Cortisol response in healthy and diseased dogs after stimulation with a depot formulation of synthetic ACTH

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Abstract: BACKGROUND The ACTH stimulation test is used to evaluate the adrenocortical reserve. Recently, the availability of the synthetic ACTH formulation was limited, causing major problems in clinical practice. OBJECTIVES The objective of this study was to evaluate poststimulation peak cortisol concentrations and the duration of the stimulatory effect of a depot ACTH preparation in dogs. ANIMALS Twenty-two healthy dogs, 10 dogs with suspected hypoadrenocorticism (HA) and 15 dogs with suspected hyperadrenocorticism (HC). METHODS Prospective study. An ACTH stimulation test using a synthetic depot tetracosactide, administered intramuscularly (5 g/kg or at least 0.1 mL) was performed. Blood samples for determination of cortisol were taken immediately before and 1, 2, 3, 4, 6, and 24 hours after stimulation. RESULTS Peak cortisol concentrations were reached after 2-4 hours in all dogs. Cortisol concentrations 1 hour after stimulation were >9 g/dL in all healthy dogs and >5 g/dL in all dogs in which HA was excluded. None of the dogs with HA showed a cortisol-increase above the detection-limit of the assay. After 6 hours, cortisol concentrations had decreased in the healthy and HC group and were back to baseline after 24 hours. CONCLUSIONS AND CLINICAL IMPORTANCE The depot formulation can be used in place of the short-acting ACTH to evaluate the adrenocortical reserve. Blood for peak cortisol concentrations should be drawn 3 hours after stimulation in cases in which HC is suspected; in HA-suspected cases, blood sampling can take place after 1 hour. As the stimulatory effect is gone after 24 hours, interference with other hormonal tests is unlikely after that time.

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Cortisol response in healthy and diseased dogs after stimulation with a depot formulation of synthetic ACTH

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Short title: ACTH stimulation with a depot formulation

Keywords: adrenocortical reserve; Synacthen Depot; canine;

List of abbreviations:
HA: hypoadrenocorticism
HC: hyperadrenocorticism
LDDS test: low-dose dexamethasone suppression test
PDH: pituitary-dependent hyperadrenocorticism

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Background: The ACTH stimulation test is used to evaluate the adrenocortical reserve. Recently, the availability of the synthetic ACTH formulation was limited, causing major problems in clinical practice.

Objectives: To evaluate post-stimulation peak cortisol concentrations and the duration of the stimulatory effect of a depot ACTH preparation in dogs.

Animals: 22 healthy dogs, 10 dogs with suspected hypoadrenocorticism (HA) and 15 dogs with suspected hyperadrenocorticism (HC).

Methods: Prospective study. An ACTH-stimulation test using a synthetic depot tetracosactide, administered intramuscularly (5 µg/kg or at least 0.1 ml) was performed. Blood samples for determination of cortisol were taken immediately before and 1, 2, 3, 4, 6 and 24 hours after stimulation.

Results: Peak cortisol concentrations were reached after 2 to 4 hours in all dogs. Cortisol concentrations 1 hour after stimulation were >9 µg/dl in all healthy dogs and >5 µg/dl in all dogs in which HA was excluded. None of the dogs with HA showed a cortisol-increase above the detection-limit of the assay. After 6 hours cortisol concentrations had decreased in all groups and were back to baseline after 24 hours.

Conclusions and clinical importance: The depot formulation can be used in place of the short-acting ACTH to evaluate the adrenocortical reserve. Blood for peak cortisol concentrations should be drawn 3 hours after stimulation in cases in which HC is suspected; in HA-suspected cases, blood sampling can take place after 1 hour. As the stimulatory effect is gone after 24 hours, interference with other hormonal tests is unlikely after that time.
The ACTH stimulation test has traditionally been recognized as an accurate measure to assess the functional reserve capacity of the adrenal cortex and serves as gold standard in diagnosing hypoadrenocorticism (HA) in dogs.\textsuperscript{1,2} The test is also used to distinguish iatrogenic from spontaneous hyperadrenocorticism (HC) and to monitor trilostane or mitotane therapy.\textsuperscript{3,4}

Several ACTH preparations are available. One of the most commonly used formulations is a synthetic polypeptide, which consists of the first 24 (of a total 39) amino acids of the ACTH, generally known as cosyntropin or tetracosactide. Tetracosactide is available in two formulations: a short-acting form for IV or IM use and a depot form for IM use only. The depot formulation contains inorganic zinc complexes, which adsorb the active substance, resulting in a protracted release. Recently, the availability of the short-acting tetracosactide was limited; in some countries it was not available at all. In contrast, the availability of the depot formulation has never been affected.

