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Effects of dermal dexamethasone application on ACTH and both basal and ACTH-stimulated cortisol concentration in normal horses

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There are no data available regarding the systemic (adverse) effects which might be induced by topical/dermal glucocorticoids (GCs) application in the horse. Besides their widespread use for the treatment of a variety of peripheral inflammatory disorders such as atopic dermatitis, eczemas or arthritis in the horse, their surreptitious application has become a concern in doping cases in competition/performance horses. Assessing both basal and ACTH-stimulated plasma cortisol as well as basal ACTH concentrations following application of dexamethsone-containing dermal ointment is necessary to determine influences on hypothalamus-pituitary-adrenal (HPA) axis. Ten clinically healthy adult standardbred horses (6 mares, 4 geldings) were rubbed twice daily each with 50 g dexamethasone-containing ointment on a defined skin area (30 × 50 cm) for 10 days. RIA and chemiluminescent enzyme immuno-metric assay were used to determine resting and ACTH-stimulated plasma cortisol and basal ACTH concentrations, respectively. HPA feedback sensitivity and adrenal function were measured by a standard ACTH stimulation test. Dermal dexamethasone suppressed significantly the resting plasma cortisol level (to 75–98%) below baseline ($P < 0.001$) within the first 2 days and decreased further until day 10. ACTH stimulation test showed a markedly reduced rise in plasma cortisol concentrations ($P < 0.001$ vs. baseline). Plasma ACTH level decreased also during topical dexamethasone application. The number of total lymphocytes and eosinophil granulocytes was reduced, whereas the number of neutrophils increased. No significant change of serum biochemical parameters was noted. Dermal dexamethasone application has the potential to cause an almost complete and transient HPA axis suppression and altered leukocyte distribution in normal horses. The effects on HPA axis function should be considered in relation to the inability of animals to resist stress situations. The data further implicate that percutaneously absorbed dexamethasone (GCs) may cause systemic effects relevant to 'doping'.

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INTRODUCTION

For many decades topical glucocorticoids (GCs) are widely used drugs for the treatment of several acute or chronic allergic or other periphery inflammatory disorders in humans and animals, owing to their anti-inflammatory and anti-proliferative effects (Smith, 1993; Curtis, 1999). Primarily, they are considered to exert local efficacy followed by reduced systemic adverse effects (Wester & Maibach, 1991; Drake *et al.*, 1996), thus would deliver a merit over parenteral or oral GCs which are generally known to induce serious side effects; also, in the horse parenteral GC-induced laminitis, adrenocortical suppression, muscle wasting, hyperglycaemia, immunsuppression are known (Cohen & Carter, 1992; French *et al.*, 2000; Johnson *et al.*, 2002; Ryu

et al., 2004). Though topical GCs represent a significant milestone in dermatologic and periphery inflammatory conditions, there is, however, widespread concern about possible systemic (adverse) effects, such as depression of the adrenal gland function and the hypothalamus-pituitary-adrenal (HPA) axis feedback mechanism triggered by topical GC application (Hanania *et al.*, 1995; Brazzini & Pimpinelli, 2002; Hengge *et al.*, 2006) this issue has not been studied in detail in the horse so far. Moreover, in the last years, the use of topical GCs has raised 'doping' relevant questions in performance/competition horses.

Therapeutic efficacy and possible systemic (adverse) effects (risk/benefit ratio) related to topical GCs are associated with steroid potency and percutaneous absorption capacity, and classified accordingly as mild, moderate, potent and very potent topical GCs (four classes) (Brazzini & Pimpinelli, 2002). Modifications to the basic steroid structure comprising 17 carbon atoms arranged in three six-membered rings and one five-membered ring have led to the development of compounds with varying potencies and adverse effects. Hydrocortisone (acetate) – with mild potency – was the first GC for topical use synthesized by simple reduction of the carbonyl group on position 11 of cortisone (Sulzberger & Witten, 1952). Additional modification of this molecule (insertion of double bond at position 1 and 2; halogenations, methylation, acetylation) led the synthesis of more potent drugs, and that means, all synthetic GCs consist of a hydrocortisone molecule. As topical GCs have to penetrate the stratum corneum to achieve enough concentrations in the epidermis, increasing the lipophilicity of the drug is an important modification such as by esterification at positions of 16, 17 and 21 or introducing long carbon side chains, e.g. acetonide, acetate, valerate or propionate. All these modifications were performed to produce GCs with high potency, but were often associated with a potential for systemic adverse effects, but depend generally too on drug vehicle, dose, frequency, area as well anatomical regional differences of application (Maibach *et al.*, 1971; Brazzini & Pimpinelli, 2002; Levin & Maibach, 2002). According to the classification of topical GCs, dexamethasone-21-acetate (0.017%) which is used as an ointment in the current study belongs to the mild group, with possible lower systemic (adverse) effects, but has to be proven in the horse.

Several studies in humans and animals have shown that dermal or ototopical GCs suppress the HPA axis activity and result in strong adrenal suppression (Garden & Freinkel, 1986; Zenoble & Kemppainen, 1987; Turpeinen, 1988; Patel *et al.*, 1995; Clark & Lipworth, 1997; Buske-Kirschbaum *et al.*, 1998; Todd *et al.*, 2002; Abraham *et al.*, 2005). Findings were documented by measuring lower resting or ACTH-stimulated cortisol secretion from the adrenal gland, and as a cross-link a decrease in ACTH release from pituitary gland (Buske-Kirschbaum *et al.*, 2002). A decreased responsiveness of the adrenal gland is recognized as a sensitive and reproducible marker of systemic adverse effects of GCs (Hanania *et al.*, 1995). An integrated cortisol determination after ACTH-challenge (250 μg Synacthen) is regarded to be sensitive for detecting adrenocortical suppression, to draw a conclusion from blunted higher centre activities, i.e. hypothalamus and pituitary gland, also in horses (Bousquet-Mélou *et al.*, 2006).

