

Urinary corticosteroids: an indicator of stress in dairy cattle

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ABSTRACT

The measurement of immunoreactive urinary corticosteroids was validated as a method of monitoring adrenocortical activity in lactating dairy cows. The physiological relevance of the technique was validated by demonstrating that (i) injection of ACTH led to a significant increase in plasma and urinary corticosteroid concentrations, and (ii) the translocation of cows from pasture to a barn facility resulted in a 1.1- to 2.5-fold increase in urinary cortisol concentrations. Plasma cortisol concentrations exhibited a 15- to 33-fold increase above basal concentrations 30 mins after ACTH administration (2.98 ± 0.35 vs 63.7 ± 3.9 ng/ml plasma; mean \pm SEM; $P < 0.001$) and remained elevated for 4.0-4.5h. Urinary corticosteroid concentrations paralleled plasma cortisol with a 1- to 2-h temporal lag and exhibited a 5.6- to 13-fold increase at 120 mins after ACTH injection (130 ± 9.9 vs 1149 ± 159 ng/mg creatinine; mean \pm SEM; $P < 0.01$). Monitoring urinary corticosteroid concentrations is a valid tool for studying and evaluating adrenal activity and acute stress in cattle.

Keywords: dairy cows; urine; cortisol; ACTH; stress.

INTRODUCTION

Due to increasing public awareness of animal welfare, there is a need to quantify potentially stressful conditions on New Zealand farms. Stress measurements need to be sufficiently benign so as not to influence the endocrine response they are intended to measure. In mammals undergoing stress, glucocorticoids (e.g., cortisol) are secreted by the adrenal cortex and excretory metabolic products can be detected in the urine and faeces. Historically, plasma cortisol has been used as an indicator of stress (Broom and Johnston, 1993). However, measurement of plasma cortisol concentrations only provides a single point-in-time estimate and the handling and restraint necessary for frequent blood sample collection may themselves act as stressors, increasing plasma cortisol concentrations (Willett and Erb, 1972; Alam and Dobson, 1986). In contrast, measuring urinary steroid excretion has the advantage of providing an integrated index of cortisol production over the period preceding sampling, with the data being less influenced by the natural episodic nature of hormone secretions (Lasley, 1985) or animal handling.

Urinary corticosteroid metabolite concentrations reflect circulating cortisol concentration that enters the urine pool via glomerular filtration by the kidneys (Biesel *et al.*, 1964). Increased urinary corticosteroids in response to an ACTH or CRH challenge have been measured in bighorn sheep (Miller *et al.*, 1991a), cats (Carlstead *et al.*, 1992), cattle (Redbo, 1993) and pigs (Hay *et al.*, 2000). Increased urinary corticosteroids have also been recorded in response to psychological stressors (Berman *et al.*, 1980; Carlstead *et al.*, 1992; Redbo, 1993) and disease (Miller *et al.*, 1991b; Schelcher *et al.*, 1999). Urinary cortisol testing is frequently used in post-racing samples from horses suspected of doping (Popot *et al.*, 1997) and to diagnose hyperadrenocorticism in dogs (Feldman, 1983).

For a metabolite excretion profile to accurately reflect a physiological event, corresponding changes in circulating hormones and the respective excreted metabolites must be established and verified. With respect to cortisol, this can

be achieved by artificially elevating circulating plasma cortisol concentrations by administering ACTH or by inducing physiological and/or psychological stress (e.g., transport, restraint, translocation, confinement).

The objectives of the experiments were to (1) validate an enzyme immunoassay (EIA) to measure immunoreactive corticosteroids in urine from dairy cows and (2) assess the biological relevance of measuring urinary corticosteroids by stimulating cortisol release from the adrenal cortex.

MATERIALS AND METHODS

All experiments were conducted at the No. 5 Dairy of the Dairying Research Corporation Ltd with the approval of the Ruakura Animal Ethics Committee. Two experiments that would result in increased adrenal activity were conducted - ACTH administration (Experiment 1) and subjecting the cows to different housing environments (Experiment 2).

Experiment 1

Animals

Five multiparous lactating Friesian cows (542 ± 14.9 kg; mean \pm SEM live weight) were housed in a semi-enclosed barn facility (consisting of outdoor individual yards allowing free movement) for a 5-day period. Cows were offered fresh-cut ryegrass/white clover pasture and fresh water *ad libitum*.

Treatment

On Day 2 each cow was fitted with an indwelling jugular catheter and treated prophylactically with antibiotics (1mg/kg Excenel, Upjohn, Auckland, New Zealand). On Day 3, the cows received two injections of ACTH₍₁₋₂₄₎ (0.05 mg i.v.; Synacthen, Novartis Pharma AG, Basle, Switzerland) administered via the catheter at a 0900 and 1100 h, using a protocol that reliably elevates plasma cortisol concentrations for 4-6 hours (Verkerk *et al.*, 1994; Stelwagen *et al.*, 1998). Catheters were removed on Day 4 and the cows returned to pasture on Day 5.

Blood samples

Pre-treatment blood samples were collected at 0900 h on Days 1 and 2 by tail venipuncture. A 6.5-h bleed was conducted on each cow on Day 3. Samples (10 ml) were collected every 30 minutes between 0830 and 1500 h with ACTH administration occurring immediately after the 0900 and 1100 h blood samples had been withdrawn. Additional samples were collected 12, 24 and 48 h after the first ACTH injection. Blood samples were collected into heparinised tubes, centrifuged and the plasma stored at -20°C until analysis for cortisol concentrations.