In human medicine, the depot tetracosactide is used for both therapeutic and diagnostic purposes. Among other things, it has been described in the treatment of rheumatoid arthritis, dermatosis (ie, pemphigus, psoriasis) or infantile myoclonic encephalopathy.\textsuperscript{5,6} To diagnose adrenal insufficiency, the depot tetracosactide is used as a 5-hour test in which cortisol concentrations are determined before and every hour after stimulation for a 5-hour period.\textsuperscript{7} According to the manufacturer the duration of the stimulatory effect of the depot tetracosactide can be expected to last between 24 and 36 hours.

In veterinary medicine, the product has been evaluated in healthy dogs using two different concentrations (5 ug/kg and 250 ug/dog) and blood sampling has been performed during the first 3 hours after stimulation.\textsuperscript{8} However, it is unknown whether peak serum cortisol concentrations in dogs are reached 3 hours after stimulation. In
addition, the information as to what time after stimulation cortisol concentrations are back to baseline levels is lacking. Knowledge about the time of peak cortisol concentration is a prerequisite for correct interpretation of the test and evaluation of adrenocortical reserve, and knowing the duration of the stimulatory effect is important in order to assess the risk of interference with other hormonal measurements. The use of the depot ACTH preparation has not yet been evaluated in dogs with adrenal diseases. Therefore, the objective of the present study was to evaluate the depot tetracosactide preparation in healthy dogs and in dogs with clinical signs consistent with HA or HC, with the aims of determining the time point of peak serum cortisol concentrations and the duration of increased cortisol concentrations after tetracosactide depot administration.
Materials and Methods

Animals

Twelve purpose-bred beagle-pinscher mixed-breed dogs (6 females and 6 males) with a median age of 2 years (range, 1-2 years) and a median body-weight of 14.8 kg (range, 13-17 kg) were included. Dogs were housed in groups in standard kennels at the research unit of the Vetsuisse Faculty of the University of Zurich, fed a standard commercial maintenance pellet diet once daily, and had ad libitum access to water. In addition, 10 healthy privately-owned dogs from hospital personnel and students, including 2 intact males and 8 females (5 spayed), with a median age of 5 years (range, 1.5 – 12 years) and a median body weight of 15.8 kg (range, 6.9 – 27 kg) were used. They were determined to be healthy on the basis of their history and results of a physical examination plus hematological and biochemical evaluation. None of the dogs had received any medication for at least 8 weeks before inclusion in the study except routine vaccination, deworming and heartworm prophylaxis. Ten dogs with a clinical suspicion of HA were prospectively enrolled. All dogs had initially been suspected of having HA based on clinical signs or laboratory findings routinely seen in dogs with HA, such as vomiting, diarrhea, weakness, lethargy, hyperkalemia and/or hyponatremia. Blood and urine samples were collected for a complete blood count, serum biochemical profile and urinalysis. Work-up included an ACTH stimulation test using the depot preparation, which excluded HA when post-ACTH cortisol concentration was > 5 µg/dl. Additional tests were abdominal ultrasonography (in particular adrenal glands) and measurement of endogenous ACTH. There were 5 males (1 castrated) and 5 females (4...
Fifteen dogs with a clinical suspicion of HC were prospectively enrolled. Dogs previously treated with glucocorticoids, mineralocorticoids, gestagens or trilostane were excluded from the study. They all underwent a complete hematological and biochemical evaluation, urinalysis including urinary culture, low-dose dexamethasone suppression test (LDDS test: 0.01 mg/kg dexamethasone IV, blood sampling before, 4 and 8 hours thereafter), measurement of endogenous ACTH and an ultrasonographic examination of the adrenal glands. HC was confirmed when the LDDS test yielded a positive result (serum cortisol concentration 8 hours after dexamethasone administration > 1.0 µg/dL\(^9\)\(^{11}\)) and treatment with trilostane showed an appropriate response (starting dose of 2 mg/kg; re-evaluations performed as described previously). Pituitary-dependent hyperadrenocorticism (PDH) was diagnosed on the basis of the dogs’ concentration of endogenous ACTH and a symmetrical ultrasonographic appearance, with or without enlargement, of their adrenal glands. There were 9 males (4 castrated) and 6 females (5 spayed). Their median age was 10 years (range, 7 – 15 years) and their median body weight 13.4 kg (range, 3.6 - 45.0 kg).