Generally, the magnitude of such GC-induced iatrogenic adrenal suppression is supposed to be associated with excessive high-dose and long-term treatment with GCs (Keller-Wood & Dallman, 1984; Fisher, 1995; Clark & Lipworth, 1997), as well in some other instances with deteriorated skin integrity, region and surface area of treated skin (for review see Levin & Maibach, 2002). An *in-vitro* study by Mills and Cross (2006) has shown that hydrocortisone can percutaneously be absorbed through normal equine skin from different regions; and an ophthalmic application of dexamethasone ointment in horses produces detectable dexamethasone concentrations in serum and urine (Spiess *et al.*, 1999). On the other hand, horses with recurrent airway obstruction treated for 7 days with aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively, resulted in decreased cortisol concentrations but adrenocortical responsiveness to ACTH challenge test was not affected (Rush *et al.*, 1998), whereas high doses of inhaled beclomethasone dipropionate impaired ACTH-stimulated cortisol release in horses with heaves (Rush *et al.*, 2000); however, basal cortisol level resumed 2–4 days after treatment cessation. Indeed, it has been shown that prolonged excessive topical use of GCs by inhalation caused suppression and delayed recovery of the HPA axis function even months or more than a year after drug withdrawal (Livanou *et al.*, 1967; Clark & Lipworth, 1997).

Furthermore, although previous studies have indicated that oral or parenteral GCs alter hepatic functions and serum biochemical parameters (Bhagwat & Ross, 1971; Rutgers *et al.*, 1995), little or no information exists concerning induction of serum liver enzyme activities, which are sensitive to GCs, by topical GC treatment in the horse. Gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) are often used enzymes as a marker of the integrity of the liver cell membranes and as putative indicators of liver dysfunction in horses (Carlson, 1996). In dogs, it has been recently shown that ototopical dexamethasone administration increases liver enzyme activities, i.e. ALP, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and GGT without obvious symptoms of liver damage (Abraham *et al.*, 2005).

The specific aim of the present study was to investigate whether the application of topical dexamethasone-containing ointment on intact skin and at therapeutic doses attenuates the HPA axis activity in the horse or not. It should be evaluated resting and ACTH-stimulated cortisol concentrations as well as basal ACTH levels, whether a potentially reduced cortisol response upon topical dexamethasone can at least partly be explained by an impaired activity of the HPA axis at the adrenal, pituitary or supra-pituitary level. Furthermore, serum biochemical (specifically liver enzyme activities) and haematological parameters during a twice daily application of dermal dexamethasone should be examined. Finally, it was sought to provide the recovery potential of eventually attenuated HPA axis activities after cessation of topical dexamethasone treatment.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the local ethical committee for animal welfare and were carried out in accordance with the guidelines of the German law relating to animal welfare.

Ten adult thoroughbred horses of mixed gender (6 mares, 4 male horses), with age range of 5–23 years (mean age: 13.40 ± 2.21), weighting between 470–660 kg were enrolled. None of the horses had any signs of neurological and endocrinological disorders as assessed by clinical examination and routine blood biochemical analyses. Except routine vaccination, none were on any medications known to alter the HPA axis function. Dermatological examination of each horse showed no symptoms of inflammation or other skin disorders. All animals were housed in a single horse pens, and were fed three times a day with oat and hay and received water *ad libitum*. Each animal served at its own control.

Study design and blood sampling

A single-blind, nonrandomized, repeated measure design with a washout period (recovery time after discontinuation of treatment) was used in the following order: (a) clinical and dermatological examinations were accomplished before starting dermal dexamethasone application (day 0); here, baseline values of resting and ACTH-stimulated cortisol and basal ACTH levels, haematological and serum biochemical parameters were determined; (b) animals become shaved a surface area of 30 × 50 cm skin on the left side of the neck, and rubbed twice daily at 9:00 AM and 5:00 PM with 50 g dexamethasone-containing (0.017%) commercial ointment for 10 days [100 g ointment formulation contained: pharmacologically active agents dexamethasone-21-acetate (0.017 g), neomycine sulphate (1.2 g) and potassium sorbate as well as other components]. Each horse received a dose of 8.5 mg dexamethasone twice daily; c) this dexamethasone phase was followed by 20 days drug withdrawal period (∼ washout period). This was attempted to investigate the recovery perpetuity of possibly dexamethasone-induced alterations of the HPA axis function and other intended parameters. The whole study was designed to take 30 days. On all occasions, clinical and skin examinations as well blood sample collection were carried out every morning before animals received dexamethasone ointment application at 8:00 AM.

For the analysis of total blood cell count, serum biochemical profile (including urea, electrolytes, liver function tests) and plasma cortisol and ACTH levels, blood samples were collected at 8:00 AM from jugularis venipuncture in EDTA or lithium-heparin containing tubes (Sarstedt, Hamburg, Germany) at different time points: baseline (day 0), during dexamethasone application (day 2, 6, 8 and 10) and drug withdrawal period (day 13, 17, 21, 24 and 30). After centrifugation, serum and plasma samples were collected and stored at −20 °C until analysis.

ACTH-challenge test

To prove the functionality of the HPA axis, an ACTH-stimulation test was performed at three occasions (baseline-day 0; during dexamethasone application-day 8; washout period-day 14). Each animal was challenged at 8:00 AM with a standard-dose (250 μg) of synthetic ACTH (Synacthen®; Novartis, Basel, Switzerland). As there is no a clear-cut argument on about the ACTH dosages which should be used in test of the HPA axis function in humans or animals (see references Nye *et al.*, 1999; Bousquet-Mélou *et al.*, 2006; Hart *et al.*, 2007 and the references therein), we adopted the 250 μg synacthen bolus i.v. (vena jugularis) application as convenient and reliable screening test for the diagnosis of possible exogenous GC-induced adrenal dysfunction. Though also there is variability in administration routes of ACTH and time of blood sampling, 1–3 h are seen as an appropriate time range to assay cortisol after i.v. administration of 250 μg of the stimulating hormone; thus, we sampled blood immediately before, and 1 h after i.v. administration of an ACTH bolus. Samples were then processed as described above.

Cortisol and ACTH assays

In all thawed 100 μL equine plasma samples, resting and ACTH-stimulated cortisol concentrations were analysed in duplicates by radioimmunoassay using [^{3}H]-cortisol (specific activity: 50–90 Ci/mmol) (Amersham Biosciences, Freiburg, Germany). The cortisol assay method is described elsewhere in detail (Abraham *et al.*, 2005). The detection limit of the assay was 0.4 nmol/L, and coefficients of variation were 8.1% (intra-assay) and 10.2% (inter-assay) at plasma cortisol levels measured in the animals.

