THE IN VITRO METABOLIC CHANGES IN THE CORTICOSTEROIDAL HORMONES IN FAECES OF RUMINANTS

By

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SUMMARY

The in vitro measurements of cortisol and corticosterone by enzyme immunoassay (EIA) were done in the faecal samples of ruminants (cattle and sheep). The study was aimed, therefore to investigate the extent of stability of corticosteroid hormones against the bacterial actions and their enzymatic activities that are naturally occurring in faeces. For this purpose, faecal samples were freshely collected from each of 10 cattle and 10 sheep. Each sample was immediately incubated in a water bath at 38°C with either 1µg of cortisol or corticosterone. The incubations were then run at time interval of 4 h, 8 h and 24 h (in cattle) and at 1 h, 2 h, 4 h, 8 h and 24 h (in sheep). The samples were extracted with methanol and the aliquots were taken after centrifugation from their supernatants and analyzed for measurement of cortisol and corticosterone by EIAs. The results of cattle faeces revealed a dramatical decline in the concentrations of the added cortisol and corticosterone. Only about 20 % of the starting levels of both hormones after 4 h in contrast to almost no cortisol with about 10 % of corticosterone after 8 h and about 5 % of corticosterone after 24 h was detected. Prolonged incubation of faecal samples of sheep decreased the immunoreactive substances measured by the 3α-hydroxy,11-oxoandrogens EIA (11-oxo-A- EIA), whereas with the 11,17 dioxoandrotanes EIA (11,17 DOA-EIA), the values were increased. This study concluded that cortisol was heavily metabolized by faecal flora being quicker in its metabolism than corticosterone in the faeces of ruminants. In addition, the present study provides a powerful evidence to the absence of native cortisol in the faeces of ruminants.

INTRODUCTION

The concentration of glucocorticoids (cortisol and corticoterone) in blood is widely used as an indicator of stress, although caution is advised, since an increase does not occur with every type of stressor *(Broom and Johnson, 1993)*. Like many other hormones, glucocorticoids have a circadian

rhythm in many species but such rhythms may be abolished by prolonged stress *(Przkop et al., 1985)*. Thus, rhythmicity and episodic secretion demand frequent sampling. One has to consider that sample collection, which often involves confinement or handling of animals may by itself be stressful and may confound the results *(Morton et al., 1995)*. Therefore, feedback-free sampling methods are preferential.

To overcome these problems, several authors have investigated non-invasive sampling procedures such as a corticoid (metabolites) determination in urine *(Hay and Mormede, 1998)*, saliva *(Cooper et al., 1989)* or milk *(Verkerk et al., 1998)*. However, there are some major drawbacks related to each: saliva or urine collection also needs some manipulation of the animal and can be used only to a limited extent in free moving animals and milk is limited to lactating animals. Above all, faecal samples offer the advantages that they can be easily collected without stressing the animals. Methods for measuring faecal metabolites of placental or gonadal origin are well established to evaluate reproductive function *(Schwarzenberger et al., 1996)*. As the measurement of physiological stress also has an importance in wildlife management, conservation biology and behavioural ecology, the measurement of faecal glucocorticoids metablites is gaining increased importance. For the development of non-invasive techniques to monitor adrenocortical activity, basic information of the metabolism and excretion of glucocorticoids is necessary. *Lindner (1972)* investigated the excretion of ^{14}C cortisol in the sheep. He stated that two-third of the radioactivity was subsequently found in the bile. The main metabolites were glucouronides of tetrahydrocortisol, tetrahydrocortisone and cortolone. Also $C_{19}O_3$ steroids (androstanes) were formed. Additionally, enterohepatic circulation of these metabolites occurred and cortisol metabolites were voided via faeces. In farm animals, infused radioactive cortisol is mainly excreted in urine *(Palme et al., 1996)*. Excretion via faeces is 28 % in sheep. These authors also described a species-specific lag time between the infusion and the peak of radioactivity in the faeces (10-12 h in ruminants). The cortisol metabolite may be further metabolized during this period. For example, the side chain of some C-21 steroids cleaved by the faecal flora of human and rats to C-19 compounds *(Cerone-McLeron et al., 1981)*.

