Wien. Tierärztl. Mschr. - Vet. Med. Austria **97** (2010), 259 - 262

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Physiological validation of a non-invasive method for measuring adrenocortical activity in goats

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received June 2, 2010
accepted for publication July 8, 2010

Keywords: goats, stress, animal welfare, faecal glucocorticoid metabolites.

Summary

Glucocorticoids can be determined as a parameter of the stress response in plasma samples, and since recently also in faecal samples. The measurement of faecal cortisol metabolites (FCM) avoids confounding effects due to the disturbance during fixation and blood collection. The aim of this study was to demonstrate that FCM can be measured to reliably and non-invasively monitor adrenocortical activity in goats. Therefore, following the morning milking, ACTH (0.5 mg) was injected into the lateral cervical muscles of 20 goats. All voided faecal samples of each individual were collected immediately before until 24 hours after injection. FCM were determined by 2 group specific 11-oxoaetiocholanolone enzyme immunoassays (EIAs). After approximately 9 hours concentrations of FCM started to increase. Peak values, which represented a ninefold increase above baseline, were reached about 13 (± 1.3) hours after ACTH administration. Afterwards, levels decreased again. However, even 24 hours after the injection, levels had not returned to baseline in all animals. The present study demonstrates that measurement of FCM by EIAs can be used to monitor adrenocortical activity in goats. This non-invasive method is a valuable tool to evaluate potentially aversive situations. It can be used in different research fields, such as ethology, livestock production, husbandry and animal welfare.

Abbreviations: ACTH = adrenocorticotrophic hormone; EIA = enzyme immunoassay; FCM = faecal cortisol metabolites

Schlüsselwörter: Ziegen, Stress, Tierschutz, Kortisolmetaboliten im Kot.

Zusammenfassung

Validierung einer nicht invasiven Methode zur Bestimmung der Nebennierenaktivität bei Ziegen

Glukokortikoide werden von der Nebennierenrinde in Belastungssituationen vermehrt ans Blut abgegeben und dort als Parameter für Stressreaktionen gemessen. Seit einiger Zeit können auch ihre Metaboliten im Kot bestimmt werden. Der Vorteil dieser nicht invasiven Methode liegt darin, dass dabei der Stress der Blutprobennahme vermieden wird. Ziel der vorliegenden Studie war es, aufzuzeigen, dass auch bei der Ziege die Messung von Kortisolmetaboliten im Kot eine geeignete Methode ist, um Stressreaktionen zu erheben. Als Versuchstiere standen 20 Bunte Deutsche Edelziegen zur Verfügung. Direkt nach dem Morgenmelken wurde den Tieren ACTH (0,5 mg) in die seitliche Halsmuskulatur appliziert. Unmittelbar vor bis 24 Stunden nach der ACTH-Verabreichung wurden von den Ziegen Kotproben nach Spontanabsatz gesammelt. Mittels zweier gruppen-spezifischer 11-Oxoätiocholanolon Enzymimmunoassays erfolgte die Bestimmung der Konzentrationen der Kortisolmetaboliten. Nach ungefähr 9 Stunden kam es zu einem Anstieg der Kortisolmetaboliten. Die maximale Konzentration wurde ca. 13 (± 1,3) Stunden nach ACTH-Applikation erreicht und lag ungefähr um das Neunfache über dem Ausgangswert. Danach sank die Konzentration wieder ab, wobei nach 24 h noch nicht bei allen Tieren das Ausgangsniveau wieder erreicht war. Mit der vorliegenden Studie wurde also gezeigt, dass auch bei Ziegen Kortisolmetaboliten im Kot messbar sind und ihre Konzentration die Nebennierenrindenaktivität widerspiegelt. Somit steht auch bei dieser Tierart ein geeignetes, nicht invasives Hilfsmittel zur Erhebung von Belastungen zur Verfügung. Verwendung könnte es in der Ethologie, in der Tierhaltung beziehungsweise im Tierschutz finden.

