PAIN CAUSES INCREASED CONCENTRATIONS OF GLUCOCORTICOID METABOLITES IN HORSE FECES

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SUMMARY

The concentration of 11,17-dioxoandrostanes (11,17-DOA), a group of cortisol metabolites, was measured using enzyme immunoassay in fecal samples of horses experiencing painful episodes. One group of horses consisted of 10 stallions castrated (samples were collected daily for 10 days); the other group was made up of 29 horses which were brought to an animal hospital because of signs of colic (samples were collected twice daily for six days).

Before castration, median concentrations of 10.5 nmol/kg feces were measured. On days 1 and 2 after castration, median 11,17-DOA values increased up to 26.2 and 50.0 nmol/kg feces, respectively, and decreased thereafter to levels lower than at the beginning of the sampling period.

High variations were measured between individual cases of colic. In animals with colic, all horses excreted more than 33 nmol 11,17-DOA/kg feces for various periods. The highest concentration measured was 885 nmol/kg feces. One animal out of the 29 colic horses did not show any clinical signs of pain upon arrival in the hospital. The 11,17-DOA values were below 17 nmol/kg feces in all those samples. From this data we conclude, that the concentration of 11,17-DOA in feces is a parameter for painful situations that have occurred one or two days earlier.

INTRODUCTION

Increased amounts of glucocorticoids are produced in the

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adrenal gland by the stimulating effect of adrenocorticotropic hormone (ACTH). The dominant adrenal steroid in the plasma of horses is cortisol, which is secreted in a circadian pattern with elevated levels occurring in the morning.[1] Plasma concentrations are also elevated with exertion[2] and during restraint.[3] Irvine and Alexander[4] describe the difficulties in determining the cortisol status of horses, since it is influenced by the time of the day, episodic fluctuations and how accustomed the horse is to the experimental procedure. Nevertheless, cortisol concentration in blood is a proven parameter for disturbance in horses.[5-7]

Horses with disturbances of the gastrointestinal tract show a broad spectrum of clinical symptoms. Greatorex[8] postulated that because of the low threshold for pain, abdominal pain (colic) is more evident in horses. Various attempts were performed to find a parameter for pain in horses. Hodson et al.[9] measured ACTH, cortisol and catecholamines in cases of grass sickness, colic and in horses before and after mild stress. No differences were detected between animals with colic and stressed control horses.

Various methods to evaluate pain after orthopedic surgery were investigated by Raekallio et al.[10] The authors indicated the difficulties associated with pain assessment in horses. They compared the concentrations of beta-endorphin, cortisol, catecholamines, heart rate and a subjective pain score before premedication up to 72 hours after surgery. Anesthesia and surgery did not change mean plasma cortisol concentrations. There was a poor correlation between the parameters measured.

Santschi et al.[11] described that in cases of medical colic cortisol values ranged between 33 and 267 ng/ml plasma, in surgical colics between 50 and 279 ng/ml. Uterine torsions caused mean cortisol values of 303 ng/ml.

Restraint and blood sample collection is often a procedure which is stressful in itself and may cause elevated cortisol levels in samples. Therefore non-invasive methods of sample

collection were investigated. Measurement of cortisol and cortisol metabolites in urine was described by Popot et al.[12,13] Urine cortisol metabolites like 20ß-dihydrocortisol are a good parameter for cortisol administration[13] and are elevated for a few hours after i.v. administration of cortisol.

In ponies, 41 % of infused radioactive cortisol is excreted via feces.[14] The authors described a lag time of 24 hours between the infusion and the peak of radioactivity in feces.

In horses, there are only a few publications concerning the measurement of glucocorticoids or their metabolites in feces. Möstl et al.[15] showed that infused ^{14}C cortisol was mainly excreted as various unconjugated metabolites. Authentic radioactive cortisol was found only in trace amounts. A cortisol enzyme immunoassay showed only very low concentrations of immunoreactive substances in the feces and this assay was not suitable for measuring cortisol metabolites in feces. Möstl and Palme[16] described that 11,17-dioxoandrostanes (11,17-DOA), which are produced as excretory products of cortisol, are present in fecal samples of various species such as horses, pigs, okapies, roe deer and rhinos. As Möstl et al.[15] showed, the formation of 11,17-DOA is also done by the bacteria of the gut because storing fecal samples at room temperature for longer than four hours caused a significant increase in 11,17-DOA concentrations.

