Serum concentrations of cortisol and cortisone in healthy dogs and dogs with pituitary-dependent hyperadrenocorticism treated with trilostane

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Abstract

The serum concentrations of cortisol and cortisone were measured in 19 healthy dogs and in 13 dogs with pituitary-dependent hyperadrenocorticism (PDH) before and one hour after an injection of synthetic adrenocorticotropic hormone (ACTH). In the dogs with PDH, the cortisol and cortisone concentrations were measured before and after one to two weeks and three to seven weeks of treatment with trilostane. The dogs with PDH had significantly higher baseline and poststimulation concentrations of cortisol and cortisone, and higher baseline cortisol:cortisone ratios than the healthy dogs. During the treatment with trilostane, the poststimulation cortisol, the baseline and poststimulation cortisone concentrations, and the baseline and poststimulation cortisol:cortisone ratios decreased significantly. The decrease in poststimulation cortisone was significantly smaller than the decrease in cortisol.
Cortisol and cortisone in healthy dogs and dogs with pituitary-dependant hypercortisolism treated with trilostane

Nadja S. Sieber-Ruckstuhl DrMedVet, DipACVIM, ECVIM-CA\(^1\); Felicitas S. Boretti DrMedVet, DipACVIM\(^1\); Monique Wenger DrMedVet, DipACVIM\(^1\); Christiane Maser-Gluth DrMed, PhD\(^2\); Claudia E. Reusch DrMedVet, DipECVIM-CA\(^1\)

\(^1\)Clinic for Small Animal Internal Medicine, Vetsuisse Faculty University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland

\(^2\)Steroid Laboratory, Institute of Pharmacology, Ruprecht-Karls-University, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany

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Title: Effect of trilostane on cortisol and cortisone concentrations in dogs with pituitary-dependant hyperadrenocorticism. Abstract No. 26, pp 197
Summary
The objectives of this study were to evaluate cortisol, cortisone and cortisol/cortisone ratios in healthy dogs and dogs with pituitary-dependant hypercortisolism (PDH) and to investigate the effect of trilostane on these parameters.

Cortisol and cortisone concentrations were measured in 19 healthy dogs and in 13 dogs with PDH before and 1 hour after injection of synthetic adrenocorticotropic hormone (ACTH). In dogs with PDH cortisol and cortisone levels were investigated prior to (t0), in weeks 1-2 (t1) and in weeks 3-7 (t2) of trilostane treatment.

Dogs with PDH had significantly higher baseline and post-stimulation cortisol, baseline and post-stimulation cortisone and baseline cortisol/cortisone ratios than healthy dogs.

During trilostane therapy post-stimulation cortisol, baseline and post-stimulation cortisone and baseline and post-stimulation cortisol/cortisone ratios decreased significantly. The decrease in post-stimulation cortisone was significantly smaller than that of cortisol.

The larger decrease in cortisol than in cortisone during trilostane therapy could be caused by an influence of trilostane, of endogenous ACTH or of steroid precursors on the interconversion of cortisol and cortisone by the 11β-hydroxysteroid-dehydrogenase (11β-HSD). A low saturation point of the 11β-HSD type 2 would also explain the findings.

To determine the underlying reason for the observed hormone alterations further studies are needed.
Introduction

Pituitary-dependant hypercortisolism (PDH) is a frequent endocrine disease in dogs. Most often it is addressed medically, and recently trilostane has been introduced as an effective and safe treatment (Ruckstuhl and others 2002, Neiger and others 2002, Braddock and others 2003, Wenger and others 2004).

In vitro studies and studies in research animals have characterised trilostane as a competitive inhibitor of the 3ß-hydroxysteroid dehydrogenase/Δ5,4- isomerase enzyme system (3ß-HSD) (Neumann and others 1970, Potts and others 1978, Schane and others 1979, Lambert and others 1984). This enzyme catalyses the conversion of the Δ5-3ß-hydroxysteroids, pregnenolone, 17α-OH-pregnenolone and dehydroepiandrostenedione (DHEA) to the Δ4-3-ketosteroids, progesterone, 17α-OH-progesterone, and androstenedione. The enzymatic action of the 3ß-HSD is therefore essential for the biosynthesis of all classes of steroid hormones, namely glucocorticoids, mineralocorticoids, progesterone, androgens, and estrogens.

