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Adrenocorticotropic hormone, but not trilostane, causes severe adrenal hemorrhage, vacuolization and apoptosis in rats

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Table of contents

1 Summary

Adrenal necrosis has been reported as a complication of trilostane application in dogs with hyperadrenocorticism. It was hypothesized that necrosis results from the increase of adrenocorticotropic hormone (ACTH) during trilostane therapy. Aim of this study was to assess the effects of ACTH and trilostane on adrenal glands in an experimental animal model. The study consisted of 2 parts (experiment 1 and experiment 2). For experiment 1, 36 rats were divided into six groups. 4 groups received ACTH in different doses (60, 40, 20, 10 µg/d), 1 group received saline and 1 no therapy. For experiment 2, 24 rats were divided into 3 groups. Two groups received trilostane mixed into chocolate pudding (5, 50 mg/kg/d) and one group received pudding alone.

At the end of the experiments, adrenal glands were assessed by histology and immunohistochemistry; levels of endogenous ACTH and nucleosomes were measured in the blood. Rats treated with 60 µg ACTH/d showed significantly more hemorrhage, vacuolization and apoptotic cells in their adrenal glands than rats treated with 20 or 10 µg ACTH/d, trilostane or saline or animals receiving no therapy. Rats treated with 60 µg ACTH/d had a significant higher amount of nucleosomes in blood compared to rats treated with 10 µg ACTH/d, trilostane or saline. We conclude that in healthy rats ACTH, but not trilostane, causes adrenal degeneration in a dose-dependent manner. Results of this study support the hypothesis that adrenal gland lesions seen in trilostane-treated dogs are caused by ACTH.

2 Introduction

Trilostane is a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/$\Delta^{5,4}$-isomerase enzyme system (3β-HSD) [1-3], which is essential for the biosynthesis of all classes of steroid hormones. Due to its reliable clinical efficacy, trilostane is widely used for the treatment of dogs with pituitary-dependent hyperadrenocorticism (PDH). Trilostane therapy decreases cortisol and, as a result of the reduced negative feedback, increases endogenous adrenocorticotropic hormone (ACTH) concentration [4-8]. When dosed optimally, side effects during trilostane therapy are rare. However, recently, adrenal necrosis has been described in trilostane-treated dogs with PDH [9-11]. Necrosis of the adrenal cortex cannot be explained by the known enzyme-inhibiting mechanism of trilostane [1]; the cell death was therefore attributed most probably to either the hypersecretion of ACTH or to a direct action of trilostane or one of its metabolites [9-11].

ACTH is secreted by the anterior pituitary gland; its primary function is to stimulate the production of glucocorticoids from the adrenal cortex. The use of a chemically synthesized analogue of the natural ACTH molecule is widespread in human and veterinary medicine to diagnose and monitor adrenal diseases. Although ACTH is an important physiologic hormone, the suspicion exists that it can have deleterious effects if present in excessive amounts in animals and humans. First, in human medicine, both, stress-mediated increases in ACTH during severe infections and septicemia and the therapeutic administration of huge doses (> 1 mg/d) of exogenous ACTH seemed associated with the risk of adrenal hemorrhage and necrosis [12-14]. Second, rats treated intermittently with high doses of a purified bovine ACTH preparation, showed degeneration, focal necrosis and hemorrhage in the adrenal glands [15]. Later studies, however, using lower doses of synthetic ACTH in rats, did not report such side effects [16-18]. These discrepancies are striking, but possible explanations are the dose differences or the chemical purity of the newer synthetic preparations.

Systematic studies on the influence of ACTH on adrenal cells using synthetic ACTH in different concentration have not been published. Furthermore, long-term studies in animals using high doses of trilostane and evaluating adrenal changes are lacking. As ACTH concentrations during trilostane therapy can increase impressively in dogs, it has been hypothesized by several researchers that adrenal necrosis seen in trilostane-treated dogs with PDH could be attributed to the increased ACTH concentration [9-11,19]. To gain further insight into the effects of ACTH and trilostane on adrenal cells, the authors intended to evaluate their effects in an experimental animal model. Therefore, the goals of the study reported here were to evaluate the effects of different doses of synthetic ACTH and the effects of different doses of trilostane on adrenal glands of healthy rats.

3 Materials and methods

3.1 Animals and experimental procedures

All studies were approved by the Cantonal Veterinary Office of Zurich and conducted in accordance with guidelines established by the Animal Welfare Act of Switzerland (permission number: 34/2008).

For experiment 1, 36 adult male Sprague-Dawley rats (Charles River, Sulzfeld, Germany), weighing 224-272 g (median 255) were used. Rats were housed in pairs, under 12:12 h light-dark cycle (light onset at 8:00 am), at 23 °C, and maintained on a standard diet (Rat Chow; Charles River) and tap water *ad libitum* for the duration of the experiment. The rats were divided into six groups. The rats of four groups were infused subcutaneously (Alzet osmotic pumps Mod 2ML4; Alzet, Cupertino, CA, USA) for up to 16 wk with synthetic human ACTH (Synacthen® Depot; concentration: 2 mg/ml; Novartis International AG, Basel, Switzerland) dissolved in 0.9 % NaCl; group 1.1 (n=9), 1.2 (n=5), 1.3 (n=5) and 1.4 (n=5) received 60, 40, 20 and 10 µg ACTH/d, respectively. Animals of group 1.5 (n=6) were infused for 16 wk with 0.9 % NaCl, and animals of group 1.6 (n=6) did not receive any treatment. For the pump implantation, the rats received 2 mg/kg meloxicam (Metacam®, Boehringer Ingelheim GmbH, Basel, Switzerland) subcutaneously and were subsequently anesthetized with isoflurane (Abbott AG, Baar, Switzerland). Pumps were exchanged every 4 wk.

