Evaluation of serum concentrations of cortisol and sex hormones of adrenal gland origin after stimulation with two synthetic ACTH preparations in clinically normal dogs

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Objective—To compare the adrenocortical response of healthy dogs to a commonly used dose of a nonadsorbed tetracosactide product (tetracosactide) with responses to 2 doses of a depot formulation of tetracosactide (depot tetracosactide).

Animals—14 dogs.

Procedures—Dogs were randomly assigned to receive tetracosactide (5 mg/kg, IV) or depot tetracosactide (250 μg, IM, or 5 μg/kg, IM). Dogs received each treatment once with a 2-week interval between treatments. Blood samples were assayed for cortisol, progesterone, 17-hydroxyprogesterone, androstenedione, and estradiol concentrations.

Results—Serum cortisol concentrations were significantly higher than the preadministration (baseline) concentrations for all treatments 60 minutes after administration of ACTH. Peak cortisol concentration was detected 180 minutes after IM administration of 250 μg of the depot tetracosactide. Serum concentrations of progesterone, 17-hydroxyprogesterone, and androstenedione did not differ significantly from baseline concentrations after stimulation with the 5 μg/kg dose of depot tetracosactide. Adrenal gland progesterone response was significantly higher than baseline concentrations at 60 minutes after administration of the 250-μg dose of depot tetracosactide, and the 17-hydroxyprogesterone and androstenedione responses were significantly higher than baseline concentrations at 120 minutes. Compared with the response to tetracosactide, adrenocortical response was higher and more sustained following administration of the depot tetracosactide, except for androstenedione concentration, which had a nonsignificant response.

Conclusions and Clinical Relevance—Except for androstenedione concentrations, a high dose of the depot tetracosactide (250 μg, IM) induced an adrenocortical response similar to that after administration of tetracosactide. Thus, depot tetracosactide may represent an alternative to the nonadsorbed tetracosactide product. (*Am J Vet Res* 2012;73:237–241)

The ACTH stimulation test is commonly used to diagnose hyperadrenocorticism, hypoadrenocorticism, and alopecia X in dogs.[1] It is used to differentiate iatrogenic hyperadrenocorticism from endogenous hyperadrenocorticism and is used to monitor treatment of hyperadrenocorticism.[2] Cosyntropin, tetracosactrin, and tetracosactide are generic names for β^{1-24} corticotropin, a synthetic polypeptide identical to the *N*-terminal 24 residues of corticotropin, and have all of the hormonal activity of corticotropin. This synthetic polypeptide has been used since aqueous preparations of porcine ACTH became unavailable.[3,4]

Various protocols that involve the use of synthetic ACTH have been recommended for the ACTH stimulation test in dogs. Initial protocols included use of a synthetic ACTH product at a dose of 250 μg/dog,[2] but findings from subsequent studies[4–7] indicated comparable stimulation of the adrenal cortex by the use of a dose of 5 μg/kg, IV or IM. Availability, cost, and formulations of synthetic ACTH differ among countries. Several proprietary formulations of synthetic ACTH are intended only for diagnostic purposes, but tetracosactide is also available as a depot formulation that can be used for treatment purposes. In the depot formulations, tetracosactide is supplied as a suspension in which the active substance is adsorbed onto an inorganic zinc phospate complex so the formulation provides a protracted release. Following an IM injection of 1 mg of a depot formulation of tetracosactide, the plasma cortisol concentration remains elevated for 24 to 36 hours.[8] Thus, depot formulations have both diagnostic and therapeutic indications in human patients and are the

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only form of tetracosactide commercially available in some countries. However, their use for an ACTH stimulation test in dogs has not been compared with that of the standard nonadsorbed tetracosactide preparations.

Cortisol, intermediary adrenocoticosteroids, and sex hormones are all produced in canine adrenal glands. Measurements of concentrations of other adrenocorticosteroids have been used to evaluate adrenocortical function and may increase the sensitivity of the ACTH stimulation test for the diagnosis of hyperadrenocorticism.[1,9,10] The purpose of the study reported here was to compare the adrenal gland responses of healthy dogs to ACTH stimulation with a nonadsorbed tetracosactide product administered IV and 2 dosages of a depot formulation of tetracosactide administered IM. In addition to cortisol concentrations, the concentration of sex hormones (estradiol and progesterone) of adrenal origin and adrenocorticosteroid intermediates (17-hydroxyprogesterone and androstenedione) was measured.