Plasma ACTH levels were measured by chemiluminescent enzyme immuno-metric assay (Immulite; DPC-Biermann; Bad Nauheim; Germany) at baseline (day 0), during dexamethasone application (day 2 and 8) and after discontinuation of drug application (day 13 and 30) as validated by Froin (1998). The lower detection limit of the assay was 5 pg/mL plasma, with the intra- and inter-assay coefficients of variation less than 7%, respectively.

Analysis of haematological and serum biochemical parameters

Electrolytes, serum liver enzyme activities such as ALP, GGT, ALT and AST, lactate dehydrogenase and creatinine, creatinine kinase, albumin, total protein, total bilirubine and urea were determined using standard methods recommended by the German Society of Clinical Chemistry (DGKC), with an automated analyzer (Hitachi 912, Boehringer Mannheim, Germany) using commercially available reagents (Roche Diagnostics Corp., Mannheim, Germany). All enzyme activities were determined at 37 °C, and assay procedures were optimized according to the manufacturer's instructions. EDTA-treated blood was analyzed for haematological constituents either directly or using semi-automated analyzer (Technicon H1, Bayer Diagnostics; Germany). Blood haemoglobin (Hb) concentration was determined by the cyano-methemoglobin procedure.

Statistical analysis

Data presented in graphs are mean ± SD (n = 10). Data before treatment (day 0), during dexamethasone application (day 2, 6, 8 and 10) and 20 days after drug withdrawal (day 13, 17, 21, 24 and 30) were compared using repeated measures analysis of variance (ANOVA). In order to examine the differences between baselines and treatment values, a Dunnett's multiple comparison tests was employed. P value <0.05 was considered to be significant.

RESULTS

Basal and ACTH-stimulated cortisol levels

The mean resting plasma and ACTH-stimulated cortisol levels before (baseline), during dexamethasone ointment and after dexamethasone withdrawal are shown in Figs 1 and 2. In all treated horses, on day 2 during dermal application of dexamethasone-containing ointment, the mean cortisol concentrations declined significantly from 63.19 ± 25.71 nmol/L (baseline – day 0) to 15.72 ± 12.98 nmol/L ($P < 0.001$). A further decrease (>95% below baseline) was observed during the subsequent treatment period with a range value of 0.18–6.38 nmol/L between day 6 and 10 (median: 2.95, 1.63 and 1.91 nmol/L on day 6, 8 and day 10, respectively). This dermal dexamethasone-induced reduction in cortisol levels was even apparent a week after discontinuation of treatment, i.e. 3 days (day 13) (3.37 ± 2.04 nmol/L) and 7 days (day 17) (33.40 ± 24.59 nmol/L) ($P < 0.001$ vs. day 0, baseline) after drug withdrawal, but reached baseline values (63.19 ± 25.01 nmol/L) 11 days (day 21) after dexamethasone withdrawal (67.90 ± 13.67 nmol/L).

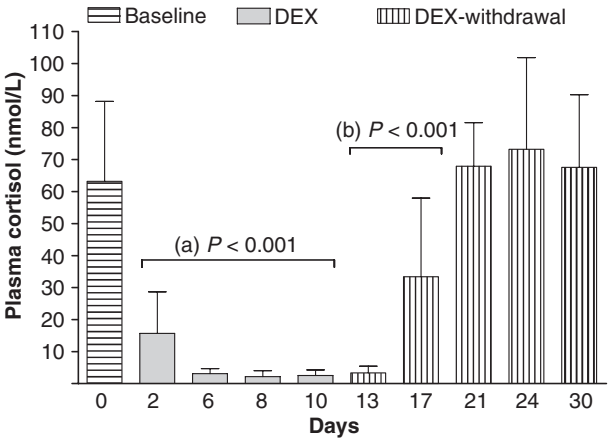

Fig. 1. Basal cortisol concentration during dermal dexamethasone application. Plasma cortisol was measured before treatment, during administration of dexamethasone containing lotion and after cessation of treatment. [a]$P < 0.001$ cortisol during DEX application vs. baseline; [b]$P < 0.001$ cortisol after DEX withdrawal vs. Baseline; DEX, dexamethasone.

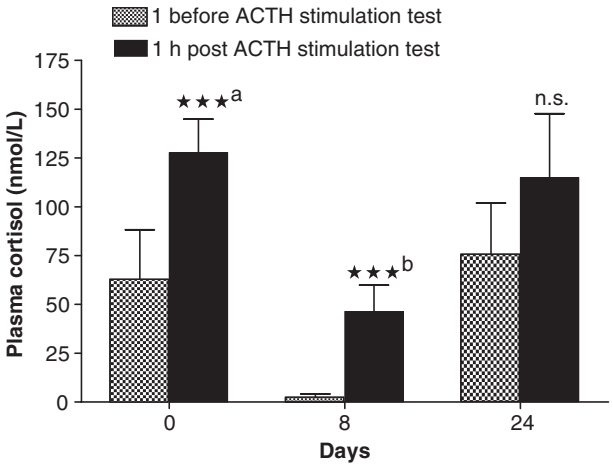

Fig. 2. Effect of dermal dexamethasone treatment on cortisol response 60 min after ACTH challenge. Before (day 0), during (Day 8) dexamethasone administration twice daily or after drug withdrawal (14 days after) horses were challenged with ACTH (250 μg). ***[a]$P < 0.001$, cortisol levels on day 0 (control), before vs. after ACTH stimulation test; ***[b]$P < 0.001$, cortisol values after ACTH response on d 0 (control) vs. ACTH response on day 8 during dexamethasone application (by Student's t-test); n.s. (not significant), comparison of stimulated cortisol values at day 0 (baseline) vs. day 14 (after drug withdrawal).

Before starting treatment, basal cortisol concentration (63.19 ± 25.01 nmol/L) was determined, and this was elevated in the adrenal cortex by ACTH challenge test to two-fold in all horses (127.59 ± 17.39 nmol/L; $P < 0.001$). During topical dexamethasone application the basal ACTH-stimulated plasma cortisol concentration (127.59 ± 17.39 nmol/L) was reduced to about 2.7-fold (46.30 ± 13.54 nmol/L; $P < 0.001$) (Fig. 2). This topical dexamethasone-induced reduction in ACTH-stimulated cortisol release reached about baseline values 2 weeks (day 24, Fig. 2) after dexamethasone withdrawal (114.82 ± 32.84 nmol/L vs. 127.59 ± 17.39 nmol/L).