Miller et al., 1991 stated that elevated level of faecal cortisol can be measured during stress in the Rocky mountain bighorn sheep. *Wasser et al. (2000)* investigated three different commercially available radioimmunoassay for cortisol and one assay for corticosterone in a variety of wildlife mammals. A corticosterone antibody (ICN Biomedicals, Costa Mesa, CA) gave best results. This antibody may not only be used for radioimmunoassay but also for EIA as shown by *Goymann et al. (1999)*. The cross-reactions of this antibody are still not fully evaluated.

In contrast, *Palme and Möstl (1997)* characterized radioactive faecal cortisol metabolites using high performance liquid chromatograghy (HPLC) and EIAs. They showed that the predominant faecal metabolites were unconjugated steroids but they could not detect immunoreactive cortisol. Especilly in ruminants, even after intravenous infusion of 1gm of cortisol, no

native cortisol could be detected in the faeces of sheep *(Palme et al., 1999). Palme and Möstl (1997) and Möstl et al. (2002)* described group specific enzyme immunoassays (EIAs) for cortisol metabolites using 11-oxoetiocholanolone as immunogen and a biotinylated steroid as label. The assay system described by *Palme and Möstl (1997)* used antibody against 11-oxoetiocholanolone coupled at position C-3. This EIA (measuring 11,17 dioxoandrotanes-11,17 DOA) proved suited for evaluating adrenocortical activity in sheep, cows and horses *(Möstl et al., 1999; Palme et al., 1999; Palme et al., 2000)* and also for some zoo and wildlife animals (e.g. roe deer, hares ad elephants; *Stead et al., 2000; Teskey-crestl et al., 2000; Dehnhard et al., 2001). Möstl et al. (2002)* raised an antibody against 11-oxoetiocholanolone coupled at C-17 (11-oxo-A). This EIA records higher concentration of cortisol metabolite in cows compared to that described by *Palme and Möstl (1997),* most probably because the new antibody has also cross reactions with $C_{21}O_4$ cortisol metabolites.

The aim of this *in vitro* study is to investigate whether the faecal flora of ruminants are capable of converting cortisol or corticosterone clarifying which of these steroids is quickly metabolized. The aim of this study was also extended to investigate the stability of cortisol in faecal samples of sheep clarifying whether or not native cortisol is present in faeces of these species.

MATERIALS AND METHODS

Experiment No. 1 in Cattle

A total of 10 faecal samples were freshly collected from healthy cattle (5 cows and 5 bulls). Forty times 0.5g faeces from each faecal sample were weighed and incubated in a water bath at 38 °C with buffer containing 1µg cortisol or corticosterone (n = 20 each; Wilton, NH, USA), respectively immediately and then at intervals after 4 h, 8 h and 24 h. Samples were deep frozen (-20°C; n = 5 each) and stored until the time of analysis (10 faecal samples × 2 steroids × 5 replicates × 4 times = 400 incubations). Afterwards all samples were extracted with 4ml methanol (80 %) and centrifuged (2500g). Then 10µl aliquot of the supernatant (after further dilution 1:100 for cortisol and 1:1000 for corticosterone) was analyzed with cortisol and corticosterone EIAs respectively.

Cortisol and corticosterone EIAs were performed according to *Palme and Möstl (1994 and 1997)* on microtitre plates using the double antibody technique and biotinylated steroids as labels. Antisera were raised in rabbits against cortisol-3-CMO and corticosterone-3-CMO respectively each coupled with bovine serum albumin (BSA). The labels were cortisol-3-CMO (for both EIAs) linked to DADOO biotin (N-biotinyl-1,8-diamino-3,6-dioxaoctane). The standards were 2 to 80 pg/well for cortisol and 2 to 500 pg/well for corticosterone.

Briefly, standards (50 µl) and samples (50 µl) were incubated in duplicate with label (100 µl) and antibody (100 µl) overnight at 4°C. Following incubation, the plates were four times washed with 0.02% Tween 20 (Merck 822184) washing solution and dry blotted, before 250 µl streptavidin

horseradish peroxidase conjugate (4.2 mU, Boehringer, No. 1089153) was added to each well. Plates were then left at 4°C in the dark on stirring tables for another 45 min.. The enzymatic reaction was stopped with 50 µl/well of 4 mol/L sulfuric acid. Absorbance was measured at a wavelength of 450 nm (referance filter: 620 nm) on an automatic plate reader (Labsystems Multiskan, MCC/340; Szabo).