For experiment 2, 24 adult male Sprague-Dawley rats (Janvier, S.A.S., Le Genest-St-Isle, France), weighing 284-334 g (median 310) were used. Rats were housed in pairs, under 12:12 h light-dark cycle (light onset at 22:00 pm) and at 23 °C. During the dark period rats were fed a chocolate pudding (Ensure plus crème, Abbott Laboratories, Saint Florent de Vallée, France) with or without trilostane in addition to the standard diet (Rat Chow; Charles River) and tap water *ad libitum*. During light period rats were fasted by removing the standard diet; tap water was available *ad libitum.* The rats were divided into three groups. Animals in

group 2.1 (n=8) and 2.2 (n=8) received 5 and 50 mg/kg trilostane/d orally mixed in the chocolate pudding and animals in group 2.3 (n=8) received chocolate pudding only.

During both experiments, the clinical condition and body weight of each rat were checked daily. The amount of food and water consumed per cage was determined every other day. If the body weight of a rat decreased to less than 30 % of the average median body weight of the control rats, the rat was euthanized before the end of the experiment because of animal welfare reasons.

At the beginning and at the end of the experiments animals were anesthetized with isoflurane and serum and EDTA-plasma was collected by sublingual venous puncture. At the end of the two experiments, the adrenal glands were collected within 30 min of euthanasia and one was fixed for 24-48 h in 4 % neutral buffered formaldehyde and the other snap frozen in liquid nitrogen and stored at -80 ºC.

3.2 Histological examinations

Adrenal glands were fixed in 4 % neutral buffered formaldehyde and embedded in paraffin by routine procedures. Sections (3-5 µm) were stained with hematoxylin and eosin by a routine procedure or used for immunostaining. The latter were mounted on positively charged glass slides (Superfrost®, Erie Scientific Company, Portsmouth, NH) and dried overnight at 37 °C. Sections through the adrenal glands were orientated on the transversal plane, and all the anatomical layers (zona glomerulosa/arcuata, zona fasciculata, zona reticularis and medulla) were evaluated histopathologically. The animals were numbered by the first author (WB) and were evaluated blinded by an experienced pathologist (FG). For statistical analysis, the histologic lesions in the adrenal cortex were quantified using a scoring system comprising a measure for hyperemia/hemorrhage (from 1-4: 1 – normal blood content; 2 – focal or multifocal hyperemia; 3 – moderate diffuse hyperemia and hemorrhage; 4 – severe diffuse hyperemia and hemorrhage), a measure for the degree of cytoplasmic vacuolization (from 1-3:

1 – normal; 2 – moderately increased; 3 – markedly increased), and a measure for the occurrence of clusters of neutrophils (from 1-2: 1 – not present; 2 – present). A cumulative score calculated by adding up the 3 single scores was determined for each animal and used for the statistical analysis.

3.3 Immunohistochemical analyses

Apoptotic cells were detected and quantified in the adrenal glands using immunohistochemical stains for cleaved lamin A and cleaved caspase-3. Antigen specific rabbit polyclonal antibodies (lamin A: 2035, Cell Signaling Technology, Bioconcept, Allschwil, Switzerland; caspase-3: AF835, R&D Systems, Abingdon, UK) were applied following a previously described immunoperoxidase protocol with minor modifications [20]. Briefly, antigen retrieval was done heating the sections for 20 min at 98 °C in EDTA buffer, pH 9 (lamin A) or for 2 min at 125 °C in citrate buffer, pH 7 (caspase-3). The primary antibody was applied for 1h at room temperature, and the reaction was visualized using a Detection Kit (Dako Cytomation, Zug, Switzerland) according to the manufacturer's instructions. Canine keratinocytes treated with UV-irradiation and rat lymph node tissue were used as positive controls, while the primary antibody was omitted as a negative control.

To evaluate the immunoreactivity for cleaved lamin A and cleaved caspase-3, 10 digital microscopic images were created from the cortical region in each adrenal gland immunostain following a predetermined scheme, using an AxioCam digital camera (Carl Zeiss AG, Feldbach, Switzerland) and a 40 x objective lens. Each microscopic image was assessed manually by counting all labelled cells displaying apoptotic morphology; groups of closely lying labelled apoptotic cell bodies were counted as one cell. For each rat, the number of apoptotic cells was determined as the average of the counts of two blinded investigators (WB, NS).

3.4 Endocrinological analyses

Blood levels of endogenous ACTH were determined using a chemiluminescent immunoassay system (DPC Immulite® One, Siemens Healthcare Diagnostics GmbH, Switzerland). The detection limit of the ACTH assay was 2.2 pmol/L.