ACTH levels

To prove whether dermal dexamethasone application affects the feedback mechanism of the HPA axis, we determined the levels of plasma ACTH. As shown in Fig. 3, 2 days after starting dermal dexamethasone application, the basal ACTH levels (37.90 ± 15.22 pg/mL) decreased but did not reach statistical significance (30.60 ± 8.78 pg/mL), but further treatment induced a significant decrease in ACTH levels (22.20 ± 12.31 pg/mL on day 8; $P < 0.05$). This value remained even significantly reduced 3 days (day 13 on Graph 3) after stopping the treatment (24.30 ± 10.98 pg/mL). A recovery of baseline values was observed 20 days (day 30 on Graph 3) after drug withdrawal (40.30 ± 11.76 pg/mL).

Haematological and serum biochemical parameters

During dermal dexamethasone application changes were observed in mean eosinophil counts, mean lymphocyte counts

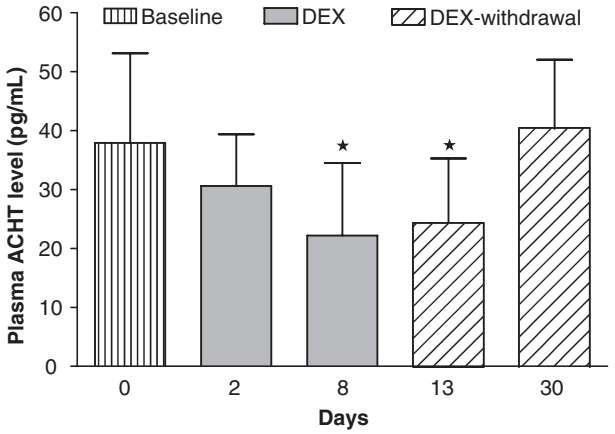

Fig. 3. Basal ACTH release during dermal dexamethasone treatment. *$P < 0.05$ vs. baseline.

and mean number of segmented neutrophils, and data are summarized in Table 1. Mean lymphocyte counts decreased significantly ($3.85 \pm 1.45 \times 10^9$/L day 0 vs. $1.74 \pm 0.21 \times 10^9$/L day 10; $P < 0.05$), and did not reach baseline values even 14 days after the study was finished ($2.26 \pm 0.29 \times 10^9$/L), whereas mean neutrophil counts increased significantly at drug use (3.11 ± 0.35 day 0 vs. 5.48 ± 0.65 day 6; $P < 0.01$), and remained at this higher level until day 3 after drug withdrawal. Mean values ($0.104 \pm 0.026 \times 10^9$/L baseline value) decreased and remained below the detection limit during treatment (day 6 and 8), but baseline values were reached three days after withdrawal.

As shown in Table 2, treatment with dermal dexamethasone was not associated with any effects on serum biochemical profiles and liver enzyme activities.

DISCUSSION

In this study, in normal horses, it was investigated the occurrence of possible systemic (adverse) effects of short-term (10 days) topical (dermal) dexamethasone application on intact equine skin. With this respect, we assessed the possible suppression of the HPA axis function; and thus examined basal and ACTH-stimulated cortisol concentration from the adrenal gland as well as ACTH release at the pituitary level. Moreover, haematological and biochemical blood parameters including white blood cell counts and liver enzyme activities were determined. Even though in horses dermal GCs are also frequently used for treating inflammatory skin disorders, the present study should be, to our knowledge, the first which aimed to demonstrate a direct correlation between *topically* applied GCs and eventually occurring effects on the HPA axis as well as liver enzyme activities, biochemical and haematological parameters. Main findings were: a twice daily dermal application of dexamethasone-containing ointment for 10 days caused a marked influence on HPA axis responses (assessed by decreased basal ACTH and resting and ACTH-stimulated cortisol levels) and white blood cellular constitution but not on liver function. The data generated here are interesting not only from therapeutic points of view but also relevant to doping control and should reinforce their consideration, specifically when topical GCs are illegally used in competition/performance horses.

The most striking abnormality upon dermal dexamethasone treatment was the strong time-dependent decrease in resting plasma cortisol levels. The earlier time to decrease cortisol response e.g. on day 2 below 95% (vs. baseline or day 0) indicates rapid impairment of the HPA axis feedback mechanism, even in relatively low topical dexamethasone dose (2×8.5 mg/day/horse). The present data from horses support strongly those found in healthy animals, where ototopical dexamethasone or dermal GC (triamcinolone acetonide, fluocinonide, betamethasone valerate) application induced marked suppression of cortisol secretion, suggesting adrenal impairment (Zenoble & Kemppainen, 1987; Abraham *et al.*, 2005). Similarly, it has been shown that the application of dermal GC-containing ointments also potentially suppress the endogenous cortisol release in man (Garden & Freinkel, 1986; Turpeinen, 1988; Patel *et al.*, 1995; Buske-Kirschbaum *et al.*,

Table 1. Effects of dermal dexamethasone on haematological parameters compared with baseline values at day 0

		Dexamethasone		Drug withdrawal	
Mean hematologicall parameters	Day 0	Day 6	Day 10	Day 3	Day 14
Hemoglobin (mM/L)	7.60 ± 0.28	7.07 ± 0.22	7.16 ± 0.15	7.43 ± 0.25	7.75 ± 0.27
Hematocrit (L/L)	0.31 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.34 ± 0.01	0.36 ± 0.11
Total cell count					
Erythrocytes (T/L)	6.95 ± 0.35	6.41 ± 0.29	6.51 ± 0.25	7.05 ± 0.31	7.36 ± 0.29
Leukocytes ($\times 10^9$/L)	6.04 ± 0.46	7.71 ± 0.55	7.12 ± 0.42	7.93 ± 0.52	6.14 ± 0.36
Thrombocytes ($\times 10^9$/L)	131.30 ± 10.41	130.50 ± 11.52	126.70 ± 11.68	166.40 ± 14.09	164.20 ± 12.66
Differential count ($\times 10^9$/L)					
Neutrophils	3.11 ± 0.35	5.48 ± 0.65**	5.00 ± 0.41*	5.10 ± 0.42**	3.43 ± 0.33
Eosinophils	0.10 ± 0.03	0	0.04 ± 0.01	0.17 ± 0.07	0.17 ± 0.07
Lymphocytes	3.85 ± 1.45	1.78 ± 0.27	1.74 ± 0.21*	2.38 ± 0.40	2.37 ± 0.23
Monocytes	0.36 ± 0.05	0.38 ± 0.08	0.35 ± 0.02	0.46 ± 0.08	0.39 ± 0.04

Significant differences in differential white blood cell count between baseline and dexamethasone treatment are indicated. Values are the mean ± SEM, *$P < 0.05$ vs. baseline, **$P < 0.01$ vs. baseline.