Informed consent of all pet owners was obtained before including the dogs in the study. Animal care was in accordance with the guidelines and directives established by the Animal Welfare Act of Switzerland. The study protocol was officially approved by the veterinary office of the canton of Zurich and was in accordance with the guidelines and directives established by the Animal Welfare Act of Switzerland (TVB 191/2013).
Analytical procedures

All dogs underwent an ACTH stimulation test using a synthetic tetracosactide hexaacetate formulation with a concentration of 1000 µg/ml. For each test 5 µg/kg or at least 0.1 ml were administered intramuscularly (supra/infraspinatus of the left/right thoracic limb or quadriceps muscle of left/right hind limb) and blood samples (jugular venipuncture) were taken immediately before and 1, 2, 3, 4, 6 and 20-24 hours after stimulation. After clot retraction at room temperature, serum was harvested by low-speed centrifugation and transferred to tubes for storage at -20°C for later hormone assay. Cortisol concentrations were measured by chemiluminescence assay.

Sensitivity of the cortisol assay was 0.2 µg/dl. All samples of each dog were analyzed in the same run to minimize interassay variation. Endogenous ACTH before ACTH stimulation was determined by a chemiluminescence assay. Blood was collected into chilled EDTA-coated tubes placed on ice and centrifuged at 4°C. Cortisol and endogenous ACTH measurements were performed in house twice a week; plasma was stored either at -20°C (cortisol) or at -80°C (ACTH) until assayed. Immediately before analysis, samples were thawed at room temperature.

Statistical analyses

Data were analyzed with non-parametric statistical methods. Differences between groups of dogs were tested by using the Kruskal-Wallis H-test and the Mann-Whitney U test. Differences between the time points of the ACTH stimulation were tested by use of Friedman’s repeated measures test and the Wilcoxon matched pairs test. All p values quoted are Bonferroni corrected. The level of significance was set at p < 0.05.
Results

No adverse reactions and no evidence of pain at the injection site after IM administration were noticed in any of the dogs during and after the stimulation test.

Healthy dogs

Pre- and post-ACTH cortisol concentrations of all time points of the healthy dogs are summarized in table 1. There was no significant difference between the values of healthy research dogs and healthy client-owned dogs at any time point; therefore, all healthy dogs were summarized in one group for further analysis.

Maximal median cortisol concentrations were reached after 3 h. Peak cortisol concentrations occurred after 1 h in 1, after 2 h in 4, after 3 h in 11 and after 4 h in 6 dogs. Twenty-four hours after stimulation cortisol concentrations had again reached baseline concentrations. Cortisol concentrations 1, 2, 3, 4 and 6 h after stimulation were significantly higher than baseline cortisol concentrations (p: <0.001, <0.001, <0.001, <0.001, 0.002) and concentrations after 24 hours (<0.001, <0.001, <0.001, <0.001, 0.002) (figure 1).