The slope of the regression equations and the r^2 were close to 1 for each dilution. The intra-assay coefficient of variation (CV) was 8.3, 1.4 and 2.3 % for samples with 3, 13.5 and 118 pmol/L ACTH, respectively. The inter-assay coefficient of variation (CV) was 22.3, 8.8 and 4.7 % for samples with 3.6, 39.4 and 117 pmol/L ACTH, respectively.

3.5 Histone-complexed DNA fragments (nucleosomes)in blood

For relative quantification of histone-complexed DNA fragments the cell death detection ELISA^{plus} (Roche Applied Science, Rotkreuz, Switzerland) was used. All measurements were done in serum.

3.6 Statistic analysis

Results were analyzed by use of nonparametric statistical methods (SPSS 11.0 for Windows, SPSS Inc, Chicago, Ill, USA and GraphPad Prism 4, San Diego, California, USA). Ranges and median values are reported. Differences between groups were tested by use of Kruskal-Wallis H test and Dunn's post test. Differences within groups between the start and the end of the experiment were tested by use of Wilcoxon matched pairs test. Differences were considered significant at values of $p \leq 0.05$.

4 Results

4.1 Body weight and clinical parameters

4.1.1 Experiment 1:

Food intake was significantly lower and water intake significantly higher in ACTH-treated rats during the experiment compared to rats treated with saline (food intake/cage/d (2 rats): ACTH-treated rats: median: 54 g, range: 46-65, rats treated with saline: median: 59 g, range: 49-68, $p<0.0001$; water intake/cage/d (2 rats): ACTH-treated rats: median: 72 ml, range: 55-84, rats treated with saline: median: 63 ml, range: 55-78, $p<0.0001$).

All ACTH-treated rats showed a significantly lower body weight gain compared to rats treated with saline ($p<0.0001$) (Figure 1a). Nine of nine, 2/5, 1/5 and 0/5 rats treated with 60, 40, 20 and 10 μg ACTH/d had to be euthanized before the end of the experiment, because the difference in body weight compared to the control animals (rats treated with saline) exceeded the set threshold (> 30 % below controls).

4.1.2 Experiment 2:

There was no difference in food or water intake and body weight gain between trilostane treated and control rats (Figure 1b). No rat had to be euthanized before the end of the experiment.

4.2 Histological examinations

4.2.1 Experiment 1

Histological changes were found only in the adrenal cortex. They were particularly pronounced in the group treated with 60 μg ACTH/d and were found only occasionally and to a minor extent in some animals of the other groups. The major changes consisted in strong dilatation of the sinusoids, associated with a very pronounced hyperemia combined, to some extent, with extravasation of erythrocytes (Figures 2). Another feature that was prominent in

several animals in the group treated with 60 µg ACTH/d and that was only occasionally evident in the low-dose (10-40 µg ACTH/d) group and absent in both control groups, was the occurrence of predominantly large cytoplasmic vacuoles filling most of the cell cytoplasm (Figures 2). Further, small clusters of neutrophils, mostly located in the dilated sinusoids and to a lesser extent outside of the capillaries were observed only in animals treated with 60 µg ACTH/d. Finally, there was a loss of basophilia of the zona reticularis, caused by an increase in the cytoplasmic volume and an apparent increase in the size of the nucleus; these cells of the zona reticularis were indistinguishable from those in the zona fasciculata. This finding was present in all animals treated with high dose of ACTH (60 µg/d), 1/5 animals treated with 40 µg ACTH/d and 2/5 animals treated with 20 µg ACTH/d. In all other animals, cells in the zona reticularis were clearly distinguishable from those in the zona fasciculata.

Overall, rats treated with 60 µg ACTH/d had a significantly higher cumulative histologic score than rats treated with 20 or 10 µg ACTH/d, rats treated with saline or rats receiving no therapy ($p=0.0001$) (Figure 3a).

4.2.2 Experiment 2

A slight degree of hyperemia in the adrenal cortex was present in 1/8 animals treated with 5 mg/kg trilostane/d, in 2/8 animals treated with 50 mg/kg trilostane/d and in 2/8 control animals. The degree of vacuolization of adrenal cortical cells was similar in all groups and similar to the control animals in experiment 1. There were no clusters of neutrophils in any of the tissues, while the zona reticularis and the zona fasciculata could be easily distinguished in all animals.

There was no difference in the cumulative histologic score between rats treated with trilostane and control rats (Figure 3b).

4.3 Immunohistochemical analyses

4.3.1 Experiment 1

Cellular structures immunolabelled for lamin A mostly consisted of small groups of apoptotic cell bodies and occasionally of individual intact adrenal cortical cells showing early features of apoptotic cell death. The former showed a diffuse labelling, while the latter showed either diffuse or nuclear labelling (Figure 4). Immunohistochemical stains for cleaved caspase-3 resulted in diffuse labelling of apoptotic bodies.

Animals treated with 60 μg ACTH/d had a significantly higher number of lamin A positive cells in the adrenal cortex than rats treated with 20 and 10 μg ACTH/d, rats treated with saline and rats receiving no therapy ($p=0.004$) (Figure 5a).