Experiment No. 2 in Sheep

Ten faecal samples were freshly collected from healthy sheep (5 ewes and 5 rams). Aliquots of 0.5g faeces in triplicates from each faecal sample were incubated in a water bath at 38°C with 1ml buffer containing 1µg cortisol. Bacterial action was stopped immediately by adding 4ml of 80% methanol and then after 1 h, 2 h, 4 h, 8 h and 24 h (10 faecal samples × 1 steroid × 3 replicates × 6 times = 180 incubations).

Afterwards all samples were extracted, centrifuged (2500g) and a 10µl aliquot of the supernatant (after further dilution 1:100) was analyzed with 11,17 DOA and 11-oxo-A EIAs which were performed according to *Palme and Möstl (1997) and Möstl et al. (2002),* on microtitre plates using the double antibody technique and biotinylated steroids as labels. Antisera were raised in rabbits against 11-oxoaetiocholanolone-3-HS:BSA and 11-oxoaetiocholanolone-17-CMO:BSA respectively. The labels were 11-oxoaetiocholanolone-3-glucuronide (for both) linked to DADOO biotin (N-biotinyl-1,8-diamino-3,6-dioxaoctane). The standards were 11-oxoaetiocholanolone (range 500 to 2 pg/well for both). The EIAs were performed as described above.

STATISTICAL ANALYSIS

Amounts of measured glucocorticoids (cortisol and corticosterone), present after a given time, were expressed as a percentage of added glucocorticoids (cattle). The concentrations of 11,17-DOA and 11-oxo-A during incubations, measured immediately after collection of the faeces were considered to be 100 % and the variations were calculated as percent increase or decrease (sheep). To determine the effect of incubation time on the measured steroid concentrations a repeated measures ANOVA was used. All tests were performed with the software package SIGMASTAT© (SPSS Inc., Chicago, IL, USA).

RESULTS

Experiment No. 1 in Cattle

In the cattle faeces (cows and bulls) a dramatical decrease ($P < 0.001$) in the concentrations of the added cortisol and corticosterone (1µg) was observed (Figure 1a and 1b; amounts of measured steroids, present after a given time are expressed as a percentage of the starting values). After 4 h about 23 % of the starting level of cortisol and 25 % corticosterone were reached (Figure 1a). After 8 h almost no cortisol could be detected but about 10 % of corticosterone was detected (Figure 1a). After 24 h about 5 % of the

starting levels of corticosterone still present. The same pattern was found in bull (Figure 1b). There was some individual variation between the five animals (of the same sex) concerning time course and the amount decreased.

Experiment No. 2 in Sheep

To demonstrate changes in the 11,17-DOA and 11-oxo-A concentrations during incubation with cortisol (1µg), the values measured immediately after collection of the faeces were considered to be 100% and the variations were calculated as percent increase or decrease (Table 1 and 2).

In sheep (both rams and ewes), there was a significant ($P < 0.01$) increase in 11,17-DOA concentrations within 1 h (Table 1). After 2 h, 4 h, 8 h and 24 h of incubation, the concentrations were significantly elevated compared to the starting values (Table 1). The increase in 11,17-DOA concentrations was significantly differed in rams and ewes at all time points of incubations (Table 1).

In sheep (both rams and ewes), there was a significant ($P < 0.01$) decrease in 11-oxo-A concentrations within 1 h (Table 2). After 2 h, 4 h, 8 h and 24 h of incubation, the concentrations were also significantly decreased compared to the starting values (Table 2). The decrease in 11-oxo-A concentrations was significantly differed in rams and ewes at 4 h, 8 h and 24 h of incubations (Table 2).