A recent study confirmed that in dogs, as in other species, trilostane is an inhibitor of the 3ß-HSD enzyme system. This inhibition, however, seems not the only effect of trilostane. It was postulated that trilostane has, in addition to the inhibitory effect on the 3ß-HSD enzyme system, an influence on the 11ß-hydroxylase and possibly on the interconversion of cortisol and cortisone by the 11ß-hydroxysteroid-dehydrogenase (11ß-HSD) (Sieber-Ruckstuhl and others 2006).

An effect of trilostane on the 11ß-HSD enzyme, leading to an activation of the conversion of cortisol to cortisone, has been shown in sheep adrenal cells (Touitou and others 1984). The 11ß-HSD enzyme catalyzes the interconversion of physiologically active cortisol and inactive cortisone. It is known to exist in different isoenzymes, of which two have been identified in humans. 11ß-HSD type 1 mainly reduces cortisone to cortisol and is widely distributed in human tissues (liver, adipose, gonads, bone, ocular tissues, vascular smooth muscle and skin),
whereas 11β-HSD type 2 inactivates cortisol to cortisone and is predominantly located in the kidneys, colon, and salivary glands (Krozowski and others 1999). Recently, also in dogs two 11β-HSD isoenzymes have been found and their tissue distribution has been studied (Sieber-Ruckstuhl and others 2007).

If trilostane has an effect on the 11β-HSD, it would not only influence the blood but also the tissue concentrations of cortisol and cortisone. This would be clinically relevant, because altered tissue concentrations of cortisol and cortisone may play a role in the development of side effects or the resistance to side effects of dogs.

The purposes of the study were, therefore, to evaluate cortisol, cortisone and cortisol/cortisone ratios in the blood of healthy dogs and dogs with PDH and to investigate the effect of trilostane on these parameters.

Material and methods

Dogs and inclusion criteria

Nineteen clinically healthy dogs, nine females (4 spayed) and 10 males (5 neutered), aged between 4.5 to 14 years (median, 8 years) with a bodyweight between 7.8 to 30.5 kg (median, 24.4 kg) were included in the study. Breeds represented included Labrador Retriever (n = 4), German Shepherd Dog (2), Dachshund (1), Jack Russell Terrier (1), Hovawart (1), Golden Retriever (1), English Cocker Spaniel (1), Beagle (1), Siberian Husky (1), German Shorthaired Pointer (1), and 5 mixed-breed dogs. The dogs were considered healthy on the basis of medical history, results of physical examination and laboratory analyses (haematology, biochemical profile, ACTH stimulation test). They were owned by students or employees of the Vetsuisse Faculty University of Zurich, Switzerland. Informed consent was obtained from all owners of these dogs and their use was approved by the Swiss Cantonal Veterinary Office.
Additionally, thirteen dogs with HC were included in the study. Eight were female (6 spayed), 5 were male (3 neutered), age ranged between 8 to 16 years (median, 9 years) and bodyweight between 2.3 and 37 kg (median, 16 kg). Breeds represented included Yorkshire Terrier (n = 1), Dachshund (1), Poodle (1), Maltese (1), Labrador Retriever (1), Beagle (1), Hovawart (1), Giant Schnauzer (1), Jack Russel Terrier (1), Tibetan Terrier (1), Bichon Frisé (1) and mix breed dog (2). In all dogs a complete haematological and biochemical evaluation, a low dose dexamethasone suppression test (LDDS test), an ACTH stimulation test and ultrasonographic examination of the adrenal glands were performed. In 10 dogs the urine cortisol-to-urine creatinine ratio from a voided urine sample collected by the owner in the morning was additionally evaluated up to three times. In 10 dogs endogenous ACTH concentrations were measured and in 6 dogs a pre- and post contrast computed tomography (CT) of the hypophyseal fossa was performed. Dogs were included in the study when consistent clinical and laboratory findings for HC were present, the LDDS and/or the urine cortisol-to-urine creatinine ratio were positive, treatment with trilostane showed an adequate response and the dog had not received other treatments (eg radiation therapy, mitotane therapy) for HC. In more detail, the following clinical signs were observed in the dogs: reduced activity (n=13), polyuria/polydipsia (12), dermatologic problems (10 - alopecia (10), hyperpigmentation (3), thin skin (3)), polyphagia (7), pendulous abdomen (7), panting (6) and hepatomegaly (4). The LDDS test revealed an 8-hour cortisol value of > 27.5 nmol/L in 12/13 dogs. The one dog showing suppression during the LDDS test (baseline cortisol 118.6 nmol/L, 4-hour cortisol 2.8 nmol/L, 8-hour cortisol 5.5 nmol/L) had a positive ACTH stimulation test (0-hour cortisol 85.5 nmol/L 1-hour cortisol 775.3 ug/dl) and three positive urine cortisol-to-urine creatinine ratios (64, 66, 52). All dogs responded favourably to trilostane treatment.