Animals treated with 60 μg ACTH/d had a significantly higher number of caspase-3 positive cells in the adrenal cortex than rats treated with 10 μg ACTH/d and control rats receiving no therapy ($p=0.003$) (Figure 5c).

4.3.2 Experiment 2

There was no significant difference in the number of lamin A or caspase-3 positive cells between trilostane treated rats and control rats (Figure 5b and d).

4.4 Endogenous ACTH concentrations

4.4.1 Experiment 1

The endogenous ACTH concentration was below the detection limit in rats treated with synthetic ACTH. In the rats treated with saline the median (range) endogenous ACTH concentration was 38.5 pmol/L (20.8-149) at the start and 24.2 pmol/L (20.5-55.6) at the end of the experiment. There was no significant difference between the two measurements. In rats receiving no therapy the median (range) endogenous ACTH concentration at the end of the experiment was 11.6 pmol/L (6.2-23.8). ACTH concentrations at the end of the experiment were significantly lower in rats with no therapy than in rats treated with saline ($p=0.03$).

4.4.2 Experiment 2

Endogenous ACTH concentrations did not change significantly during trilostane therapy. Median (range) endogenous ACTH concentrations at the start of the study were 29 pmol/L (23.8-52.6), 43.6 pmol/L (14.2-53.7), and 53.1 pmol/L (20.1-100) for rats treated with 5 mg/kg trilostane/d, 50 mg/kg trilostane/d and control rats, respectively. Median (range) endogenous ACTH concentration at the end of the study were 18.2 pmol/L (10.8-61.1), 21.7 pmol/L (6.7-50), and 49.2 pmol/L (22-85) for rats treated with 5 mg/kg trilostane/d, 50 mg/kg trilostane/d and control rats, respectively.

4.5 Levels of histone-complexed DNA fragments (nucleosomes) in blood

4.5.1 Experiment 1

Rats treated with 60 µg ACTH/d had a significantly higher amount of histone-complexed DNA fragments in the blood compared to rats treated with 10 µg ACTH/d or saline ($p=0.03$) (Figure 6).

4.5.2 Experiment 2

There was no difference in the amount of histone-complexed DNA fragments in the blood between trilostane-treated rats and control rats.

5 Discussion

Treatment of rats with ACTH led to severe hemorrhage and vacuolization within the adrenal cortex in a dose-dependent manner. These findings are in agreement with a previous study, where treatment of rats with purified bovine ACTH resulted in similar histologic alterations [15]. In contrast, later studies in rats did not report adrenal damage after ACTH administration [16,18]. Of note, in the latter studies (as in our study), synthetic ACTH was used and additionally much lower doses (µg ACTH/d) than the ones used in the study of Wilbur and Rich (mg ACTH/d) were administered. Hence, it seemed unclear, if the reported deleterious effects of the purified bovine ACTH in the study of Wilbur and Rich were related to the nature of the product or to the dose of ACTH. Our study proves that synthetic ACTH is capable to induce severe adrenal damage, and that the adrenal changes caused by ACTH are dose-dependent. The dose dependency may explain why doses up to 20 µg ACTH/d, as used in the study of Miskowiak et al. and that of Sawchenko and Arias, did not induce adrenal damage [16,17]. That the ACTH dose is a crucial factor for the induction of adrenal damage is supported by the human literature. In humans, intravenous administration of huge doses of ACTH (1.2 mg/d) seemed associated with the risk of adrenocortical hemorrhagic necrosis and hypocortisolism [12,13].

In addition to the hemorrhage and vacuolisation, treatment with ACTH resulted in a significant increase in the numbers of apoptotic cells within the adrenal cortex. Presence of apoptosis was assessed by immunohistochemistry against neoepitopes of cleaved lamin A and cleaved caspase-3 [21]. Caspase-3 is a critical effector caspase, expressed in most cells and activated through cleavage in response to nearly all apoptosis triggers [22]. Lamin A is a ubiquitously expressed nuclear protein cleaved in a caspase-dependent manner during apoptosis [23,24]. Both methods showed significant differences between groups. However, immunolabeling of cleaved caspase-3 was more difficult to handle and to score and resulted in a higher inter-investigator variability than immunolabeling of cleaved lamin A. In addition,

the latter method indicated that apoptotic cells comprised adrenalcortical cells. Thus, immunohistochemistry for cleaved lamin A appears to be a simple and reliable method to detect apoptotic cells within adrenal glands.

Clinically the ACTH-treated rats showed a significantly decreased food intake and a significantly blunted weight increase compared to the rats treated with saline. As both the blunted weight gain and the adrenal changes were dose-dependent a direct association between the two seems most likely. Indeed, the absence of glucocorticoids in rats (induced by adrenalectomy) was shown to reduce food consumption and body weight gain [25-27].

To evaluate the effect of trilostane on adrenal glands, rats were treated with two different doses of trilostane in a second experiment. Our results show that in rats, trilostane in a dose up to 50mg/kg, does not induce adrenal necrosis. Unfortunately, 50 mg/kg was the highest trilostane dose accepted by the rats within the chocolate pudding. The authors are completely aware that a higher trilostane dose would have been desirable to definitively prove that trilostane doesn't induce adrenal damage. Other researchers, however, used doses up to 300 mg/kg trilostane/d in rats and reported a dose-dependent increase in adrenal weight, but no adrenal necrosis [1,28,29]. Notably, our study is the first study to report long-term therapy with trilostane (4 months), as in all other studies trilostane was administered for only 7-21 days.