Introduction

During the recent years the number of dairy goats has steadily increased both in Austria and other EU-countries due to a growing demand for goat milk products. In addition, public concern about farm animal welfare has increased, visible for example in the results of the Eurobarometer and in EU commission activities (COMMISSION OF THE EUROPEAN COMMUNITIES, 2006). Only few studies exist regarding effects of different housing and management conditions on dairy goat welfare and thus research in this area is necessary. In animal welfare research different methods and parameters are used to assess stress reactions and welfare. Besides behaviour, pathology and production, also physiological parameters are widely utilised. For proper evaluation of potentially

stressful situations the combination of several parameters is recommended (e.g. DANTZER and MORMÈDE, 1983; TERLOUW et al., 1997). Numerous publications have shown that during many adverse situations glucocorticoid production is increased. Therefore plasma glucocorticoids are frequently measured as a parameter of stress (MÖSTL and PALME, 2002; MORMÈDE et al., 2007). However, (repeated) venipuncture itself is a source of stress and can interfere with the results (HOPSTER et al., 1999). For these reasons some authors have investigated other sample materials, such as saliva, milk, urine and faeces (see reviews: MÖSTL and PALME, 2002; MORMÈDE et al., 2007). Except for sampling of spontaneously voided faeces collection of all other materials mentioned above include some handling of the animals. Faecal cortisol metabolites (FCM) as a parameter of stress have already been utilized in domestic livestock including ruminants such as cattle and sheep (PALME et al., 1999, 2000; MERL et al., 2000; MÖSTL et al., 2002; TOUMA and PALME, 2005). As there is a species-specific delay in their excretion via the faeces (PALME et al., 1996), FCM reflect adrenocortical activity a certain period before the sampling. In cattle and sheep this delay time is about 12 hours (PALME et al., 1999; MÖSTL and PALME, 2002; TOUMA and PALME, 2005).

Because, even in closely related species, clear differences regarding the metabolism and excretion of glucocorticoids exist, a careful validation for each species is obligatory (PALME et al., 2005; TOUMA and PALME, 2005). As glucocorticoids are heavily metabolized and per se not present in the faeces (PALME and MÖSTL, 1997; MÖSTL et al., 2002; LEXEN et al., 2008), group specific immunoassays, which recognize several FCM are favoured for their determination (MÖSTL et al., 2005). In different ruminant species, 2 different 11-oxoaetiocholanolone enzyme immunoassays (EIA; PALME and MÖSTL, 1997; MÖSTL et al., 2002) have been used successfully (PALME et al., 1999; DEHNHARD et al., 2001; HUBER et al., 2003, TOUMA and PALME, 2005; PESENHOFER et al., 2006). The aim of this study was to demonstrate that faecal cortisol metabolites can also be measured in goats to monitor adrenocortical activity.

Materials and methods

Animals and treatment

In the present study 20 clinically healthy dairy goats were used.The goats were all of the same breed (Coloured German Improved Fawn Goat - Bunte Deutsche Edelziege) and aged between 2 and 6 years. The goats were housed at the Institute of Organic Farming, Johann Heinrich von Thünen-Institute, Federal Research Institute for Rural Areas, Forestry and Fisheries, in Trenthorst, 23847 Westerau, Germany (animal experiment number V 312-72241.123-7). During this study they were kept indoors, sharing a straw yard. They had permanent access to hay and water, and each animal received an individual portion (related to milk yield; up to a maximum of 0.5 kg) of mixed provender (40 % wheat, 35 % oats, and 25 % field beans). The animals were milked twice a day (around 6 a.m. and 4 p.m.). These goats were well accustomed to human manipulation. All goats were randomly assigned to 3 subgroups (n = 7, 7 and 6, respectively). An ACTH stimulation test was performed for each of these groups on a separate day. Immediately after the morning milking, 0.5 mg ACTH (Synacthen®, Novartis Pharma, Basel, Switzerland) were administered into the lateral cervical muscles of each animal.

Sampling and analysis of FCM

During a period of 24 hours, including one sample directly before injection, all voided faecal samples of each goat were collected immediately after defecation and the respective time of voidance was recorded. Samples were frozen immediately to avoid further metabolism by bacteria (MÖSTL et al., 2005; PALME et al., 2005; TOUMA and PALME, 2005; LEXEN et al., 2008) and stored at -20 °C until analysis, which was performed according to PALME and MÖSTL (1997). Briefly, 0.5 g of each well homogenized sample was extracted with 5 ml of methanol (80 %). After shaking (30 min) and centrifugation (2.500 g; 10 min), the amounts of cortisol metabolites in the supernatant were determined by 2 group specific 11-oxoaetiocholanolone EIAs (assay A, MÖSTL et al., 2002, measures metabolites with a 5ß-3α-hydroxy-11-oxo structure and assay B, PALME and MÖSTL, 1997, measures 11,17-dioxoandrostanes, 11,17-DOA). Details of the used 11-oxoaetiocholanolone EIAs are given elsewhere (PALME and MÖSTL, 1997; MÖSTL et al., 2002). Intra- and inter-assay coefficients of variation of a high and low level pool were between 8.1 % and 10.3 %.