PDH was diagnosed on the basis of the concentration of endogenous ACTH, results of the computed tomography and a symmetrical appearance, with or without enlargement of the
adrenal glands during ultrasonographic examination. Informed consent was obtained from the owners of all dogs.

**Endocrinologic analyses**

Before performing the ACTH stimulation test, blood was collected into chilled EDTA-coated tubes placed on ice for measurement of plasma endogenous ACTH concentration. After centrifugation at 4°C plasma was stored at -80°C until assayed. Measurement of 2 samples was performed at the University of Utrecht, Netherlands by use of a commercially available two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands) as described elsewhere (Bosje and others 2002). Measurement of 8 endogenous ACTH samples was performed in-house also with a commercially available two-site immunoradiometric assay (IRMA) (DiaSorin, Stillwater, Minnesota, USA). Sensitivity of this ACTH assay was 1.5 pg/ml. Intra assay coefficient of variation for ACTH was 6.75 % (4.7-12.5). Recovery by mixing two plasma samples with different ACTH concentrations was 115 % (109-121.6).

The ACTH stimulation test was performed by collecting blood samples for determination of serum concentrations of cortisol and cortisone before and 1 hour after IM injection of 0.25 mg of synthetic ACTH (Synacthen®, Novartis Pharma Schweiz AG, Bern, Switzerland). The serum was separated and stored at -80°C until assayed. Extracted cortisol and cortisone concentrations were measured by a specific in-house radioimmunoassay (RIA) established at the Steroid Laboratory of the University of Heidelberg, Germany using titrated steroid (Amersham Biosciences, Freiburg, Germany). Antibodies were raised and characterized in the steroid laboratory, as described elsewhere (Vecsei 1979). Prior to RIA a recovery-corrected extraction and chromatographic purification was performed, thereby efficiently removing cross-reacting steroids. More specifically the chromatographic separation on microcolumns modified from the method previously described (Abraham and others 1972) was carried out using Celite (Celite 545 AW, Sigma Aldrich, Taufkirchen, Germany) as an inert support for
partition chromatography. Sensitivities were 1.4 nmol/L for cortisol and cortisone. Intra- and inter-assay coefficients of variation for the RIA were 9.0 % and 11.8 %, 4.8 % and 8.1 % for cortisol and cortisone, respectively. For routine evaluations, the ACTH-stimulation test was performed as described above. Cortisol concentration was determined by use of chemiluminescence method (ADVIA Centaur® System, Bayer (Schweiz) AG, Zurich, Switzerland). A cortisol concentration > 552 nmol/l in the sample 1 hour after ACTH administration was considered abnormal. A LDDS test was performed in each dog. It consisted of collection of blood samples before and 4 and 8 hours after injection of dexamethasone (0.01 mg/kg, IV) (Dexadreson, Virbac AG, Küsnacht, Switzerland). A cortisol concentration of $\geq 27.5$ nmol/l in the sample collected 8 hours after dexamethasone administration was considered to reflect a lack of suppression and was consistent with hypercortisolism. A urine cortisol-to-urine creatinine ratio $> 10 \times 10^{-6}$ was considered abnormal and consistent with hypercortisolism (Ruckstuhl and others 2002, Wenger and others 2004).