Before the results of this study are applied to dogs, two drawbacks have to be discussed. The first and most obvious is a potential species-specific effect. It is known that the sensitivity to a specific drug varies among species. Trilostane inhibits the 3β-HSD in dogs and rats and leads to a decrease in cortisol/corticosterone concentrations and an increase in (17α-OH-) pregnenolone concentrations [1,8]. Rats, however, seem to tolerate much higher doses, as no signs of hypoadrenocorticism occurred with doses up to 300 mg/kg/d for 2 wk [1,28]. In dogs, the recommended dose to treat PDH is 2-5 mg/kg/d. In the present study we tried to take this

different sensitivity into account, by using two trilostane doses, one that is recommended for clinical use in dogs and a second one which is 10times higher.

The second drawback of the study presented here is the use of healthy rats. Normal adrenal glands may react differently to a specific drug than hyperplastic adrenal glands, as seen in animals with PDH. However, because impressive differences were found between the treatment with ACTH and that with trilostane, the authors strongly believe that this study gives enough evidence that not trilostane per se but ACTH is responsible for the adrenal damage seen in dogs with PDH treated with trilostane.

In this study synthetic human ACTH was used. One may argue that endogenous, species-specific ACTH may not have the same deleterious effects. However, the suspicion that even endogenous ACTH in high concentrations can be dangerous comes from human medicine. Stress-mediated increased ACTH-secretion is presumed to be one factor leading to adrenal hemorrhage and necrosis in individuals with severe infections, septicemia or other illnesses such as burns [14].

Endogenous ACTH concentration can increase remarkably in dogs with PDH treated with trilostane. In the rats of this study ACTH concentration seemed to decrease during trilostane therapy, although the change was not statistically significant. This contrasts the results of a study with young male rats, which had been treated with 30 mg/kg trilostane PO daily for 3 wk. Trilostane therapy led to a significant decrease in basal corticosterone and a significant increase in ACTH concentration [29]. However, two other studies demonstrate that the adrenal response to trilostane in rats is variable. First, trilostane did not influence basal corticosterone levels in doses up to 200 mg/kg in one study [1]. A second study showed that sex differences in the adrenal response to trilostane exist. In young female rats trilostane lowered peak plasma corticosterone concentrations in a dose-dependent manner. In male rats, plasma corticosterone levels were reduced only by a very high dose of trilostane (200 mg/kg), while lower doses (2-8 mg/kg) actually increased them [30]. The lacking increase of ACTH

concentrations in the rats of our study may indicate that the basal corticosterone concentrations were not significantly influenced by the two trilostane doses used. As discussed before the highest trilostane dose accepted by the rats within the chocolate pudding was 50 mg/kg. That a higher dose of trilostane (e.g. 300 mg/kg) would have induced adrenal damage can't be excluded. However, this seems less likely, as on one hand, adrenal damage induced by ACTH was very rapid in onset and grave and on the other hand, adrenal necrosis hasn't been reported with short-term trilostane doses up to 300 mg/kg. The lacking increase in ACTH concentrations in this study can also be seen as benefit, as the effects of trilostane and ACTH on adrenal cells could thus be truly distinguished.

In the clinical situation it would be very important to recognize patients with a risk for adrenal necrosis as soon as possible. Hence, a blood derived marker directing the clinician's attention towards adrenal necrosis would be helpful. Histone-complexed DNA fragments or nucleosomes are cell death products that are elevated in serum of patients with diseases that are associated with massive cell destruction (e.g. malignant tumors, acute inflammation, autoimmune disease) [31-33]. To test if adrenal cell death induced by ACTH causes an increase in nucleosomes in blood, the concentration of nucleosomes was assessed in this study. Rats treated with 60 µg ACTH/d had a significantly higher amount of nucleosomes within the blood than rats treated with 10 µg ACTH/d or rats treated with saline. These are promising results as they may indicate that the measurement of nucleosomes in the peripheral blood may be a clinically useful marker to early recognize adrenal damage.

In conclusion, this study shows that treatment of rats with synthetic ACTH leads to severe adrenal hemorrhage and vacuolisation and increased circulating levels of histone-complexed DNA fragments. Further, the results of this study support the hypothesis that the adrenal gland lesions seen in trilostane treated dogs with PDH may be caused by elevated ACTH and not by trilostane per se. Further studies are needed to bring these findings into a clinical setting in

dogs and to evaluate nucleosomes as a blood-marker for trilostane-induced adrenal degeneration.

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7 Tables and Figures

Figure 1:

Body weight development in rats treated with either ACTH (a) or trilostane (b). The symbols represent the median of each group.

In the first experiment, the body weight development over time was significantly different between all groups except between the rats treated with 20 and 10 µg ACTH/d.

Figure 2:

Histological section of the adrenal cortex of a rat treated with 60 µg ACTH/d (a) and a control rat (b).

a: Diffuse hyperemia and hemorrhage and severe focal vacuolization is seen.

