitary dependent hyperadrenocorticism in dogs. J. Vet. Intern. Med. 32, 1897–1902.

Norman, E.J., Thompson, H., Mooney, C.T., 1999. Dynamic adrenal function testing in eight dogs with hyperadrenocorticism associated with adrenocortical neoplasia. Vet. Rec. 144, 551–554.

Nuvacthen depot [package insert], 2018. Barcelona, España: Alfasigma Pharmaceuticals.

Papich, M.G., 2016. Cosyntropin. In: Handbook of Veterinary Drugs Small and Large Animal, 4th edn. Saunders-Elsevier, St. Louis, MO, USA, pp. 188–189.

Peterson, M.E., 1982. Plasma cortisol response to exogenous ACTH in 22 dogs with hyperadrenocorticism caused by adrenocortical neoplasia. Journal of American Veterinary Medical Association 180, 542–544.

Peterson, M.E., 2007. Diagnosis of hyperadrenocorticism in dogs. Clinical Techniques in Small Animal Practice 22, 2–11.

Reusch, C.E., Feldman, E.C., 1991. Canine hyperadrenocorticism due to adrenocortical neoplasia. Pretreatment evaluation of 41 dogs. J. Vet. Intern. Med. 5, 3–10.

Schofield, I., Brodbelt, D.C., Niessen, S.J.M., Church, D.B., Geddes, R.F., Kennedy, N., O'Neill, D.G., 2020. Development and internal validation of a prediction tool to aid the diagnosis of Cushing's syndrome in dogs attending primary-care practice. J. Vet. Intern. Med. 34, 2306–2318.

Sieber-Ruckstuh, N.S., Burkhardt, W.A., Hofer-Inteeworn, N., Riond, B., Rast, I.T., Hofmann-Lehmann, R., Reusch, C.E., Boretti, F.S., 2015. Cortisol response in healthy and diseased dogs after stimulation with a depot formulation of synthetic ACTH. J. Vet. Intern. Med. 29, 1541–1546.

van Bokhorst, K.L., Kooistra, H.S., Boroffka, S.A.E.B., Galac, S., 2019. Concurrent pituitary and adrenocortical lesions on computed tomography imaging in dogs with spontaneous hypercortisolism. J. Vet. Intern. Med. 33, 72–78.