Dogs with clinical suspicion of HA

Two dogs were diagnosed with primary HA (all post-ACTH cortisol concentrations < 0.2 µg/dl, cACTH > 1250 pg/ml) and 1 dog with iatrogenic HA (all post-ACTH cortisol concentrations < 2.5 µg/dl, methylprednisolone application by private veterinarian 4 weeks previously). In 7 dogs HA was excluded (post-ACTH > 5 µg/dl; non-HA group). Pre- and post-ACTH cortisol concentrations of the dogs with primary HA, with iatrogenic HA and with non-HA are summarized in table 1.
In the non-HA group, maximal median cortisol concentrations were reached after 4 hours. Peak cortisol concentrations occurred after 2 h in 1, after 3 h in 1 and after 4 h in 5 dogs. Cortisol concentrations 1 and 3 hours after stimulation were significantly higher than baseline cortisol concentration (p=0.036, 0.036) (figure 2).

**Dogs with clinical suspicion of HC**

Pre- and post-ACTH cortisol concentrations of the dogs with suspicion of HC are summarized in table 1. Maximal median cortisol concentrations were reached after 3 hours. Peak cortisol concentration occurred after 1 h in 1, after 2 h in 5, after 3 h in 6 and after 4 h in 2 dogs (1 dog missing). By 24 hours after stimulation cortisol concentrations had again reached baseline concentration. Cortisol concentrations 1, 2, 3, 4 and 6 hours after stimulation were significantly higher than baseline cortisol concentrations (p=0.002, 0.002, 0.002, 0.006, 0.026) and concentrations after 24 hours (p=0.01, 0.01, 0.016, 0.02, 0.034) (figure 3A).

In 10 dogs HC was confirmed (all PDH; HC group) and in 5 dogs HC was excluded (negative LDDS-test result; non-HC group). Two of these dogs were diagnosed with a hormonally inactive adrenal mass, one suffered from diabetes mellitus and 2 dogs had PU/PD of unclear origin. Cortisol concentrations at 24 hours after stimulation were significantly different between the HC and the non-HC group (p=0.02) (figure 3B).

**Comparison between groups (healthy, HC, non-HC, non-HA)**

The most distinctive differences were seen between the healthy dogs and the HC dogs (all PDH). Baseline cortisol and cortisol concentrations 1, 2, 3, 4, 6, and 24 hours after
stimulation were significantly higher in dogs with HC compared with those in healthy dogs (p: < 0.001, < 0.001, < 0.001, < 0.001, 0.002, 0.002, < 0.001) (figure 4).

In the non-HC group, baseline and cortisol concentrations 1 and 24 hours after stimulation (p: < 0.001, 0.02, 0.01) and in the non-HA group, baseline cortisol and cortisol concentrations 1 hour after stimulation were significantly different from those of the healthy dogs (p: 0.004, 0.048) (figure 4). Comparing the HC and the non-HA groups, cortisol concentrations at 1 and 2 hours after stimulation were significantly different (p= 0.01, 0.032) (figure 4). Between the non-HC and the non-HA groups no differences were detected.
Discussion

One of the goals of the present study was to evaluate peak cortisol concentrations after stimulation with depot tetracosactide in healthy dogs and dogs with suspicion of adrenal diseases. Peak median serum cortisol concentrations were reached after 3 hours in healthy dogs and dogs with suspected HC and after 4 hours in dogs suspected of having HA.

However, time point of peak cortisol concentration varied individually lying between 1-4, 1-4 and 2-4 hours in the healthy, non-HA group and in dogs suspected of having HC, respectively. It is therefore impossible to recommend an optimal sampling time point after stimulation with which the peak cortisol concentrations of all adrenal diseases can be detected. However, considering the objective in performing an ACTH stimulation test, it is different for hypo- and hyperfunctioning adrenal glands. In dogs suspected of suffering from HA, the goal is either to exclude HA with a sufficient stimulation (post-ACTH cortisol > 5 µg/dl) or to confirm HA by an insufficient stimulation (post-ACTH cortisol < 2 µg/dl). This goal would have been reached in our study, if blood had been taken already 1 hour after stimulation. All dogs suspected of having HA but in which it was excluded had a 1-hour cortisol concentration of > 9 µg/dl; in those with confirmed HA it was < 0.2 µg/dl. In addition, all healthy dogs had a 1-hour cortisol concentration > 5 µg/dl. Therefore, it could be concluded that for the diagnosis of HA in dogs, using the depot tetracosactide, a blood sample before and 1 hour after stimulation with 5 ug/kg is sufficient. This information is of great importance, because if there is high suspicion of HA and the dog is in critical clinical condition, administration of corticosteroids should not be delayed. Exogenous synthetic corticosteroids, however, can cross-react with cortisol assays or bear the risk of adrenal gland suppression due to the negative feed-
back mechanism, which as a consequence would lead to blunted stimulation and misinterpretation of the test.