Figure 3:

Scatter plots of the cumulative histologic score, comprising hyperemia/hemorrhage, vacuolization and clusters of neutrophils in ACTH-treated rats compared to control rats (a) and in trilostane-treated rats compared to control rats (b); the line represents the median of each group. ∗ Significant difference.

Figure 4:

Histological section of the adrenal cortex of a rat treated with 60 µg ACTH/d. Lamin A positive cells are stained red.

Figure 5:

Scatter plots of the number of lamin A positive cells in ACTH-treated rats compared to control rats (a) and in trilostane-treated rats compared to control rats (b) and of caspase-3 positive cells in ACTH-treated rats compared to control rats (c) and in trilostane-treated rats compared to control rats (d); the line represents the median of each group. ∗ Significant difference.

Figure 6:

Scatter plots of the amount of histone-complexed DNA fragments (nucleosomes) in ACTH-treated rats compared to control rats; the line represents the median of each group. ∗ Significant difference.

Figure 1:

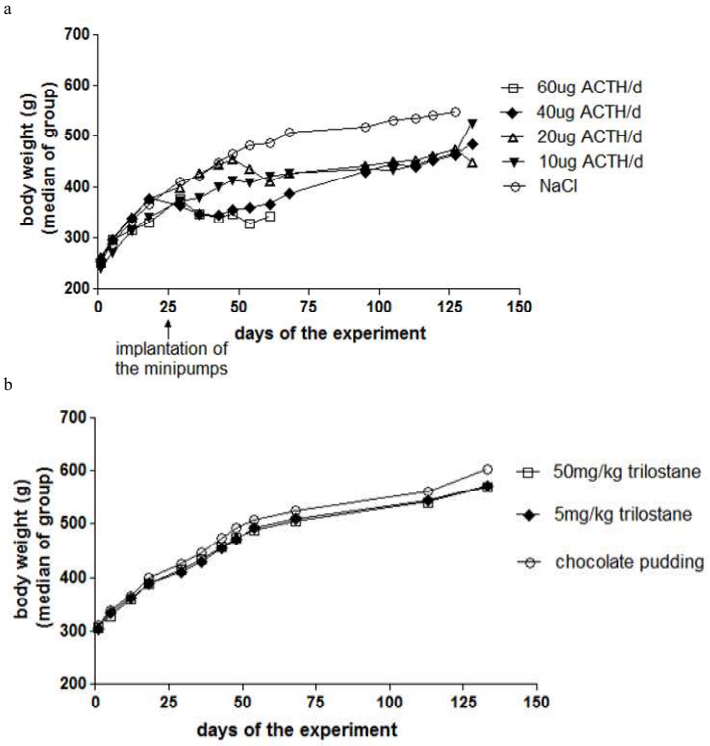

a
700
600
500
400
300
200
body weight (g)
(median of group)
0
25
50
75
100
125
150
days of the experiment
implantation of
the minipumps
60ug ACTH/d
40ug ACTH/d
20ug ACTH/d
10ug ACTH/d
NaCl
b
700
600
500
400
300
200
body weight (g)
(median of group)
0
25
50
75
100
125
150
days of the experiment
50mg/kg trilostane
5mg/kg trilostane
chocolate pudding

Figure 2a:

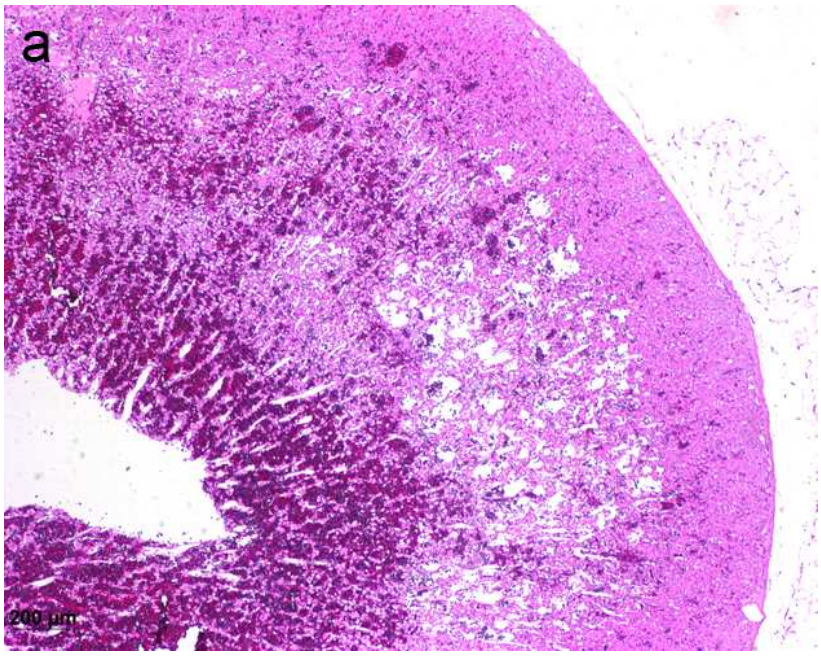
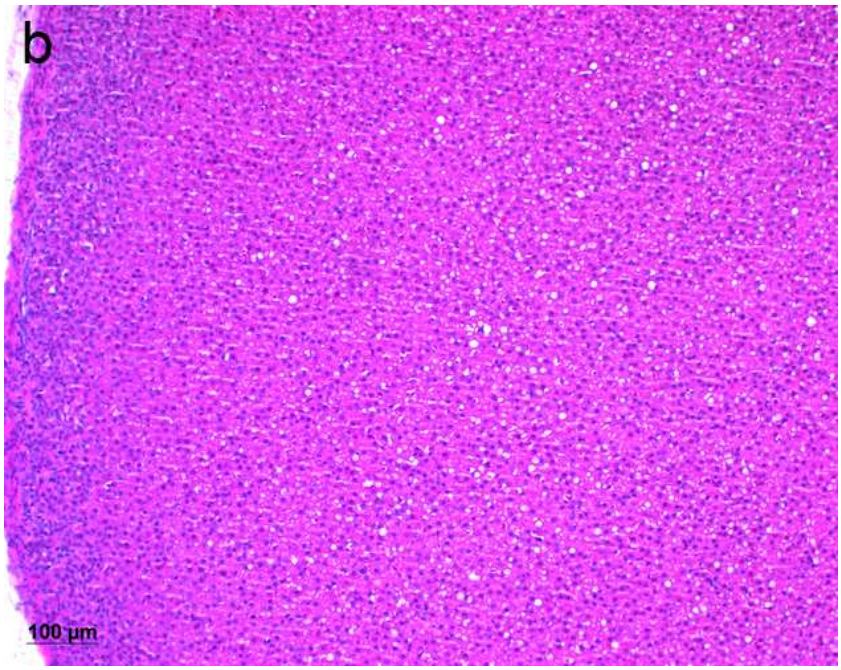