Table 2. Effects of dermal dexamethasone on serum biochemical parameters compared with baseline values at day 0

		Dexamethasone		Drug withdrawal	
Mean biochemical parameters	Day 0	Day 6	Day 10	Day 3	Day 14
Protein (g/L)	66.57 ± 1.68	69.84 ± 1.56	67.65 ± 1.32	66.77 ± 1.25	65.36 ± 1.10
Albumine (g/L)	31.99 ± 0.97	29.78 ± 3.44	33.88 ± 1.01	32.66 ± 0.95	31.89 ± 0.79
Total bilirubins (M)	22.68 ± 2.65	23.09 ± 2.95	25.85 ± 2.25	23.01 ± 2.26	23.69 ± 2.4
Urea (mM)	5.68 ± 0.33	5.12 ± 0.06	5.18 ± 0.53	5.17 ± 0.62	5.83 ± 0.52
Creatinine (μM)	103.8 ± 4.9	94.60 ± 3.44	91.30 ± 5.30	91.50 ± 3.94	123.0 ± 6.83
LDH (U/L)	736.9 ± 69.0	675.2 ± 55.64	600.8 ± 54.26	586.4 ± 48.85	647.1 ± 53.95
Creatinine kinase (U/L)	201.2 ± 9.74	180.34 ± 4.83	172.63 ± 10.31	181.39 ± 6.87	185.77 ± 14.05
Na (mM)	140.6 ± 0.37	141.0 ± 0.76	140.9 ± 0.90	140.6 ± 0.62	143.20 ± 0.79
K (mM)	3.22 ± 0.27	3.02 ± 0.16	3.45 ± 0.19	3.32 ± 0.22	3.04 ± 0.20
Cl (mM)	103.44 ± 0.61	104.08 ± 0.59	103.42 ± 0.59	103.82 ± 0.63	109.86 ± 0.14
Ca (mM)	3.10 ± 0.06	3.13 ± 0.03	3.22 ± 0.05	3.26 ± 0.02	3.17 ± 0.05
P (mM)	0.82 ± 0.05	0.95 ± 0.09	1.21 ± 0.07	1.22 ± 0.07	0.99 ± 0.08
ALP (U/L)	310.8 ± 37.95	312.7 ± 41.67	322.0 ± 45.31	327.7 ± 48.28	339.7 ± 27.95
ALT (U/l)	7.71 ± 0.78	9.56 ± 1.53	8.63 ± 1.21	9.13 ± 0.85	7.19 ± 0.83
AST (U/L)	404.27 ± 62.49	512.01 ± 129.24	445.33 ± 98.89	404.66 ± 97.47	368.17 ± 41.95
GGT (U/L)	32.4 ± 9.42	51.23 ± 20.11	59.12 ± 24.93	62.28 ± 28.33	54.51 ± 19.67

No significant changes were observed. Values are the mean ± SEM. LDH, lactate dehydrogenase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate amintransferase; GGT, gamma-glutamyltransferase.

1998). Indeed, it is supposed that in diseased skin the degree of HPA axis responsiveness and prompt endogenous cortisol secretion should already be attenuated by enhanced percutaneous absorption of the drug (Turpeinen *et al.*, 1986; Wester & Maibach, 1992). In the horse, though dermal dexamethasone in an ointment based vehicle was rubbed on healthy skin (neck region), the transdermal drug absorption and penetration through the intact skin structure should be assumed to be large enough to hamper endogenous cortisol production. In-vitro studies by Mills and Cross (2006) that have shown the transdermal absorption of e.g. hydrocortisone through normal equine skin model support the present argument. Thus, concentration of dexamethasone absorbed through intact equine skin, even if we did not measure, seems to be quite enough to suppress cortisol secretion from the adrenal gland.

In addition, the reduction in ACTH-stimulated cortisol release strengthens that treated normal horses have developed adrenal suppression (cf. Fig. 2; Turpeinen *et al.*, 1986; Turpeinen, 1989; Abraham *et al.*, 2005). By definition, adrenal suppression denotes sustained deficiency of the HPA axis function ensuing exposure of the organism to excessive and prolonged administration of exogenous GCs. This means that it is a suprapituitary mechanism exerted by GCs via negative feedback which causes long-term deprivation of ACTH and consequently adrenal cortex atrophy (Keller-Wood & Dallman, 1984; Chrousos, 2001). Decreased cortisol response received with exogenous ACTH during application of dermal dexamethasone confirms the occurrence of secondary adrenal insufficiency as well suppressed HPA axis activity (Crowley *et al.*, 1993; Bousquet-Mélou *et al.*, 2006). Even if each horse was used as it's own control, the direct influence of exogenous ACTH on cortisol test results can be ruled out, as ACTH challenge test was run in a week-interval [day 0-basline; day 8-dex; 14 days after Dex withdrawal (day 24)] and the test is effective in proofing development of adrenal insufficiency during GC treatment (and also primary adrenal suppression). In GC nontreated individuals, repeated and high dose ACTH administration would lead to hyperplasia of the adrenal gland presumably with increased cortisol secretion. Our findings are in good agreement with the data obtained from dogs (Zenoble & Kemppainen, 1987; Abraham *et al.*, 2005) and asthmatic patients treated with inhaled GC (also topical) where upon the ACTH-stimulated cortisol production was markedly blunted (Brus, 1999; Lipworth, 1999), but in the latter case, supra-physiological GC doses were used. The corticotropin releasing factor (CRF) test would be an alternative assay which enables measuring simultaneously both basal GC-induced cortisol and ACTH changes, thus, can be used to assess an impairment of the HPA axis in horses receiving parenteral/topical/inhaled GCs during heaves, as shown in human asthmatics (Clark & Lipworth, 1997).