The objective of an ACTH stimulation test in dogs suspected of having HC and in which trilostane therapy has to be monitored is different. In these situations knowledge of the peak cortisol concentration after stimulation is the important information. From our data blood for the diagnosis of HC should be drawn between 2 and 4 hours after stimulation, as within this time period the likelihood of getting the peak cortisol concentration is highest. Blood sampling after one hour in these cases might lead to a falsely low test result. Based on our data, no conclusion can be drawn as to which time point should be chosen for monitoring the trilostane therapy, as these dogs were not evaluated. It is merely speculative that in these cases the 1-hour value might also be too early to assess the maximal suppression of the cortisol synthesis during trilostane therapy. Further studies are needed to address this issue.

Another aspect of peak cortisol concentration is the magnitude of the peak. Two of the healthy dogs had post-ACTH cortisol values above the originally established normal reference interval, with values of 23.2 and 26.3 µg/dl. Also, in individual dogs with suspected HA, cortisol concentrations of up to 30 µg/dl could be observed. Although these high values could be interpreted as being consistent with HC, none of the healthy and certainly none of the HA-suspected dogs had clinical signs suggestive of HC. In agreement with our data, the study of Ginel et al. evaluating depot tetracosactide in healthy Greyhounds revealed peak cortisol concentrations with values up to 30 µg/dl.8 One might assume that the depot product itself could lead to higher cortisol concentrations compared to the short-acting ACTH. However, a study evaluating short-
acting ACTH for the diagnosis of HA in dogs reported similar results.\textsuperscript{12} Values for their
dogs, using a dose of 5 µg/kg, reached cortisol concentrations of up to 27.3 µg/dl; one
dog even had a value of 41.6 µg/dl without any evidence of HC and with a final
diagnosis of inflammatory bowel disease. Therefore we think it is more likely that the
increased response could have been caused by stress (white-coat effect) of the
hospitalization and the blood sampling, or it could be a sign of the concurrent illness of
the dogs.

A further interesting and important finding was that peak cortisol concentrations reached
values of >22 µg/dl in 2 of the 5 dogs where HC was suspected but not confirmed, which
according to published reference values should be considered abnormal.\textsuperscript{3,13,14} However,
these reference values were established using older and different assays. Care must be
taken in adapting these reference values, as it is possible that the magnitude of peak
concentration can reflect differences in the assays used to measure cortisol. In human
medicine it was shown that cortisol results from the same ACTH test are quite different
when measured by four commonly used assays.\textsuperscript{15} For the same cut-off value, it was
possible to have a negative test result with one and a positive result with another assay.
The authors concluded that establishing “normal peak” responses rely on the assay that
is used. This is likely to be the case in veterinary medicine as well. Therefore, criteria for
test interpretation probably need to be reestablished and the cut-off level for an
abnormal test result should be newly defined with the currently used assays. This has
already been suggested in the consensus statement of the ACVIM about the diagnosis
of spontaneous canine HC.\textsuperscript{4}
A second goal of the present study was to assess the duration of increased cortisol concentrations after depot tetracosactide administration. We were able to show that after 24 hours the plasma cortisol concentrations were back to baseline levels in both the healthy dogs and patient groups. This is of importance, as especially in dogs with suspected HC, a long duration of increased cortisol concentrations after ACTH administration carries the risk of interference with a LDDS test that may be performed the next day. In human medicine, plasma ACTH concentrations have been determined in healthy volunteers after the administration of depot ACTH. While with the use of the short-acting ACTH preparation, ACTH concentrations dropped to undetectable levels two hours after injection, the use of the depot preparation led to increased plasma concentrations up to 8 hours after administration. However, levels were undetectable after 24 hours in all subjects. This finding is somewhat reflected in our data: although we did not determine plasma ACTH concentrations, we observed a substantially decreased cortisol concentration after 6 hours as compared to 3 hours and, as mentioned above, values were back to baseline concentrations after 24 hours.