DISCUSSION

The metabolism of Glucocorticoids include oxidation at C-11; reduction at C-3, and/or C-20, and/or C21; hydroxylation at C-6; as well as formation of ring A saturated steroids *(Brownie (1992)*. This metabolism takes place mainly in the liver, and the metabolites are subsequently excreted as conjugates (sulphates or glucuronides) via the urine and the bile *(Lindner, 1972)*. In addition, intestinal bacteria in the gut can affect the structure of these steroids. For example, a side-chain cleavage was found for cortisol, resulting in the formation of androstanes which in contrast to androgen metabolites, still bear an oxygen at position C-11 *(Macdonald et al., 1983)*. In ruminants at least 21 cortisol metabolites (with $C_{21}O_4$ or $C_{19}O_3$ structure) were detected in faecal samples using HPLC/mass spectroscopy *(Möstl et al., 2002)*.

In the present study, incubation of faecal samples (0.5g; n = 20) with high dose of cortisol or corticosterone (1µg), for several hours, revealed a quick metabolism, reflected by a rapid decrease of glucocorticoids concentration to negligible amounts after a few hours (4 h; Figure 1). The faecal flora which are capable of metabolizing steroids in the faeces of human and rats as described by *Cerone-McLeron et al. (1981)* may be the cause for such decrease of cortisol and corticosterone in faeces of ruminants.

The present study also demonstrated that, cortisol was quickly metabolized in faeces of ruminants than corticosterone (Figure 1). This might be due to the action of faecal flora which contains a desmolase enzyme which removes the side chain from some C-21 steroids as described in the faces of

human and rats by *Cerone-McLeron et al. (1981).* This interpretation can be underlined by the fact that a 17α-hydroxy group is a prerequisite for a steroid to be undergo desmolase side chain cleavage *(Cerone-McLeron et al., 1981).* In this respect cortisol which contain 17α-hydroxy group was a preferred substrate for desmolase enzyme and subsequently more quickly metabolized than corticosterone which has no 17α-hydroxy group.

The profound and quick changes of cortisol by faecal flora of cattle (almost no cortisol could be detected after 8 h of incubation; Figure 1) was also confirmed by the second experiment in faeces of sheep as prolonged incubation of faecal samples of sheep (ewes and rams) decreased the immunoreactive substances measured by the 11-oxo-A-EIA, whereas with the 11,17-DOA enzyme immunoassay, the values were increased (Table 1 and 2).

This may be explained by the effect of bacterial side-chain cleavage, forming an increased amount of $C_{19}O_3$ steroids. The 11-oxo-A EIA cross-reacts with some $C_{21}O_4$ steroids, whereas the 11,17- DOA EIA does not. Hence, an explanation for the decrease in immunoreactive substances using 11-oxo-A EIA may be that the assay has more cross-reactions with some $C_{21}O_4$ steroids than with the $C_{19}O_3$ metabolites formed from the precursors during incubation. An explanation for the increase in immunoreactive substances using 11,17-DOA EIA may be that the assay has more cross-reactions with some $C_{19}O_3$ steroids than with the $C_{21}O_4$ metabolites formed from the precursors during incubation. The increase and the decrease in the concentration of 11,17-DOA and 11-oxo-A, respectively indicated that cortisol was heavily metabolized by bacterial side chain cleavage caused most probably by desmolase activity *(Cerone-McLeron et al., 1981).* No explanation for the sex variation in the increase in 11,17-DOA concentrations (Table 1) and the decrease in 11-oxo-A concentrations (Table 2).

This finding was found to be in agreement with *Palme and Möstl (1997)* who showed that, the predominant faecal metabolites were unconjugated steroid and they could not detect immunoreactive cortisol. The same author concluded that even after intravenous infusion of 1g cortisol, no native cortisol could be detected in the faeces of sheep.

The present results was also closely related with the results of *Möstl et al. (1999)* who found an increase in immunoreactive 11,17-DOA after incubating faecal samples at room temperature which was probably caused by bacteria.

However, this findings disagreed with *Miller et al. (1991)* who described that elevated level of faecal cortisol can be measured during stress in the Rocky mountain bighorn sheep. The findings also disagreed with *Wasser et al. (2000)* who investigated three different commercially available radioimmunoassay for cortisol and one assay for corticosterone in a variety of wildlife mammals. A corticosterone antibody (ICN Biomedicals, Costa Mesa, CA) gave best results. This antibody may not only be used for radioimmunoassay but also for EIA as shown by *Goymann et al. (1999).* The cross-reactions of this antibody are still not fully evaluated. The present study concluded that cortisol is heavily metabolized by faecal flora and seems

quicker in its metabolism than corticosterone in the faeces of ruminants. In addition, the present study provide a powerful evidence that no native cortisol could be detected in the faeces of ruminants.