**Experimental design**

The prospective study was performed between January 2001 and May 2006. The initial dose of trilostane for dogs with PDH was determined on the basis of 3 categories for bodyweight ($< 5$ kg, 30 mg, PO, q 24h; 5 to 20 kg, 60 mg, PO, q 24h; and $> 20$ kg, 120 mg, PO, q 24h). ACTH stimulation tests were performed prior to trilostane treatment (t0), 1-2 weeks (t1) and 3-7 weeks (t2) thereafter. At t1 and t2 the test was performed 2-6 hours after the daily dose of trilostane. At each re-evaluation trilostane doses were adjusted according to the previously described protocol (Ruckstuhl and others 2002, Wenger and others 2004). Healthy dogs were evaluated only once during the study. For that purpose blood was analyzed and an ACTH stimulation test was performed in the morning after a 12 hour fast.
Statistical analysis

Results were analyzed by means of nonparametric statistical methods (SPSS 11.0 for Windows, SPSS Inc, Chicago, Ill, USA and GraphPad Prism 2, San Diego, California, USA). Ranges and median values are reported. A Kruskal-Wallis test was used to detect differences in baseline and post-stimulation concentrations among categories of sex. The Wilcoxon matched pairs test was used to detect differences between baseline and post-stimulation values within sex. Differences between healthy dogs and dogs with PDH were tested by means of the Mann-Whitney-U-test. Changes during trilostane therapy were tested by use of Friedman’s repeated measures test and Dunn’s post test. Linear correlation was calculated by Spearman nonparametric correlation. Differences were considered significant at values of $p \leq 0.05$. For values below detection limit the mean between 0 and the detection limit was entered for statistical analysis.

Results

Healthy dogs

Cortisol, cortisone and cortisol/cortisone ratios before and after ACTH stimulation did not differ in healthy dogs of different sex or neutering status (data not shown). Hormone values of all healthy dogs were therefore combined for further evaluations and for comparison with hormone levels of dogs with PDH.

Cortisol, cortisone and cortisol/cortisone ratios increased significantly after ACTH stimulation (Fig 1).

Dogs with PDH before trilostane treatment

Cortisol, cortisone and the cortisol/cortisone ratios increased significantly after ACTH stimulation in dogs with PDH (Fig 1).
Comparison of dogs with PDH and healthy dogs

Cortisol and cortisone concentrations before and after ACTH stimulation were significantly higher in dogs with PDH compared to healthy dogs (Fig. 1).

Cortisol/cortisone ratios before ACTH stimulation of dogs with PDH were significantly higher than ratios of healthy dogs, whereas cortisol/cortisone ratios after ACTH stimulation did not differ significantly (Fig. 1).

Dogs with PDH during trilostane therapy

Baseline cortisol concentrations did not change significantly during trilostane treatment.

Baseline cortisone concentrations were significantly lower at t1 compared to t0. Post-stimulation cortisol and cortisone concentrations were significantly lower at t1 and t2 compared to t0 (Fig. 2).

The baseline cortisol/cortisone ratios were significantly lower at t2 compared to t0 (Fig. 2).

Post-stimulation cortisol/cortisone ratios were significantly lower at t1 and t2 compared to t0 (Fig. 2).

Compared to t0, post-stimulation cortisol concentrations decreased by 66.3-99.1 % (median: 87.1) at t1 and by 57.8-97.9 % (median: 85.5) at t2. Post-stimulation cortisone concentrations decreased by 15.4-84.6 % (median: 64.8) at t1 and by 42.9-84.6 % (median: 64) at t2. The decrease in post-stimulation cortisone concentrations was significantly smaller than the decrease in cortisol concentrations (Fig. 3).

Clinical signs

After starting trilostane treatment all dogs with PDH improved clinically within the first 1 to 3 weeks.
Discussion

The study reported here consisted of two parts; in the first part cortisol, cortisone and cortisol/cortisone ratios of healthy dogs were evaluated and compared to the corresponding values of dogs with PDH.