Moreover, dermal dexamethasone application produced pronounced decrease in basal plasma ACTH levels, supporting that chronic failure of ACTH release will lead to quiescent adrenal gland and hence, to an inadequate cortisol response to exogenous ACTH (Hanania *et al.*, 1995). These findings strongly suggest the first time that dermal dexamethasone greatly hampers the feedback sensitivity of the HPA axis in the horse by decreasing ACTH release (Alexander *et al.*, 1993; Bugajski *et al.*, 2001). Two days after dermal dexamethasone application cortisol concentration declined significantly, and also the mean ACTH levels were low but did not reach statistical significance, indicating discordance between measured ACTH and cortisol levels at a given time point during dexamethasone application (cf. Fig. 1 and 3). Although adrenal secretion of cortisol is dependent on the pituitary ACTH release, which itself is triggered by CRF release, in some cases, however, adrenal cortisol production is dissociated from ACTH levels. GCs modulate ACTH secretion through negative-feedback loops at several sites within

the brain and pituitary, operating in rapid (short)-, intermediate- and long-term time domains ranging from seconds to days (Keller-Wood & Dallman, 1984). Our results indicate that there is a temporal lag between GC-induced changes in equine ACTH and cortisol levels, and as studied in most species, as ACTH and cortisol are secreted in a pulsatile or episodic fashion under basal and stress conditions, the dissociation between plasma ACTH and cortisol might have well occurred in nonoverlapping pulses (for review see Bornstein *et al.*, 2008). Therefore, even under conditions in which adrenal cortisol responses might be governed completely by ACTH stimulation, the timing and frequency of sampling will influence the apparent fidelity of the ACTH–corticol relationship.

It was also worth assessing the duration of HPA axis recovery after dermal dexamethasone withdrawal. Even if among commonly and topically used GCs, dexamethasone, prednisone and methylprednisolone, dexamethasone with the longer half-life (36–54 h) suppresses ACTH at the longest (Krasner, 1999); the resting plasma cortisol concentrations almost recovered by day 11 after treatment cessation and reached baseline. In all horses, suppressed adrenal response reflected by lowered mean plasma cortisol values after ACTH challenge returned to baseline within 2 weeks. Although the HPA axis can be suppressed rather by a short treatment, as in our study, clinically evident adrenal impairment is perhaps rare in patients treated for less than a week. Thus, it can be suggested that if short-term treatment do suppress the HPA axis, the suppression would last for only a few days (Streck & Lockwood, 1979). In our study, complete HPA axis recovery was evident weeks after cessation of daily dexamethasone administration. Similarly, in horses with heaves treated with inhaled GCs for 5–8 days, basal and ACTH-stimulated cortisol levels returned to baseline within less than a week (Rush *et al.*, 1998, 2000). In marked contrast, there is evidence that the recovery of suppressed adrenal response after long-term and high-dose GC treatment may take more than a year (Henzen *et al.*, 2000).

Furthermore, the daily dermal dexamethasone administration did, in part, significantly alter the white blood cell counts. A typical decrement of lymphocytes was observed, but this might not be attributable to a cytotoxic GC effect, but rather may result from redistribution of mature circulating cells and a decreased liberation from bone marrow (Bloemena *et al.*, 1990). The decrease in GC-induced eosinophils should be interpreted with care, as in the horse counting eosinophil granulocytes is usually critical. The significant increase in neutrophil granulocytes might be attributed to dexamethasone effect to stimulate the liberation of matured neutrophils from bone marrow and to induce an inhibition of apoptosis. These findings were in accordance with studies shown for ototopical (Abraham *et al.*, 2005) or sub-conjunctival application (Roberts *et al.*, 1984) of dexamethasone in dogs; thus, confirming the immunosuppressive effect of the drug. Finally, despite the anti-inflammatory and immune suppressive effects mediated by GCs, their prolonged and high-dose usage can, indeed, cause hepatic disturbances, as a result biochemical profiles, but with differences in individual and species susceptibility (Rutgers *et al.*, 1995). Even though dogs upon ototopical dexamethasone administration showed a marked alteration of the liver enzymes ALP, GGT, ALAT and ASAT (Abraham *et al.*, 2005), we could not observe significant trends for dexamethasone-induced horse specific liver enzyme activities. In contrast, one case report has shown that systemic (oral or parenteral) GC administration resulted in morphological liver changes with a disruption of the hepatocytes microfilament network with increased liver enzyme activities in a horse (Cohen & Carter, 1992). GGT and ALP are increased in horses with acute and chronic active hepatitis whereas an enhanced release of AST can be observed in acute liver damage (Carlson, 1996). After dermal dexamethasone treatment in horses, however, no evidences of hepato-cellular damage and liver enzyme induction were observed. In addition, we could not demonstrate any change in serum levels of electrolytes, urea, bilirubin and albumin during dexamethasone treatment. In general, however, it can strongly be assumed that a species-dependence in GC-induced alteration in liver enzyme activities should exist.

In summary, this study provides evidence that the application of dermal dexamethasone with mild potency on intact skin of normal horses alters the HPA axis function and leukocyte counts. Treated animals exhibited consistently decreased resting and an ACTH stimulated plasma cortisol as well as basal ACTH levels, suggesting acute adrenal impairment and suppressed hypothalamic-pituitary-mediated negative feedback mechanism without overt clinical symptoms. Concomitantly, dexamethasone altered white blood cell counts indicating characteristic GC (adverse) consequences. In conclusion, the data indicate the potential risk for systemic (adverse) effects even at recommended doses of dermal GCs in the horse. Thus, when topical GCs are used at higher doses or with higher potency and long-term, one should weigh benefits and risks and consider the inability of animals to resist stress situations. Moreover, the data further indicate that percutaneous absorption of dermal dexamethasone though the skin may cause systemic effects being of relevance with regard to doping cases. As topical GCs are often used in a variety of peripheral inflammatory disorders, their systemic adverse effects should be further studied in horses with disturbed skin integrity. The present study is limited to normal horse with no dermatological alterations.