Materials and Methods

Dogs—Fourteen dogs (8 males [1 neutered] and 6 females [4 neutered]) between 3 and 8 years old and weighing between 13.5 and 33 kg (median, 25 kg) were included in the study. There were 11 Greyhounds and 3 Beagles; 6 dogs were owned by a canine club, and the rest were owned by the Veterinary Teaching Hospital at Córdoba, Spain. Dogs were determined to be healthy on the basis of results of a physical examination, CBC, serum biochemical analysis, and urinalysis. All dogs were seronegative for *Leishmania* and *Ehrlichia* organisms. At the time of the ACTH stimulation tests, all sexually intact females were in anestrus. Dogs did not receive any medication or adrenocorticosteroid preparation for the 3 months preceding and during the study. Informed consent was obtained from the owners, and all study procedures were approved by the Committee of Animal Ethics of the University of Córdoba.

Study design—The study was designed as a blinded, crossover clinical trial in which the investigator analyzing the serum hormone concentrations was unaware of the treatment used for the ACTH stimulation tests. Dogs were assigned via a computer-generated list of random numbers to initially receive synthetic ACTH consisting of a zinc phosphate depot formulation of tetracosactide[a] (depot tetracosactide) at a dose of 250 µg, IM (dose range, 7.6 to 18.5 µg/kg; treatment A) or 5 µg/kg, IM (treatment B) or a standard nonadsorbed tetracosactide product[b] (tetracosactide) at a dose of 5 µg/kg, IV (reference treatment). Tests were repeated at 2-week intervals until each dog received each ACTH stimulation treatment. Tests were performed in the morning after food had been withheld for 12 hours. Blood samples were obtained by use of cephalic venipuncture before (baseline) and at various times after ACTH administration. For treatments A and B, poststimulation blood samples were obtained 60, 120, and 180 minutes after ACTH administration. For the reference treatment, adrenal gland response was evaluated 60 and 90 minutes after ACTH administration. Because investigators of other studies[4–6,11] found that response to IV administration of tetracosactide is maximized after 90 minutes, no additional samples were obtained after that time for the reference treatment. Blood samples were allowed to clot at room temperature (21°C) and then centrifuged, and serum was harvested and stored at –20°C until assayed for cortisol, progesterone, 17-hydroxyprogesterone, androstenedione, and estradiol concentrations.

Serum cortisol, progesterone, androstenedione, and estradiol concentrations were measured by use of commercial, solid-phase, competitive chemiluminescent enzyme immunoassay kits.[12,c] The analytic thresholds reported by the manufacturer for cortisol, progesterone, androstenedione, and estradiol chemiluminescent assays were 0.2 µg/dL, 0.2 ng/mL, 0.3 ng/mL, and 15 pg/mL, respectively. Serum 17-hydroxyprogesterone concentration was measured by use of a competitive immunoenzymatic colorimetric assay[d] that had an analytic threshold of 0.1 ng/mL. The validation of all the tests included the analyses of samples at various dilutions and recovery assays with 3 reference solutions to determine their accuracy. Samples were assayed in duplicate, and each hormone was analyzed in a single assay. Intra-assay coefficients of variation were < 10% for all measurements and assays. Cross-reactivity with other hormones was not tested, but the manufacturers[13,14] reported no relevant cross-reactivity with other adrenocorticosteroids, except for prednisolone in the cortisol assay (cross-reactivity, 49%).

Statistical analysis—A 1-way repeated-measures ANOVA and Tukey multiple comparison posttest were used to compare hormone concentrations among the sample collection times and ACTH treatments. When data were not normally distributed, the nonparametric Friedman repeated-measures ANOVA and the Dunn posttest for multiple comparisons were used. A value of $P < 0.05$ was considered significant. Statistical analyses were performed by use of a statistical software package.[e]

Results

Administration of the synthetic ACTH products resulted in significantly higher serum cortisol concentrations by 60 minutes, compared with baseline concentrations (**Table 1**). Peak concentration of cortisol was detected 180 minutes after administration with treatment A, but the mean serum cortisol concentrations did not differ significantly between samples obtained at 120 and 180 minutes. The reference treatment induced the fastest response, achieving the highest mean serum cortisol concentration by 60 minutes after administration; the serum cortisol concentration then plateaued, and there was a nonsignificant increase from 60 to 90 minutes. The maximum mean serum cortisol concentration differed significantly ($P = 0.012$) between treatment A 180 minutes after administration, compared with that of the reference treatment 90 minutes after administration.