Statistical analysis

All statistical analyses were performed using SigmaStat 3.1. Individual baseline FCM values were calculated as the median concentration of all samples defecated by a goat until 9 hours after ACTH injection. For statistical comparisons all faecal samples were grouped into 3 hours intervals. If more than 1 sample of an animal was present within a certain time interval, the mean concentration of FCM of these samples was calculated. Differences in FCM of the 3 h time intervals were measured with a RM ANOVA (assay A) or a Friedman RM ANOVA (assay B; normality test failed), respectively and a Tukey test to evaluate differences between specific time intervals. Correlations between FCM measured by assay A and B in a given animal were determined by running a Pearson correlation. If not otherwise stated, results are given as mean ± SD or as median plus range (from min to max), if the data were not normally distributed.

Results

Assay A measured about 2.5 times higher FCM values than assay B. However, a significant positive correlation was recorded between both assays in all 20 animals (r ranging from 0.779 to 0.995; median: 0.950; $p<0.0001$ in all cases). Baseline concentrations varied between individuals. They were 316 ± 116 ng/g and 124 ± 34 ng/g faeces for assays A and B, respectively. Levels started to increase about 9 hours and peaked 13.1 ± 1.2 or 12.9 ± 1.3 hours after ACTH injection in both assays (Fig. 1). FCM concentrations (both EIAs) were significantly higher from 9 until 21 hours after ACTH administration in comparison to the other intervals (for details see Fig. 1). Differences in FCM values between intervals were more pronounced, if analysed with assay A (see superscripts in Fig. 1). In this EIA levels between 12-15 hours were significantly higher

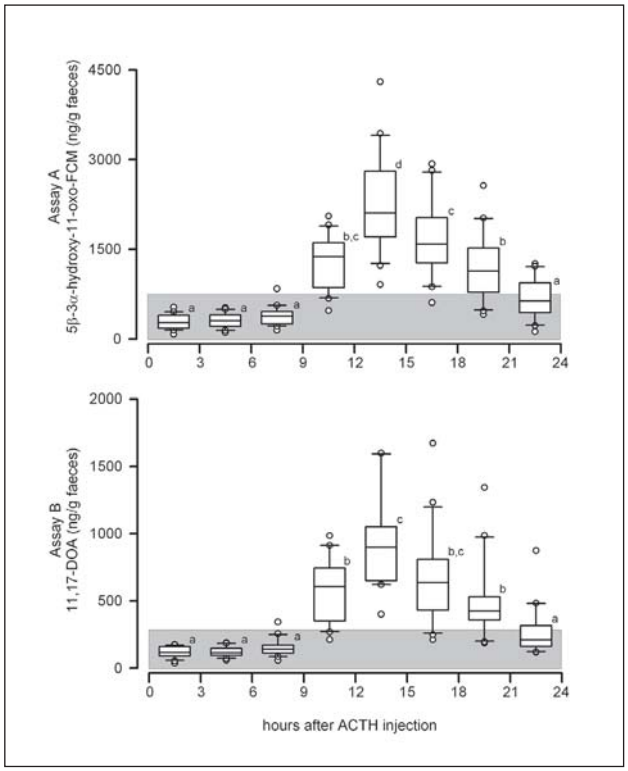

Fig. 1: Boxplots of concentrations of faecal cortisol metabolites (FCM) in female goats (n=20) measured with 2 different 11-oxoaetiocholanolone EIAs; the grey area indicates the mean ± 3 SD of baseline values. Different superscripts beside the boxes indicate significant differences ($p<0.01$ and $p<0.05$ in Assay A and B, respectively) between respective intervals.