REFERENCES

Abraham, G., Gottschalk, J. & Ungemach, F.R. (2005) Evidence for ototopical glucocorticoid-induced decrease in hypothalamic-pituitary-adrenal axis response and liver function. *Endocrinology*, **146**, 3163–3171.

Alexander, S.L., Irvine, C.H., Livesey, J.H. & Donald, R.A. (1993) The acute effect of lowering plasma cortisol on the secretion of corticotropin-releasing hormone, arginine vasopressin, and adrenocorticotropin as revealed by intensive sampling of pituitary venous blood in the normal horse. *Endocrinology*, **133**, 860–866.

Bhagwat, A.G. & Ross, R.C. (1971) Prednisolone-induced hepatic injury. Ultrastructural and biochemical changes in rabbits. *Archives of Pathology*, **91**, 483–492.

Bloemena, E., Weinreich, S. & Schellekens, P.T. (1990) The influence of prednisolone on the recirculation of peripheral blood lymphocytes in vivo. *Clinical and Experimental Immunology*, **80,** 460–466.

Bornstein, S.R., Engeland, W.C., Ehrhart-Bornstein, M. & Herman, J.P. (2008) Dissociation of ACTH and glucocorticoids. *Trends in Endocrinology and Metabolism*, **19,** 175–180.

Bousquet-Mélou, A., Formentini, E., Picard-Hagen, N., Delage, L., Laroute, V. & Toutain, P.L. (2006) The adrenocorticotropin stimulation test: contribution of a physiologically based model developed in horse for its interpretation in different pathophysiological situations encountered in man. *Endocrinology*, **147,** 4281–4291.

Brazzini, B. & Pimpinelli, N. (2002) New and established topical corticosteroids in dermatology: clinical pharmacology and therapeutic use. *American Journal of Clinical Dermatology*, **3,** 47–58.

Brus, R. (1999) Effects of high-dose inhaled corticosteroids on plasma cortisol concentrations in healthy adults. *Archives of Internal Medicine*, **159,** 1903–1908.

Bugajski, J., Gadek-Michalska, A. & Bugajski, A.J. (2001) A single corticosterone pretreatment inhibits the hypothalamic-pituitary-adrenal responses to adrenergic and cholinergic stimulation. *Journal of Physiology and Pharmacology*, **52,** 313–324.

Buske-Kirschbaum, A., Jobst, S. & Hellhammer, D.H. (1998) Altered reactivity of the hypothalamus-pituitary-adrenal axis in patients with atopic dermatitis: pathologic factor or symptom? *Annals of the New York Academy of Sciences*, **840,** 747–754.

Buske-Kirschbaum, A., Geiben, A., Hollig, H., Morschhauser, E. & Hellhammer, D. (2002) Altered responsiveness of the hypothalamus-pituitary-adrenal axis and the sympathetic adrenomedullary system to stress in patients with atopic dermatitis. *The Journal of Clinical Endocrinology & Metabolism*, **87,** 4245–4251.

Carlson, G.P. (1996) Clinical chemistry tests. In *Large Animal Internal Medicine*. Ed. Smith, P.S., pp. 441–469. Mosby, St Luis, USA.

Chrousos, G.P. (2001) Adrenal suppression versus clinical glucocorticoid deficiency in the premature infant: no simple answers. *The Journal of Clinical Endocrinology & Metabolism*, **86,** 473–474.

Clark, D.J. & Lipworth, B.J. (1997) Evaluation of corticotropin releasing factor stimulation and basal markers of hypothalamic-pituitary-adrenal axis suppression in asthmatic patients. *Chest*, **112,** 1248–1252.

Cohen, N.D. & Carter, G.K. (1992) Steroid hepatopathy in a horse with glucocorticoid-induced hyperadrenocorticism. *Journal of the American Veterinary Medical Association*, **200,** 1682–1684.

Crowley, S., Hindmarsh, P.C., Honour, J.W. & Brook, C.G. (1993) Reproducibility of the cortisol response to stimulation with a low dose of ACTH (1-24): the effect of basal cortisol levels and comparison of low-dose with high-dose secretory dynamics. *The Journal of Endocrinology*, **136,** 167–172.

Curtis, C. (1999) Use and abuse of topical dermatological therapy in dogs and cats. Part 2. *In Practice*, **21,** 448–454.

Drake, L.A., Dinehart, S.M., Farmer, E.R., Goltz, R.W., Graham, G.F., Hordinsky, M.K., Lewis, C.W., Pariser, D.M., Webster, S.B., Whitaker, D.C., Butler, B., Lowery, B.J., Raimer, S.A., Krafchik, B.R., Olsen, E. & Weston, W.L. (1996) Guidelines of care for the use of topical glucocorticosteroids. *American Academy of Dermatology*, **35,** 615–619.

Fisher, D.A. (1995) Adverse effects of topical corticosteroid use. *The Western Journal of Medicine*, **162,** 123–126.

French, K., Pollitt, C.C. & Pass, M.A. (2000) Pharmacokinetics and metabolic effects of triamcinolone acetonide and their possible relationships to glucocorticoid-induced laminitis in horses. *Journal of Veterinary Pharmacology and Therapeutics*, **23,** 287–292.

Froin, H.R. (1998) *Diagnosis of Equine Cushing's Syndrome by ACTH Measurement*. DPC® Technical Report. pp. 1–3. Diagnostic Products Corporation, Los Angeles.

Garden, J.M. & Freinkel, R.K. (1986) Systemic absorption of topical steroids. Metabolic effects as an index of mild hypercortisolism. *Archives of Dermatology*, **122,** 1007–1010.

Hanania, N.A., Chapman, K.R. & Kesten, S. (1995) Adverse effects of inhaled corticosteroids. *The American Journal of Medicine*, **98,** 196–208.

Hart, K.A., Ferguson, D.C., Heusner, G.L. & Barton, M.H. (2007) Synthetic adrenocorticotropic hormone stimulation tests in healthy neonatal foals. *Journal of veterinary internal medicine*, **21,** 314–321.

Hengge, U.R., Ruzicka, T., Schwartz, R.A. & Cork, M.J. (2006) Adverse effects of topical glucocorticosteroids. *Journal of the American Academy of Dermatology*, **54,** 1–15.