In healthy dogs, cortisol, cortisone and cortisol/cortisone ratios increased significantly after ACTH stimulation. The increase in cortisone levels is contrary to results in healthy humans, where mean cortisone concentrations either decrease or remain unchanged after ACTH administration (Walker and others 1992, Morineau and others 1997, Dötsch and others 2001, Vogeser and others 2001). Some investigators explain these findings through an up-regulation of the 11β-HSD type 1 activity (leading to increased conversion of cortisone to cortisol), others through an inhibition of the 11β-HSD type 2 activity (causing decreased production of cortisone from cortisol) by synthetic ACTH (Walker and others 1992, Morineau and others 1997, Vogeser and others 2001). Independent of the type of influence ACTH exerts on the 11β-HSD isoforms in humans, the different course of cortisone levels seen in dogs possibly indicates that the two 11β-HSD isoforms of dogs react differently to ACTH than those of humans.

Comparing hormone values between healthy dogs and dogs with PDH revealed that baseline and post-stimulation cortisol and cortisone concentrations were higher in dogs with PDH. Cortisol/cortisone ratios before ACTH stimulation were also significantly higher in dogs with PDH. Humans with Cushing’s disease have significantly elevated cortisol concentrations compared to healthy humans, but cortisone and cortisol/cortisone ratios are not consistently increased (Dötsch and others 2001, Morita and others 2004). This is again explained by an influence of ACTH on either the 11β-HSD type 1 or the 11β-HSD type 2 (Morineau and others 1997, Dötsch and others 2001, Morita and others 2004). The sensitivity of the two 11β-
HSD isoforms to ACTH or the activity of the two 11β-HSD isoforms may vary in different individuals, leading to a variable course of the cortisone concentrations and the cortisol/cortisone ratio. In dogs with PDH cortisone concentrations seem more consistently elevated. This could point once more to a different sensitivity of the canine 11β-HSD isoforms towards ACTH.

The second part of the study involved the evaluation of cortisol, cortisone and cortisol/cortisone ratios of dogs with PDH during trilostane therapy. Due to precursor changes observed in a recent study, it was postulated that 3β-HSD inhibition is not the only effect of trilostane. An additional influence on the interconversion of cortisol and cortisone by the 11β-HSD seemed possible (Sieber-Ruckstuhl and others 2006). This has been seen in sheep adrenal cells, where trilostane affected the 11β-HSD type 2, leading to an activation of the conversion of cortisol to cortisone (Touitou and others 1984).

A significantly larger decrease in cortisol than cortisone concentrations, resulting in a significant fall in the cortisol/cortisone ratio was seen during trilostane therapy in dogs. Several explanations can be given for these hormone alterations.

First, similar to effects seen in sheep adrenal cells, trilostane could activate the 11β-HSD type 2 in dogs, causing a higher conversion rate of cortisol to cortisone and therefore a smaller decrease in cortisone levels (Touitou and others 1984).

Second, the activity and especially the saturation point of the 11β-HSD type 2 in dogs have to be considered. A low saturation point of this enzyme could explain the observed hormone changes. If the 11β-HSD type 2 is saturated at a low cortisol level, a large decrease in cortisol concentrations to below the saturation point would have to occur in order to result in a decrease in cortisone values. The cortisol level at which the 11β-HSD type 2 is saturated in dogs is not known.
Third, as discussed in the first part, endogenous or exogenous ACTH is thought to alter the activity of the 11β-HSD isoforms in humans (Walker and others 1992, Morineau and others 1997, Vogeser and others 2001). In dogs, endogenous ACTH concentrations increase during trilostane therapy (Witt and Neiger 2004, Sieber-Ruckstuhl and others 2006). Assuming the same influences of ACTH on the 11β-HSD isoforms as in humans, cortisol should decrease to a lesser extent than cortisone, contrary to the results observed. However, once more, the 11β-HSD isoforms of dogs could have a different sensitivity to ACTH than those of humans, and therefore elevated ACTH levels could cause an increase in cortisone concentrations in dogs.