Figure 2b:



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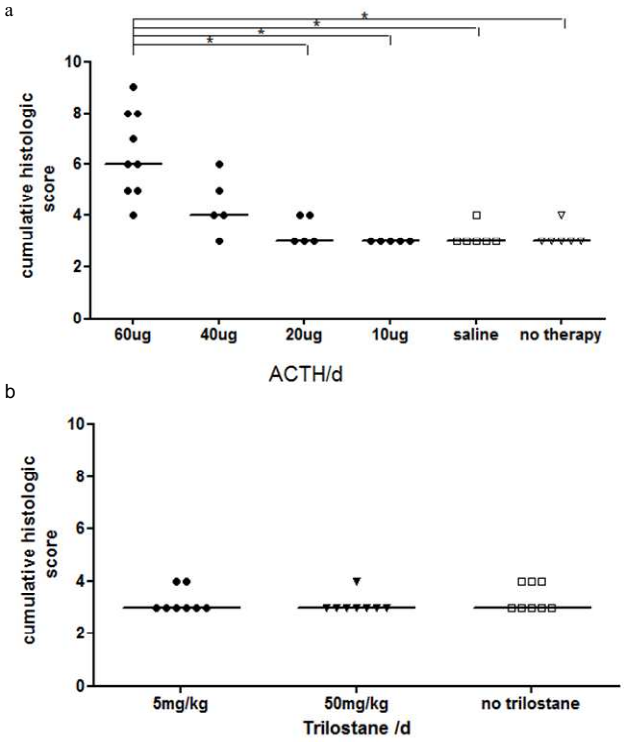

a
cumulative histologic score
10
8
6
4
2
0
60ug
40ug
20ug
10ug
saline
no therapy
ACTH/d
b
cumulative histologic score
10
8
6
4
2
0
5mg/kg
50mg/kg
no trilostane
Trilostane /d

Figure 4:

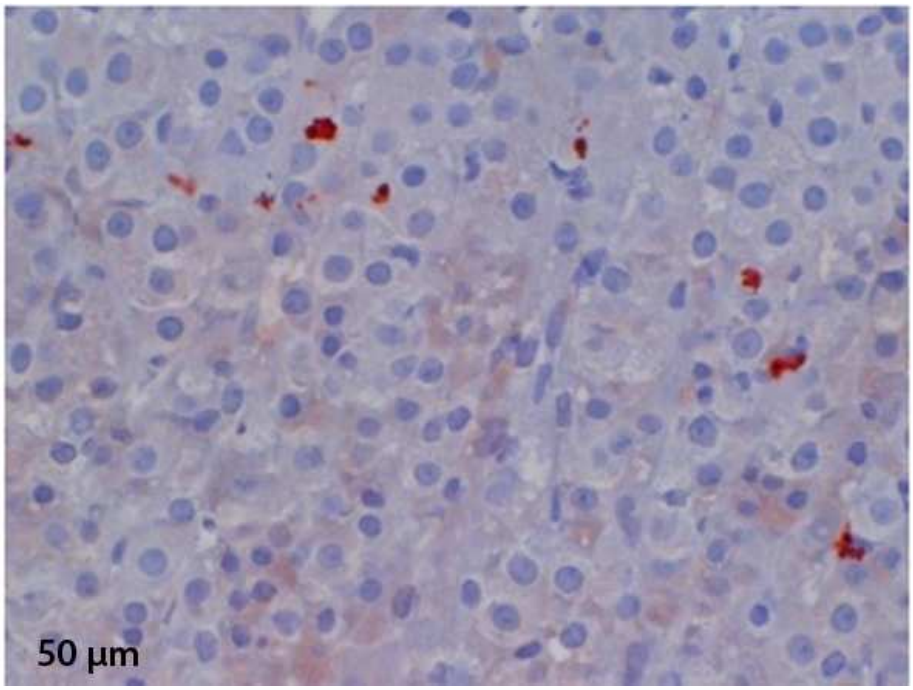


Figure 5:

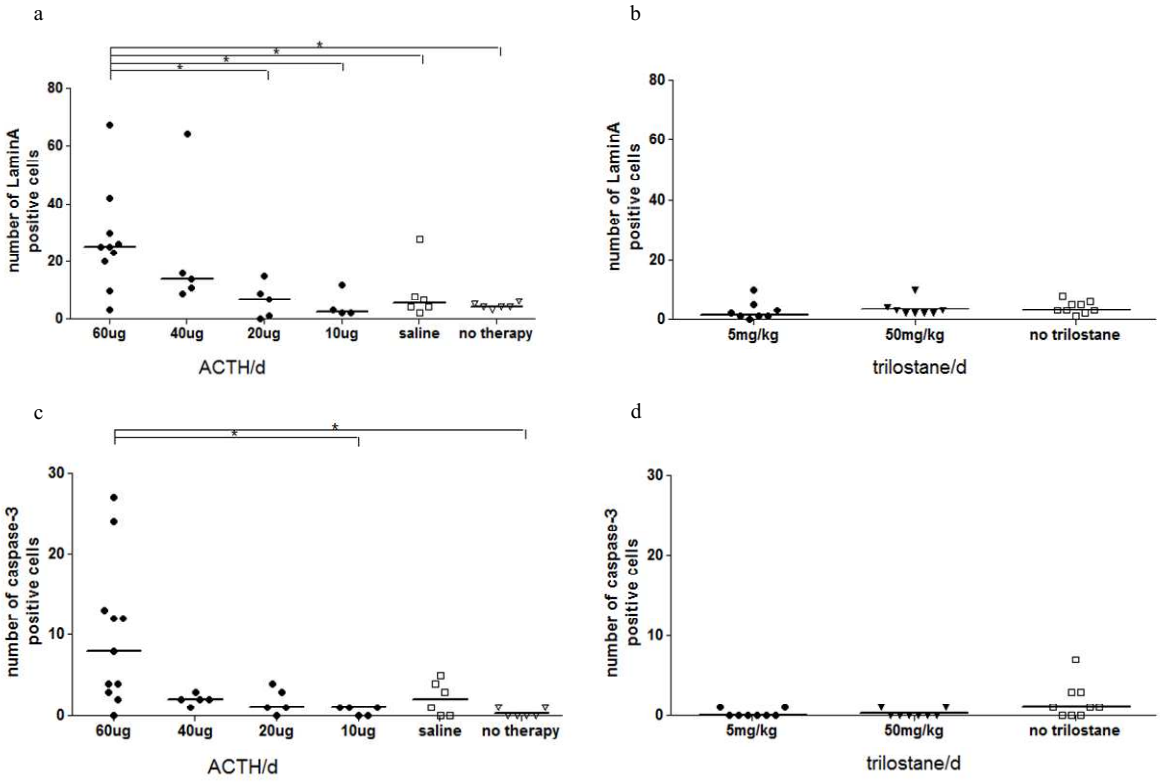
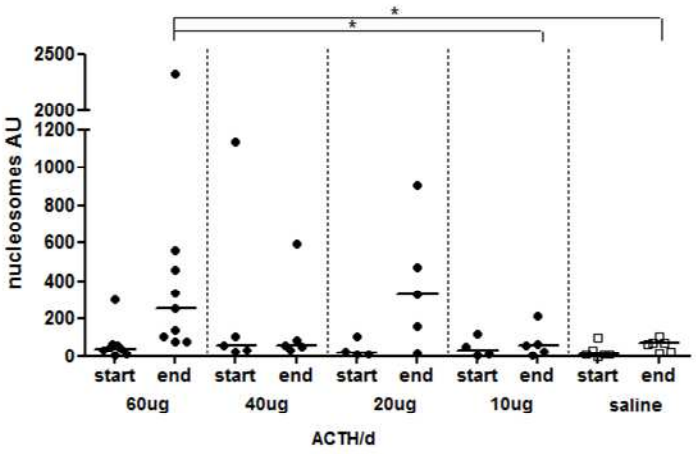

a
number of LaminA positive cells
60ug
40ug
20ug
10ug
saline
no therapy
ACTH/d
b
number of LaminA positive cells
5mg/kg
50mg/kg
no trilostane
trilostane/d
c
number of caspase-3 positive cells
60ug
40ug
20ug
10ug
saline
no therapy
ACTH/d
d
number of caspase-3 positive cells
5mg/kg
50mg/kg
no trilostane
trilostane/d

Figure 6:


*
*
2500
2000
1200
1000
800
600
400
200
0
nucleosomes AU
start
end
start
end
start
end
start
end
start
end
60ug
40ug
20ug
10ug
saline
ACTH/d

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