($p<0.001$) than of all other groups. Maximum concentrations ranged from 1,191 to 4,302 ng/g (median: 2,423) and from 459 to 2,486 ng/g (median: 1,096) for assays A and B, respectively. They represented a 9-fold (5.4 to 30.5 and 5.1 to 31.6-fold, respectively) increase above baseline levels. Afterwards levels decreased again. However, even 24 hours after the injection, levels had not returned to baseline in all individuals, although FCM of the last interval were not significantly different from those of the first 3 intervals (Fig. 1).

Discussion

Monitoring adrenocortical activity by using faecal glucocorticoid metabolite analysis gives several advantages. Feces can be collected very easily, and the sampling is feedback free. This method allows the monitoring of short-term hormonal changes in reaction to specific situations, as well as assessing day-to-day changes or even long-term endocrine profiles. It has been successfully applied to various species of animals (for review see: TOUMA and PALME, 2005). In cattle and sheep, measurement of FCM has already been established as a valid, non-invasive method for evaluating challenging situations such as transport, fixation and regrouping (PALME et al., 1999, 2000; MORROW et al., 2002; MÖSTL et al., 2002; PESENHOFER et al., 2006; LEXEN et al., 2008). A physiological validation of an EIA for measuring FCM, which is achieved by an ACTH challenge test, is obligatory for each species (PALME, 2005; TOUMA and PALME, 2005).

In this study, we used 2 different 11-oxoaetiocholanolone EIAs. Individual differences in baseline levels of FCM were found with both assays, and are in accordance with findings in cattle and sheep (PALME et al., 1999). Assay A (MÖSTL et al., 2002) yielded higher values than assay B (PALME and MÖSTL, 1997), but the concentrations were well correlated. Both EIAs utilize the same standard (11-oxoaetiocholanolone). However, the antibodies used are directed against different parts of the steroid. Thus they recognize different groups of metabolites (5ß-3α-hydroxy-11-oxo steroids versus 11,17-DOA). Therefore, the higher values measured by assay A reflect a higher amount or degree of crossreacting FCM present in goat faeces. A similar situation was also described in cattle (MÖSTL et al., 2002) and sheep (LEXEN et al., 2008). Higher amounts of measured FCM allow for a higher dilution of the supernatants following extraction. This attenuates interfering substances and thus reduces possible matrix effects (MÖSTL et al., 2005).

The time delay between peak concentrations in blood glucocorticoids and their related metabolites in the faeces resembles the gut passage time (duodenum to rectum) in a given species (PALME et al., 1996, 2005). During their experiments with cattle and sheep, PALME et al. (1999) measured peak levels around 12 hours after i.v. injection of ACTH. After administration of ACTH (i.m.) in roe deer (DEHNHARD et al., 2001) and red deer (HUBER et al., 2003) maximum concentrations were reached earlier (6-9 hours) or later (about 18 hours), respectively. In this study, peak concentrations of FCM were measured between 11.3 and 15 hours (mean: 13 h) after ACTH injection with both EIAs, although differences in FCM between intervals (especially around peak concentrations) were more expressed with assay A. Therefore, in goats, faecal samples should be collected around 12 - 15 hours after an acute stressor for its monitoring. Peak concentrations were not always measured in exactly the same sample with both assays. LEXEN et al. (2008) found in sheep that the pattern of excreted FCM changed over time with a trend towards more polar metabolites. This, in connection with the above mentioned differences in antibody characteris-

tics of the 2 EIAs, possibly accounts for the differences in peak samples. Increases in FCM levels above baseline (medians of about 900 %) after ACTH stimulation were similar in both EIAs, and within the range reported for other ruminants (PALME et al., 1999; HUBER et al., 2003). In other animals, such as male elephants and Mountain hares, only one of both 11-oxoaetiocholanolone EIAs (assay A) was able to reflect adrenocortical activity adequately (GANSWINDT et al., 2003; REHNUS et al., 2009). In contrast, in goats both EIAs turned out to be comparably suited.

Taken together, our study demonstrates that it is possible to measure the concentration of cortisol metabolites in the faeces of goats in order to monitor adrenocortical activity and thus stress. Due to more pronounced differences in FCM values between intervals (especially the 12 to 15 hours one) and higher levels of FCM measured, assay A may be favoured. This non-invasive method can be used in goats as a valuable tool to evaluate potentially stressful situations in different research fields, such as ethology, livestock production, husbandry and animal welfare.

Acknowledgments

The study was funded by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management and the Federal Ministry for Health (Project: 100191).

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