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Table 1. Increase (%) in 11,17-DOA concentrations (mean ± SD) after incubation of faecal samples of sheep with cortisol (1µg) for 1-24 h

	Incubation hours					
Animal	0	1	2	4	8	24
Rams	100^{Ea}	164 ± 36^{Da}	198 ± 87^{Ca}	235 ± 97^{Ba}	235 ± 75^{Bb}	277 ± 104^{Ab}
Ewes	100^{Fa}	136 ± 14^{Eb}	175 ± 33^{Db}	221 ± 97^{Cb}	274 ± 83^{Ba}	332 ± 105^{Aa}
t-value	1.55^{NS}	40.55^{**}	35.46^{**}	37.97^{**}	42.93^{**}	49.45^{**}

Capital letter: indicated that means within the same raw carrying different letter are significantly differed at (P< 0.01).
Small letter: indicated that means within the same column carrying different letter are significantly differed at (P< 0.01). NS: non significant. **: highly significant at (P< 0.01).

Table 2. Decrease (%) in 11-oxo-A concentrations (mean ± SD) after incubation of faecal samples of sheep with cortisol (1µg) for 1-24 h

	Incubation hours					
Animal	0	1	2	4	8	24
Rams	100^{Aa}	59 ± 13^{Ba}	47 ± 13^{Ca}	35 ± 17^{Db}	25 ± 13^{Ea}	20 ± 12^{Fa}
Ewes	100^{Aa}	59 ± 16^{Ba}	49 ± 15^{Ca}	40 ± 17^{Da}	16 ± 9^{Eb}	12 ± 7^{Fb}
t-value	3.24^{NS}	2.22^{NS}	5.57^{NS}	15.56^{*}	38.49^{**}	37.40^{**}

Capital letter: indicated that means within the same raw carrying different letter are significantly differed at (P< 0.01).
Small letter: indicated that means within the same column carrying different letter are significantly differed at (P< 0.01).
NS: non significant. **: highly significant at (P< 0.01). *: significant at (P< 0.05).

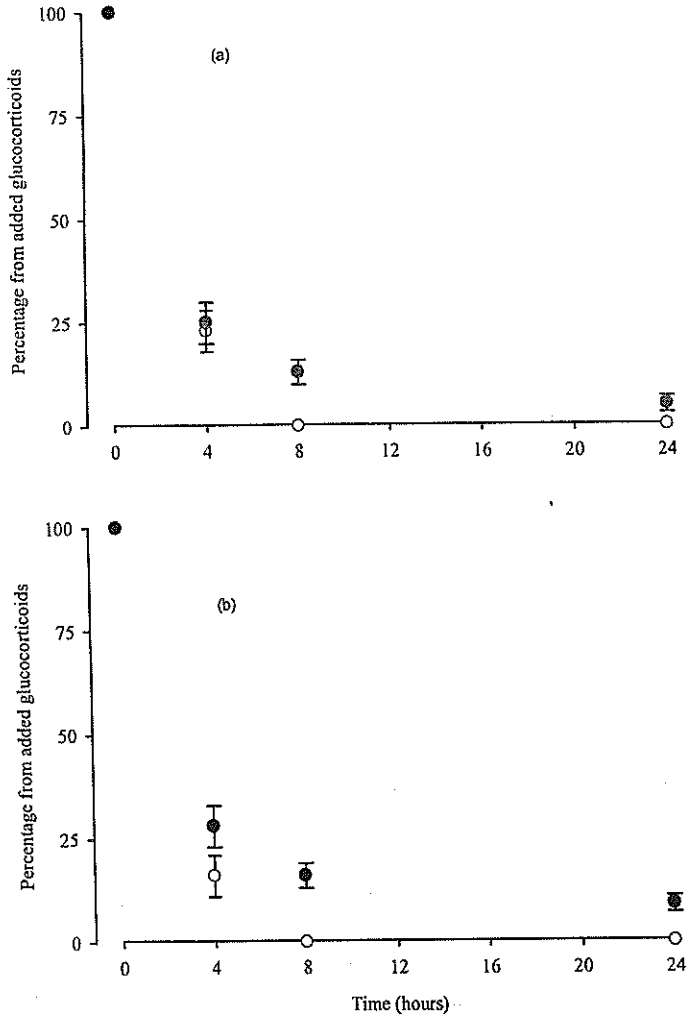

Figure 1. Metabolic changes of cortisol (1µg; □) and corticosterone (1µg; □) in fresh faecal sample of cows (a) or bulls (b; n = 5, mean ± SE)