Serum concentrations of progesterone were not significantly different from baseline concentrations at any sample collection time for treatment B. The other treatments induced a significant ($P = 0.002$) response 60 minutes after administration, but no further increase

Table 1—Mean ± SD serum concentrations of cortisol, progesterone, 17-hydroxyprogesterone, and androstenedione before (baseline [time = 0]) and at various sample collection times after administration of synthetic ACTH (250 µg of a depot formulation of tetracosactide, IM [treatment A]; 5 µg/kg of the same depot formulation of tetracosactide, IM [treatment B]; and 5 µg/kg of a nonadsorbed tetracosactide product, IV [reference treatment]) to 14 clinically normal dogs.

		Time (min)				
Hormone	**Treatment**	**0**	**60**	**90**	**120**	**180**
Cortisol (ng/mL)	A	3.6 ± 1.7	14.0 ± 4.4*	—	19.9 ± 5.2*	24.2 ± 8.6*[a]
	B	4.1 ± 2.0	11.4 ± 3.6*	—	15.5 ± 4.3*	20.0 ± 6.7*
	Reference	3.4 ± 1.7	16.1 ± 4.0*	16.9 ± 5.2*[b]	—	—
Progesterone (ng/mL)	A	0.4 ± 0.2	1.4 ± 0.6*	—	2.0 ±1.3*[a]	1.8 ± 1.1*
	B	0.4 ± 0.1	0.8 ± 0.4	—	1.1 ± 0.7[b]	1.2 ± 0.7
	Reference	0.4 ± 0.2	1.4 ± 0.4*	1.4 ± 0.5*	—	—
17-hydroxyprogesterone (ng/mL)	A	2.4 ± 2.6	6.1 ± 4.7	—	8.2 ± 4.9*	7.8 ± 4.7*
	B	2.2 ± 2.3	4.5 ± 4.9	—	4.9 ± 3.8	5.3 ± 5.7
	Reference	1.8 ± 2.4	7.5 ± 5.9*	5.0 ± 5.0	—	—
Androstenedione (ng/mL)	A	0.4 ± 0.3	1.1 ± 1.2	—	1.0 ± 0.9*	1.2 ± 0.7*
	B	0.6 ± 0.5	0.9 ± 0.7	—	1.0 ± 0.8	1.1 ± 1.1
	Reference	0.5 ± 0.4	1.6 ± 0.8*	0.8 ± 0.8*	—	—

*Within a row, means are significantly ($P < 0.05$) different from baseline concentrations. Each dog was administered each of the 3 treatments; there was a 2-week interval between treatments.
— = Not determined.
[a,b]Within a hormone, means with different superscript letters differ significantly.

was recorded at 90 minutes for the reference treatment. Mean serum progesterone concentration for treatment A peaked at 120 minutes and then plateaued. This maximum progesterone concentration was significantly ($P = 0.004$) higher, compared with that of treatment B at 120 minutes; however, it did not differ significantly from the progesterone concentration of the reference treatment at 90 minutes after administration.

Concentrations of 17-hydroxyprogesterone increased significantly ($P = 0.015$) by 60 minutes after administration for the reference treatment and by 120 minutes for treatment A ($P = 0.017$); treatment B failed to induce a significant increase in 17-hydroxyprogesterone concentration. Similar to progesterone results, no further increase in the 17-hydroxyprogesterone concentration was found at 90 and 180 minutes for the reference treatment or treatment A, respectively. The highest response was reported for treatment A at 120 minutes after administration. No significant differences among the 3 treatments were found at any of the sample collection times.