Henzen, C., Suter, A., Lerch, E., Urbinelli, R., Schorno, X.H. & Briner, V.A. (2000) Suppression and recovery of adrenal response after short-term, high-dose glucocorticoid treatment. *Lancet*, **355,** 542–545.

Johnson, P.J., Slight, S.H., Ganjam, V.K. & Kreeger, J.M. (2002) Glucocorticoids and laminitis in the horse. *The Veterinary clinics of North America Equine practice*, **18,** 219–236.

Keller-Wood, M.E. & Dallman, M.F. (1984) Corticosteroid inhibition of ACTH secretion. *Endocrine Reviewes*, **5,** 1–24.

Krasner, A.S. (1999) Glucocorticoid-induced adrenal insufficiency. *The Journal of the American Medical Association*, **282,** 671–676.

Levin, C. & Maibach, H.I. (2002) Topical corticosteroid-induced adrenocortical insufficiency: clinical implications. *American Journal of Clinical Dermatology*, **3,** 141–147.

Lipworth, B.J. (1999) Systemic adverse effects of inhaled corticosteroid therapy: a systematic review and meta-analysis. *Archives of Internal Medicine*, **59,** 941–955.

Livanou, T., Ferriman, D. & James, V.H. (1967) Recovery of hypothalamo-pituitary-adrenal function after corticosteroid therapy. *Lancet*, **2,** 856–859.

Maibach, H.I., Feldmann, R.J., Milby, T.H. & Serat, W.F. (1971) Regional variation in percutaneous penetration in man. *Archives of Environmental Health*, **23,** 208–211.

Mills, P.C. & Cross, S.E. (2006) Regional differences in the in vitro penetration of hydrocortisone through equine skin. *Journal of Veterinary Pharmacology and Therapeutics*, **29,** 25–30.

Nye, E.J., Grice, J.E., Hockings, G.I., Strakosch, C.R., Crosbie, G.V., Walters, M.M. & Jackson, R.V. (1999) Comparison of adrenocorticotropin (ACTH) stimulation tests and insulin hypoglycemia in normal humans: low dose, standard high dose, and 8-hour ACTH-(1-24) infusion tests. *The Journal of Clinical Endocrinology and Metabolism*, **84,** 3648–3655.

Patel, L., Clayton, P.E., Addison, G.M., Price, D.A. & David, T.J. (1995) Adrenal function following topical steroid treatment in children with atopic dermatitis. *British Journal of Dermatology*, **132,** 950–955.

Roberts, S.M., Lavach, J.D., Macy, D.W. & Severin, G.A. (1984) Effect of ophthalmic prednisolone acetate on the canine adrenal gland and hepatic function. *American Journal of Veterinary Research*, **45,** 1711–1714.

Rush, B.R., Worster, A.A., Flaminio, M.J., Matson, C.J. & Hakala, J.E. (1998) Alteration in adrenocortical function in horses with recurrent airway obstruction after aerosol and parenteral administration of beclomethasone dipropionate and dexamethasone, respectively. *American Journal of Veterinary Research*, **59,** 1044–1047.

Rush, B.R., Raub, E.S., Thomsen, M.M., Davis, E.G., Matson, C.J. & Hakala, J.E. (2000) Pulmonary function and adrenal gland suppression with incremental doses of aerosolized beclomethasone dipropionate in horses with recurrent airway obstruction. *Journal of the American Veterinary Medical Association*, **217,** 359–364.

Rutgers, H.C., Batt, R.M., Vaillant, C. & Riley, J.E. (1995) Subcellular pathologic features of glucocorticoid-induced hepatopathy in dogs. *American Journal of Veterinary Research*, **56,** 898–907.

Ryu, S.H., Kim, B.S., Lee, C.W., Yoon, J. & Lee, Y.L. (2004) Glucocorticoid-induced laminitis with hepatopathy in a Thoroughbred filly. *Journal of Veterinary Science*, **5,** 271–274.

Smith, E.W. (1993) Do we need new and different glucocorticoids? A re-appraisal of the various congeners and potential alternatives. *Current Problems in Dermatology*, **21,** 1–10.

Spiess, B.M., Nyikos, S., Stummer, E., Sahin, A. & Naegeli, H. (1999) Systemic dexamethasone concentration in horses after continued topical treatment with an ophthalmic preparation of dexamethasone. *American Journal of Veterinary Research*, **60,** 571–576.

Streck, W.F. & Lockwood, D.H. (1979) Pituitary adrenal recovery following short-term suppression with corticosteroids. *The American Journal of Medicine*, **66,** 910–914.

Sulzberger, M.B. & Witten, V.H. (1952) The effect of topically applied compound F in selected dermatoses. *The Journal of Investigative Dermatology*, **19,** 101–102.

Todd, G.R., Acerini, C.L., Buck, J.J., Murphy, N.P., Ross-Russell, R., Warner, J.T. & McCance, D.R. (2002) Acute adrenal crisis in asthmatics treated with high-dose fluticasone propionate. *European Respiratory Journal*, **19,** 1207–1209.

Turpeinen, M. (1988) Influence of age and severity of dermatitis on the percutaneous absorption of hydrocortisone in children. *British Journal of Dermatology*, **118,** 517–522.

Turpeinen, M. (1989) Adrenocortical response to adrenocorticotropic hormone in relation to duration of topical therapy and percutaneous absorption of hydrocortisone in children with dermatitis. *European Journal of Pediatrics*, **148,** 729–731.

Turpeinen, M., Salo, O.P. & Leisti, S. (1986) Effect of percutaneous absorption of hydrocortisone on adrenocortical responsiveness in infants with severe skin disease. *British Journal of Dermatology*, **115,** 475–484.

Wester, R.C. & Maibach, H.I. (1991) In vivo percutaneous penetration. In *Dermatotoxicology*. Eds Marzulli, F.N. & Maibach, H.I., pp. 75–96. Hemisphere Publishing, Washington, DC.

Wester, R.C. & Maibach, H.I. (1992) percutaneous absorption in diseased skin. In *Topical Corticosteroids*. Eds Maibach, H.I. & Surber, C., pp. 128–141. Karger, Basel.

Zenoble, R.D. & Kemppainen, R.J. (1987) Adrenocortical suppression by topically applied corticosteroids in healthy dogs. *Journal of American Veterinary Medical Association*, **191,** 685–688.