الملخص العربى

التغيرات الأيضيه المعمليه لهرمونات الكورتيكوستيرويد فى براز المجترات

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فى هذه الدراسة تم توضيح مدى تأثيرالبرازعلى عمليات الايض المعملى للكورتيزول والكورتيكوستيرون فى براز الابقار وكذلك توضيح ما اذا كان الكورتيزول مازال يوجد فى براز الاغنام بعد فتره تحضين من 1– 24 ساعة. ولذلك تم تجميع عشرة عينات براز من 10 ابقار (خمسة اناث وخمس ذكور) وتم وزن عدد40 مرة (5 و جم براز) من كل حيوان على حدة وتم تحضينها مع محلول عيارى يحتوى على 1 ميكروجرام كوريتيزول وكذلك كورتييكوستيرون (ن = 20 لكل نوع على الترتيب) فى حمام مائى عند 38 ° م فورا وبعد 4 ساعات و8 ساعات و24 ساعة. تم تجميد العينات (–20 م ، ن= 5 لكل نوع) و تخزينها حتى وقت التحليل. بعد ذلك تم استخلاص كل العينات بواسطة الكحول الميثيلى 80% وتم عمل طرد مركزى لها وتم تحليل 10 ميكروليتر من السائل الفوقى بواسطة التحليل المناعى الأنزيمى للكورتيزول والكورتيكوستيرون. واشتملت الدراسة ايضا على تجميع عشرة عينات براز من 10 اغنام (خمسة اناث وخمس ذكور) حيث تم تحضين 5 و جم من البراز فى ثلاثيات من كل حيوان على حدة مع محلول عيارى يحتوى على 1 ميكروجرام كوريتيزول فى حمام مائى عند 38 ° م. تم وقف عمل البكتيريا بأضافة الكحول الميثيلى 80% فورا ثم بعد ساعة وبعد ساعتين وبعد 4 ساعات و8 ساعات و24 ساعة على التوالى. تم تجميد العينات (–20 م ، ن= 3 لكل حيوان) و تخزينها حتى وقت التحليل. بعد ذلك تم استخلاص كل العينات وتم عمل طرد مركزى لها وتم تحليل 10 ميكروليتر من السائل الفوقى بواسطة التحليل المناعى الأنزيمى لل–11و17 داى–اوكسواندروستان و 3–هيدروكسى–11–اوكسواندروجينز. واوضحت النتائج انه فى براز الأبقار يوجد انخفاض شديد فى تركيز الكورتيزول والكورتيكوستيرون المضاف حيث انة وجد ما يقرب من 20 % فقط من تركيز الكورتيزول والكورتيكوستيرون المضاف بعد 4 ساعات من التحضين بينما بعد 8 ساعات من التحضين لم يوجد الكورتيزول على الأطلاق بل وجد ما يقرب من 10 % فقط من تركيز الكورتيكوستيرون المضاف بينما بعد 24 ساعة من التحضين وجد ما يقرب من 5 % فقط من تركيز الكورتيكوستيرون المضاف.أما فى براز الاغنام فقد ادى تحضين البراز لفترة طويلة مع الكورتيزول الى زيادة المادة المتفاعلة مناعيا و المقاسة بواسطة التحليل المناعى الأنزيمى لل– 3–هيدروكسى–11–اوكسواندروجينز كما أدى الى انخفاض المادة المتفاعلة مناعيا و المقاسة بواسطة التحليل المناعى الأنزيمى لل– 11و17 داى–اوكسواندروستان. و مما سبق يتضح ان عمليات الايض للكورتيزول تتم بصورة مكثفة واسرع من الكورتيكوستيرون وأن الكورتيزول لم يتم تعيينه فى براز المجترات.