Serum concentrations of androstenedione increased significantly by 60 minutes after administration for the reference treatment ($P < 0.001$) and by 120 minutes for treatment A ($P = 0.038$). The androstenedione concentration for treatment B did not differ from baseline concentrations at any sample collection time. The maximum increase in androstenedione concentration was detected 60 minutes after administration of the reference treatment, but the concentration had decreased by 90 minutes. Although the response to the depot tetracosactide was slower and more sustained for all the hormones analyzed, the androstenedione response was weak, compared with the response of the other hormones assayed. This was evident particularly for treatment B (Table 1). No significant differences in androstenedione concentration were found among the 3 treatments at any of the sample collection times.

Serum estradiol concentrations did not increase after stimulation with any treatment. No adverse effects were observed during the study.

Discussion

In the study reported here, we compared the adrenal gland responses in healthy dogs that received 2 doses of a depot tetracosactide formulation IM and a reference treatment of a nonadsorbed tetracosactide product administered IV. All 3 treatments induced significantly higher serum cortisol concentrations, compared with baseline concentrations; but in contrast to results for the other 2 treatments, treatment B did not induce significant increases in any of the adrenal gland sex hormones analyzed, compared with baseline concentrations. Generally, IV administration resulted in maximum serum concentrations of the respective hormones by 60 minutes after injection, whereas IM administration did not result in peak concentrations until 120 (progesterone and 17-hydroxyprogesterone) or 180 (cortisol and androstenedione) minutes after injection.

Currently, serum is assayed to determine cortisol and adrenocorticosteroid concentrations before and after IV or IM administration of a 5 µg/kg dose of a short-acting synthetic ACTH. This dose stimulates maximum cortisol, sex hormones of adrenal gland origin, and adrenocorticosteroid intermediates 1 hour after ACTH administration.[5,6] The results from the present study are in agreement with those of other studies[4–6,11] and support the use of tetracosactide at a dose of 5 µg/kg to stimulate the adrenal glands. Also consistent with results of previous studies,[5,6] the hormone concentrations at 90 minutes after administration of 5 µg/kg, IV, in our study were similar to (cortisol and progesterone) or lower than (androstenedione and 17-hydroxyprogesterone) the concentrations at 60 minutes.

Depot tetracosactide formulations are inexpensive and provide an alternative to the use of tetracosac-

tide preparations for IV administration. Although the tetracosactide preparation for IV administration currently is readily available, there is always the possibility of decreased availability of the product in the future. If various drugs cause similar secretion of adrenocortical reserves, then the drugs can be used interchangeably in ACTH stimulation tests without altering the cutoffs for interpreting the results of such tests.[15] Maximum cortisol concentration was achieved the fastest following adrenocortical stimulation with tetracosactide given IV, but the 250-µg dose of the depot tetracosactide administered IM induced similar cortisol concentrations 60 minutes after administration.

Even though hormone concentrations between treatments did not differ significantly, it is possible that the small sample size in our study resulted in a lack of power to detect significant differences. It is likely that with a larger sample size, the cortisol concentrations achieved with 5 µg/kg, IM, of depot tetracosactide would have been significantly lower, compared with those of the reference treatment.

Also, it is probable that the peak cortisol response was not identified following IM injection of the depot tetracosactide. In the study reported here, peak cortisol concentrations were detected 180 minutes after IM administration, but the sustained action of this preparation will likely result in further cortisol increases after 180 minutes. However, collection of additional samples was not considered necessary because mean cortisol concentrations 120 and 180 minutes after IM administration were higher than those for the reference treatment 90 minutes after administration.

In humans, depot tetracosactide administered IM is used in a 5-hour test to determine the functional reserve of the adrenal cortex when the 30-minute test with tetracosactide administered IV yields inconclusive results for the diagnosis of adrenocortical insufficiency.[16] It would be interesting to investigate whether an extended test that used depot tetracosactide, similar to the 5-hour test used in humans, would improve the diagnostic value of the ACTH stimulation test in dogs. The sustained stimulation observed with this ACTH preparation is likely related to the depot nature of the preparation and also to the higher dose used. Similar cortisol concentrations have been reported 60 minutes after tetracosactide stimulation in healthy dogs when the entire vial was used (250 µg, IV), compared with administration of only 5 µg/kg, IV[17]; however, the 250-µg dose resulted in a more sustained cortisol response.[5]