The biological relevance of this non-invasive method has been proven in ruminants following stimulation (ACTH) or suppression (dexamethasone) of cortisol release by the adrenal cortex.[18] In the horse, Möstl et al.[15] showed that ACTH (1 mg) caused increased concentrations of 11,17-DOA, reaching maximum values about one day after injection. Dexamethasone (30 mg) diminished the concentrations of these cortisol metabolites in feces. Cortisol is extensively metabolized before excretion via feces. As there is a lag time between the increase of glucocorticoids in blood and the rise of the metabolites in feces,[14,15] fecal sampling offers the possibility to gather information concerning events that have happened about one day before in healthy horses.

The aim of the study was to evaluate whether glucocorticoid metabolites in fecal samples can be measured for monitoring discomfort or pain in horses.

MATERIAL AND METHODS

Animals
Castration

Mature Haflinger stallions (n = 14) owned by the Austrian army were castrated at the training area Hochfilzen in a stall. For premedication, Domosedan® (detomidine, 0.1 - 0.2 ml/ 100 kg body mass) was administered. Anesthesia was induced with Thiopental/Myolaxin® (thiopental sodium/ glycol glycerine ether), afterwards with an halothane-oxygen mixture.

The castration was done in lateral recumbency. Fecal samples were collected daily from the rectum for 10 days starting on the day of castration and were stored at -24°C until analysis. The experiment and the results of measuring gonadal steroids in these fecal samples were described earlier by Palme et al.[19]

Colic

Horses of various breeds (n = 29), which were taken to the animal hospital Mitterndorf due to signs of abdominal pain, were used for these investigations. The colic was classified according to the clinical signs[20] as mild (n = 4), moderate (n = 19) or severe (n = 5). Only horses that survived were considered in this study. One horse showed no clinical signs of pain at the animal hospital. Every 12 hours fresh fecal samples were collected for six days, starting with the arrival of the horse at the clinic. The samples were taken directly from the bedding or rectally and frozen at - 24°C till analysis.

Colics were treated with conservative methods (n = 13) or surgery (n = 16).

Extraction of samples

The method described earlier[15] was modified to improve the separation between the diethylether and the water-methanol phase. In brief, 0.5 g feces plus 1 ml water and 4 ml methanol were vortexed for 30 minutes and centrifuged (2500g/15 min). One ml of the supernatant was mixed with 5 ml diethylether and 0.5 ml 5% $NaHCO_3$ for 10 seconds. Thereafter, 4 ml water were added and the tube was turned upside down four times. The aqueous phase was frozen at -24°C and then ether decanted and dried down. The extract was redissolved in assay buffer and the concentration of 11,17- DOA was measured as described earlier.[17]

Statistics

The 11,17-DOA values in the samples collected after castration were not normally distributed. Therefore, the data are shown in the figure as a boxplot diagram.

In cases of colic, the samples were assigned to time periods according to the case report of the owner. Starting point was the time when the colic was first observed. If there was a longer period than 12 hours between observation of pain and the arrival in the clinic, the data were missing.

As in castrated horses, in animals with colic the values were also not normally distributed. Therefore median 11,17-DOA concentrations were calculated.

The median 11,17-DOA concentrations were calculated for the three groups of horses with colic in each time period. To check for differences in concentrations of cortisol metabolites between the three groups the Mann–Whitney U-test was used; the Wilcoxon signed rank test was used to check for differences between the days before and after castration.
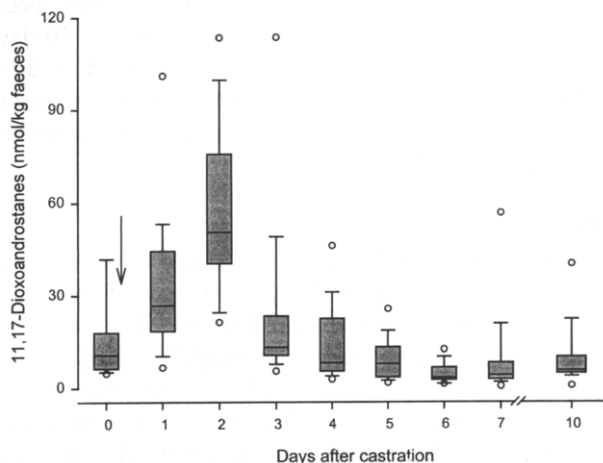
Figure **1.** Boxplot of fecal 11,17-DOA (nmol/kg) concentrations before and after castration. Each box shows the median and the upper and lower quartile value; the whiskers, the 10th and 90th percentiles of the 11,17-DOA values. The circles represent data points that are outside the percentiles.