Fourth, changes in cortisol, cortisone or cortisol precursor levels could inhibit or activate the 11β-HSD isoforms. In humans, increased cortisone concentrations have been demonstrated to cause a retroinhibition of the 11β-HSD type 2 activity (Steward and others 1995). Corticosterone, 18-OH-corticosterone and 11β-OH-androstenedione showed a significant inhibition of the 11β-HSD type 2 activity (Diederich and others 1996). During trilostane therapy in dogs several precursor changes have been observed (Sieber-Ruckstuhl and others 2006). An influence of these changes on the interconversion of cortisol and cortisone should therefore also be considered for the larger decrease in cortisol than in cortisone during trilostane therapy.

One has to raise the question if blood hormone concentrations are a good indicator of 11β-HSD activity. Concentrations of circulating hormones are affected by production and removal rates. To measure cortisol and cortisone concentrations in tissues or the 11β-HSD action itself would predict the enzyme activity more accurately. To do this, however, tissue biopsies would have been necessary, which is difficult to justify in client-owned dogs. Therefore, to gain conclusive results about the effect of trilostane on 11β-HSD activity in dogs cell culture experiments will be necessary.
In conclusion, healthy dogs and dogs with PDH show several interesting differences in cortisone levels compared to humans; this points to a possibly distinct sensitivity of the 11β-HSD isoforms to ACTH in the two species.

During trilostane therapy the decrease of cortisol was more pronounced than that of cortisone. To determine if these findings are due to an effect of trilostane, of endogenous ACTH or of steroid precursors on the interconversion of cortisol and cortisone by the 11β-HSD or are caused by a low saturation point of the 11β-HSD type 2 further studies are needed.

References


SCHANE, H. P., POTTTS, G. O. & CREANGE, J. E. (1979) Inhibition of ovarian, placental, and adrenal steroidogenesis in the rhesus monkey by trilostane. Fertility and Sterility, 32(4), 464-467


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Tables:

Table 1:

Cortisol, cortisone and cortisol/cortisone ratios in 19 healthy dogs and in 13 dogs with PDH

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Healthy dogs (n=19)</th>
<th>Dogs with PDH (n=13)</th>
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<tr>
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<tr>
<td>Cortisol (ug/dl)</td>
<td>27.6 (8.3-110.4)</td>
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<td>Cortisone (ug/dl)</td>
<td>13.9 (6.9-40.2)</td>
<td>27.7 (2.8-44.4)</td>
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<td>Cortisol/ cortisone</td>
<td>1.8 (0.4-4.8)</td>
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# Indicates significant difference to corresponding hormone value in healthy dogs
* Indicates significant change in relation to the corresponding value before treatment.

Median (range) cortisol, cortisone and cortisol/cortisone ratios before (0) and after (1) ACTH stimulation in 19 healthy dogs and in 8 dogs with PDH before (t0), 1-2 weeks (t1) and 3-7 weeks (t2) after starting trilostane therapy.
Figure 1

**Cortisol, cortisone and cortisol/cortisone ratios in healthy dogs and dogs with PDH**

Point plots of baseline (0) and post-ACTH stimulation (1) serum cortisol, cortisone and cortisol/cortisone ratios in healthy dogs and dogs with PDH. * Indicates significant increase compared to baseline value. ** Indicates significant difference between the groups (p ≤ 0.05).

Figure 2

**Cortisol, cortisone and cortisol/cortisone ratios in dogs with PDH during trilostane therapy**

Point plots of baseline (0h) and post-ACTH stimulation (1h) serum cortisol, cortisone and cortisol/cortisone ratios before (t0), 1-2 weeks (t1) and 3-7 weeks (t2) after starting trilostane treatment in dogs with PDH. * Indicates significant difference compared to baseline value (p ≤ 0.05).

Figure 3

**% decrease of cortisol and cortisone during trilostane therapy**

Point plots of the decrease in serum cortisol and cortisone 1-2 weeks (t1) and 3-7 weeks (t2) after starting trilostane treatment in dogs with PDH.

* Indicates significant difference between the groups (p ≤ 0.05).
Figure 1 (N. Sieber-Ruckstuhl)

Cortisol nmol/L

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Cortisone nmol/L

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Cortisol/cortisone

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Figure 2 (N. Sieber-Ruckstuhl)
Figure 3 (N. Sieber-Ruckstuhl)

% decrease of cortisol and cortisone

- Cortisol
- Cortisone