Interestingly, dogs with confirmed HC still had higher cortisol concentrations after 24 hours compared to dogs in which HC was excluded. This shows that in dogs with hyperfunctioning adrenal glands, exogenous ACTH can result in protracted cortisol secretion in comparison with dogs with normal adrenal function. Alternatively, it can reflect a decreased cortisol clearance in dogs with HC.

In conclusion, our data shows that the depot tetracosactide may be used in place of the short-acting product for evaluation of the hypothalamic-pituitary-adrenal axis in dogs with adrenal malfunction. Blood sampling can take place 1 hour after stimulation in dogs with suspected HA. However, special attention has to be paid in the diagnostic
evaluation of HC, as blood sampling one hour after stimulation does not reveal maximal
stimulation. Nonetheless, the LDDS test can be performed 24 hours after depot
tetracosactide administration without risk of interference.
Footnotes

a Synacthen Depot®, Novartis Pharma Schweiz AG, Bern, Switzerland

b DPC Immulite® 1000, Siemens Schweiz AG, Zurich, Switzerland

c GraphPad Prism6, Graph Pad Software, San Diego, CA, USA

d SPSS 22.0 for Windows, SPSS Inc, Chicago, IL USA
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**Figure legends**

**Figure 1:**
Serum cortisol concentrations before and after ACTH stimulation with a synthetic ACTH depot formulation in healthy dogs (n=22). Each box represents the interquartile (ie, 25th to 75th percentile) range, the horizontal line within the box represents the median value, the bars represent the 5th to 95th percentile, and the circles represent outlying data points. a-f: different letters indicate statistically significant differences between the time points (P < 0.05)

**Figure 2:**
Serum cortisol concentrations before and after ACTH stimulation with a synthetic ACTH depot formulation in dogs with initial suspicion of hypoadrenocorticism (HA), but in which HA was excluded (n=7). Each box represents the interquartile (ie, 25th to 75th percentile) range; the horizontal line within the box represents the median value; the bars represent the 5th to 95th percentile; the circles represent outlying data points. a-c: different letters indicate statistically significant differences between the time points (P < 0.05)

**Figure 3:**
3A:
Serum cortisol concentrations before and after ACTH stimulation with a synthetic ACTH depot formulation in dogs with clinical suspicion of hyperadrenocorticism (HC) (n=15). Each box represents the interquartile (ie, 25th to 75th percentile) range; the horizontal line within the box represents the median value; the bars represent the 5th to 95th
percentile; the circles represent outlying data points. a-f: different letters indicate statistically significant differences between the time points (P < 0.05)

3B:
Median and range of serum cortisol concentrations before and after ACTH stimulation with a synthetic ACTH depot formulation in dogs with confirmed hyperadrenocorticism (HC, n=10) and dogs with suspicion of HC but normal LDDS test (non-HC, n=5).

* Significant difference between the two groups.

Figure 4:
Serum cortisol concentrations before and after ACTH stimulation in healthy dogs (healthy, n=22), dogs with HC (HC, n=10), dogs with initial suspicion of HC, but negative LDDS test results (non-HC, n=5), dogs with initial suspicion of HA, but normal ACTH-stimulation (non HA, n=7), dog with iatrogenic HA (iHA, n=1) and dogs with primary HA (primary HA, n=2). For healthy, HC, non-HC and non-HA, median and range are reported. For iHA and HA, single values of each individual are reported. Statistical comparison was performed between healthy, HC, non-HC and non-HA, only.

a: significant difference to healthy dogs, b: significant difference to non-HA dogs.

#: a) applies to HC, non-HC and non-HA. ##: a) belonging to HC and non-HC.