Figure **2.** Examples of the concentration of 11,17-DOA (nmol/kg) in feces of individual horses with colic.

RESULTS

Concentration of glucocorticoid metabolites after castration

Before castration the median value of the 11,17-DOA concentration was 10.5 nmol/kg feces. The values were significantly (p < 0.001) increased for two days after castration compared to those from the day of surgery (Fig.1), reaching a median concentration of 49 nmol/kg feces. On days 3, 4 and 5 after castration the 11,17-DOA concentrations in the feces were in the same range as on day 0, whereas the samples collected on days 6, 7 and 10 contained significantly (p < 0.007) lower 11,17-DOA concentrations compared to day 0.

Concentration of glucocorticoid metabolites during and after colic

In one horse, no signs of colic were seen when it arrived at the clinic. 11,17-DOA concentrations higher than 17 nmol/kg feces were never measured in the fecal samples of this horse. In the other patients, there were considerable variations in 11,17-DOA concentrations among the individual cases of abdominal pain, but all had periods with concentrations
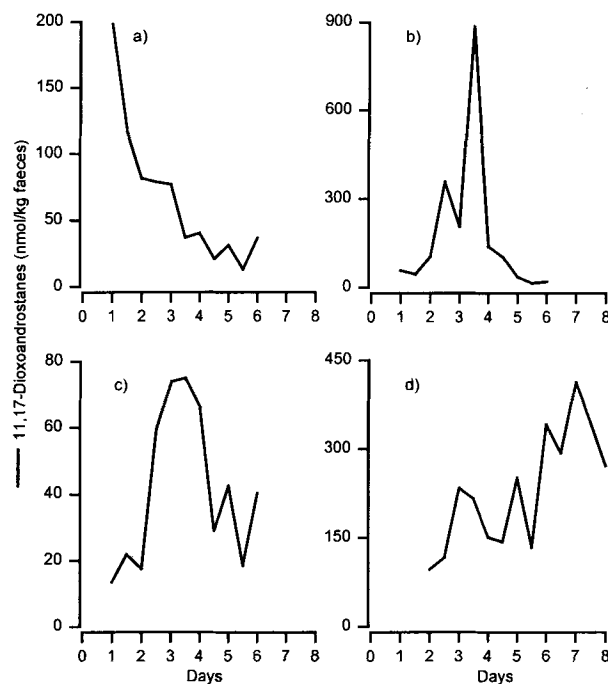
higher than 32 nmol/kg feces. In the samples of two out of four horses with mild colic symptoms, values of more than 65 nmol/kg were measured. In cases of moderate colic symptoms (n = 19) 16 horses with higher fecal values than 65 nmol/kg were seen. In all cases of severe colic there were time periods when the horses excreted more than 65 nmol/kg 11,17-DOA.

Most of the animals were brought to the clinic between 12 and 24 hours after the owners had noticed signs of pain. As only few samples could be collected in the first 12 hours after the onset of colic, these data are not given in Table 1, which shows the median values of the three groups. The differences in 11,17-DOA concentrations between the groups were not significant. Most of the animals excreted the highest concentrations of cortisol metabolites 84–96 hours after the colic was noticed by the owner. Because of high individual differences, four individual time courses of the 11,17-DOA excretion are shown in Figure 2 as examples.

In a case of severe colic, surgery had to be done on a 12-year-old standardbred gelding showing abdominal

Table **1.** Median concentrations of 11,17-DOA (nmol/kg) in fecal samples of horses with colic.

Symptoms		Hours after observation of pain								
	-	24	36	48	60	72	84	96	108	120
mild	-	15.7	32.5	17.4	51.5	29.2	52.8	37.1	23.9	42.3
moderate	-	26.4	32.8	83.3	83.3	64.9	57.1	101.1	71.2	41
severe	-	44	102	78.4	78.4	76.7	119.7	94.7	89.5	56.6

discomfort for eight hours before he arrived at the clinic after a long transport of six hours. He had continuous colic and the referring veterinarian had administered 20 ml of Novasul® (metamizol sodium) once. The gelding was sweating all over his body and was shaking in his forehand from muscle fasciculation. The rectal examination indicated surgery. The diagnosis "colon torsion" was ascertained during the intervention. In the first sample collected in the clinic, a peak value of 197 nmol/kg feces was measured (Fig. 2a). The concentration decreased after treatment was started.