An adrenocortical response of longer duration would be desirable clinically to avoid false-negative results when the interval to sample collection is delayed for a few minutes.[4] Because the response with the 250-µg depot tetracosactide administered IM was sustained, samples could be obtained 60 or 120 minutes after injection. Analyses of cortisol responses for individual dogs for treatment A revealed that 2 dogs had serum cortisol concentrations within the reference range 60 minutes after administration, but those concentrations continued to increase and exceeded the commonly recommended cutoff value (22 µg/dL) by 120 minutes. Although there was good agreement between results for treatment A and the reference treatment at 60 minutes after administration, it would be advisable to establish a reference range for samples obtained 120 minutes after stimulation with the depot tetracosactide. This was beyond the scope of the present study and would require a population of dogs with adrenocortical disease and a larger control group of age-matched clinically normal dogs.

In the present study, serum concentrations of all adrenal gland sex hormones assayed after administration of treatment B failed to increase significantly from baseline concentrations; this is an important criterion that must be met by any stimulation test protocol.[15] This weaker adrenocortical stimulation with the same dose as for the reference treatment may be explained by the route of administration or by differences in the tetracosactide preparations. In cats,[18] synthetic ACTH given IV induces a significantly greater and more prolonged adrenocortical stimulation than does IM administration of the same dose. The increased stimulation of the adrenal cortex can be explained by the higher circulating ACTH concentration attained with IV administration, compared with the concentration attained with IM administration.[18] However, the effect of route of administration is controversial. Other researchers[18,19] have concluded that although higher plasma ACTH concentrations are achieved after IV injection of synthetic ACTH, the plasma cortisol response is identical after IV or IM administration. Nevertheless, analysis of our results suggested that the depot tetracosactide preparation cannot be recommended for use in the ACTH stimulation test at the same dose as the short-acting standard tetracosactide administered IV.

Large variation was detected in the concentrations of 17-hydroxyprogesterone and androstenedione in the present study. This was expected because the reproductive status differed among the study dogs, and differences in the response of intermediary sex hormones to administration of synthetic ACTH have been reported[6,9] for sexually intact female and male and neutered dogs of either sex. After synthetic ACTH administration, significantly higher concentrations of progesterone in sexually intact females, androstenedione in sexually intact males, and 17-hydroxyprogesterone in both sexually intact females and males were reported.[6] The variability in the reproductive status of the dogs in the present study likely accounted for the lack of significant differences among the treatments at the same collection times. For example, the androstenedione concentration 60 minutes after ACTH administration was almost 50% higher for the reference treatment, compared with that of treatment A. However, the heterogeneous reproductive status of the dogs included in the present study did not affect the main objective of our study, which was to determine whether a depot tetracosactide formulation could induce a significant adrenocortical response.

The serum estradiol concentration did not increase after administration of any of the treatments evaluated. This finding is consistent with that[6] described in sexually intact and neutered dogs after administration of the same product and dose used in our reference treatment. The present study revealed a similar lack of estradiol release when a depot tetracosactide was administered IM.

Except for androstenedione, the present study indicated that a high dose (250 µg) of depot tetracosactide administered IM induced an adrenocortical response similar to that for the 5 µg/kg dose of tetracosactide administered IV, although the depot tetracosactide preparation resulted in stronger and more prolonged adrenocortical stimulation. Because there are no contraindications to the use of 250 µg of tetracosactide, these 2 treatments could be interchanged without a reduction in the confidence of the test results. However, the 250-µg dose should be evaluated in dogs with adrenal gland disease. Depot tetracosactide administered IM was able to stimulate the adrenal cortex and represents an alternative to the IV administration of tetracosactide when that product is not available or an IV injection is not feasible.

a. Nuvacthen depot, Novartis Farmaceútica, Barcelona, Spain.
b. Synacthen, Novartis Farma, Origgio, Italy.
c. Immulite, Siemens Medical Solutions, Diagnostics Ltd, Caernarfon, Gwynedd, Wales.
d. 17-α-OH progesterone EIA well, Radim Ibérica, Barcelona, Spain.
e. GraphPad Prism, version 5.03, GraphPad Software Inc, San Diego, Calif.

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