In a 12-year old standardbred stallion with an inguinal hernia on the left side surgery had to be done. According to clinical signs, the colic was classified as moderate. The concentration of cortisol metabolites (Fig. 2b) steadily increased, reaching maximum 11,17-DOA concentrations (900 nmol/kg) 84 hours after the owner had noticed the colic. This was the highest concentration measured during these investigations. Afterwards the values decreased.

A long period of elevated fecal 11,17-DOA levels (Fig. 2c) were seen in a 12–year–old Standardbred gelding (a wind sucker), which showed signs of mild colic. In the entrance examination it was ascertained, that the caecum and the colon were severely bloated. A rectal examination 69 hours after the beginning of colic was negative. As in most cases, the first samples showed relatively low values.

A case of moderate colic (6-year-old Standardbred gelding) with a long period of high concentrations of glucocorticoid metabolites is shown in Fig. 2d. The rectal examination revealed an entrapment of the colon in the nephrosplenic space. About 70 hours after surgery the body temperature rose to 39.1°C. During the rest of the sampling period temperatures between 39.8 and 38.6°C were measured. The 11,17-DOA concentration was already 97 nmol/kg feces when the animal arrived at the clinic (36 hours after the beginning of colic). In contrast to all other cases, the values of the cortisol metabolites in feces remained elevated.

Surgically treated horses excreted significantly (P = 0.009) higher peak values of 11,17-DOA (median 79.6 nmol/kg feces, n = 16) compared to nonsurgically treated animals (median 200.6/kg feces, n = 16).

DISCUSSION

Pain is a state which is hard to measure. Meyer[21] describes qualitative and quantitative methods to identify pain and concludes that all methods for quantifying pain are inexact.

Measuring cortisol or cortisol metabolites in blood or urine samples is one parameter for measuring discomfort or pain. But blood sampling may disturb the horses and urine sampling is not as easy as collecting fecal samples. Therefore, we tested a recently published method[15] to test if increased concentrations of cortisol metabolites are present in fecal samples following pain.

In our investigations, the 11,17-DOA excretion via feces increased in castrated animals one day after surgery. The values were similar to that described by Möstl et al.[15] The authors found maximum values of cortisol metabolites of about 100 nmol/kg after ACTH injection (1 mg). Peak values were seen about one day after application. After castration, the highest values were measured about 36 hours after surgery in most of the horses. The longer time interval observed compared to the results described earlier[15] may be explained by a retarded defecation. This is in agreement with the data of Palme et al.[19] The authors showed that the concentration of unconjugated estrogens in fecal samples of these animals started to decrease on day two after castration. The retarded defecation may cause higher 11,17-DOA concentrations as Möstl et al.[15] described an increase of 145 ± 27 % in the concentrations measured by the immunoassay after incubation of fecal samples for four hours at room temperature. The higher 11,17-DOA values on day 0 compared to the values measured at the end of the sampling period may have been caused by the preparation of the stallions for castration, as the animals were housed in stables unknown to these animals. This may have led to a disturbance of the stallions.

In horses with colic, the 11,17-DOA values showed considerable differences between the individual animals but in all horses with clinical signs of colic, 11,17-DOA values higher than 32 nmol/kg feces were measured for an individual time period. The high variation of the concentration of the cortisol metabolites in feces is similar to that described for cortisol concentrations in blood. High levels and considerable variations were measured during medical and surgical diseases.[11] The higher 11,17-DOA excretion in surgical treatment is most probably caused by the fact that more painful cases are treated surgically.

There were no statistical differences between the 11,17-DOA concentrations in cases of mild, moderate or severe colic. The potential differences in the three groups may be masked by the additional stress of transport and medical examination.

In contrast to values obtained after castration, there were also variations in the time period between the signs of colic and the increase in fecal 11,17-DOA values. This may be explained by changes in the motility of the intestines. The prolonged period between bile secretion and excretion of feces may be an additional reason for the elevated levels of these glucocorticoid metabolites, as it was shown[15] that after storing fecal samples at room temperature the 11,17-DOA values increased.

Stress increases plasma cortisol levels within a short time, urine levels of cortisol or cortisol metabolites can remain elevated for some hours.[13] Unlike most endocrine methods, fecal sampling offers the possibility of gathering information on cortisol concentrations one to two days before sampling. The method can, therefore, be used to detect the pain a horse had already experienced one to three days before.

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