Urine samples

Urine samples (10 ml) were collected by perineal stimulation on Days 1-2 (pre ACTH treatment), at 0830 h on Day 3 and approximately every hour for 6 h after the initial ACTH injection on Day 3 (0900-1500 h). Additional samples were collected at 12, 24 and 48 h after the first ACTH injection. Urine samples were centrifuged and stored -20°C until analysis for corticosteroid and creatinine concentrations. The times of spontaneous urinations were recorded for each cow during a 24-h period beginning 0615 h on Day 4.

Experiment 2

Four of the five cows utilised in Experiment 1 were subjected to two housing environments; the semi-enclosed barn facility (as in Expt 1), and individual indoor stalls where movement was constrained by a tether. Following removal from pasture on Day 1, the cows were fed cut pasture in the barn facility on Days 2-7 and 16-18, and in indoor stalls on Days 8-15. Daily urine samples were collected by perineal stimulation or from the bulk daily collection while in the indoor stalls.

Cortisol analysis

Plasma cortisol concentrations were measured in duplicate using a commercially available I^{125} radioimmunoassay kit (Coat-a-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA, USA).

Urine corticosteroid concentrations (UCC) were measured using a competitive cortisol enzyme immunoassay (EIA) developed by Munro and Stabenfeldt (1985). The assay was validated for bovine urine by demonstrating: (i) parallelism between serial dilutions of bovine urine and the standard curve; and (ii) recovery of exogenous cortisol added to bovine urine. Urine samples were diluted 1:20 (Expt 1) or 1:10 (Expt 2) in EIA phosphate buffer (phosphate-buffered saline, 0.1% BSA; pH 7.0) and analysed in duplicate. Standards consisted of purified cortisol in absolute ethanol, pipetted to yield 5, 10, 25, 50, 100, 250, 500 and 1000 pg/well (50 μl). The antibody (R4866, raised in rabbit against cortisol-3-carboxymethylloxime:BSA) cross reacted with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, compound S 6.2%, cortisone 5.0%, corticosterone 0.7% and dexamethasone 0.3%. A 1:9,000 antiserum solution of 0.05M Na_2CO_3 - NaHCO_3 (pH 9.6) was pipetted (50 μl /well) into microtitre plates, incubated overnight at 4°C and discarded. Plates were washed five times in normal saline with 0.5% Tween 20 (BDH), 50 μl of sample, standard or

control was incubated with 50 μl of cortisol-horseradish peroxidase conjugate (CHRP) at 1:30,000 and incubated at room temperature for 60 minutes. The mixture was discarded and plates washed as before. A substrate that consisted of 0.4mM ABTS (Sigma) and 1.6mM H_2O_2 in 0.05M citric acid (pH 4.0) was added (100 μl /well) and incubated 25-30 minutes at room temperature. Plates were read on a Biorad 3550 Microplate reader at 405 nm (reference 570 nm). The inter- and intra-assay coefficients of variation for bovine urine control samples were 9.4 and 8.7%, respectively.

The creatinine concentration of each urine sample (1:10 in saline) was determined using a Hitachi 717 autoanalyser using the Roche Diagnostics test (Jaffe method; Roche Package insert, 1997). To control for variations in urine volume and concentration, hormone concentrations were corrected for creatinine concentrations. Accordingly, urine corticosteroid concentrations are expressed as ng cortisol/mg creatinine.

DATA ANALYSIS

Results are reported as mean \pm SEM. In Expt 1, mean basal corticosteroid concentrations were calculated from morning samples on Days 1, 2, 4, 5. The total adrenal response was calculated as the area under the curve for the 6-h period following the first ACTH injection and adjusted for basal corticosteroid concentrations. Hormone concentrations were compared using Students t-test for paired means.

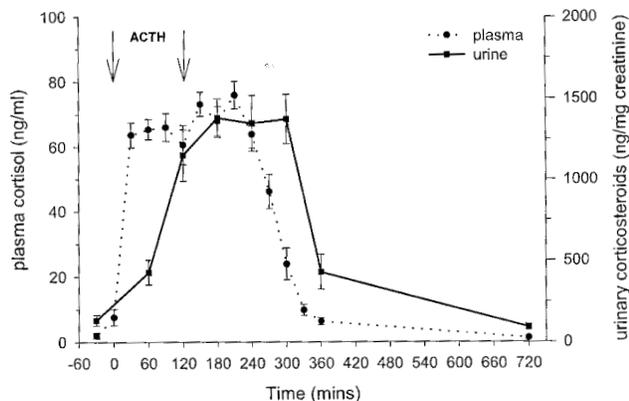
RESULTS

Binding inhibition curves of serially diluted bovine urine (1:8–1:1024) were parallel to the standard curve. Recovery of exogenous cortisol (5–500 pg) added to bovine urine was $y=1.04x-31.9$ ($r=0.99$; $P<0.001$). Assay sensitivity was 5 pg/well. Urinary creatinine concentrations ranged from 0.1 to 0.7 mg/ml urine.

Expt 1: Basal plasma cortisol concentrations were 3.0 ± 0.4 ng/ml. At the first sample, 30 mins after ACTH injection, plasma cortisol concentrations exhibited a 15.1- to 33.3-fold increase above basal concentrations (63.7 ± 3.9 ng/ml; $P<0.001$, Figure 1). Plasma cortisol concentrations were elevated for 240-270 mins after the initial ACTH injection and exhibited a bi-phasic response curve (Figure 1). Maximum plasma cortisol concentrations (77.8 ± 3.3 ng/ml) were recorded 150-210 mins after the initial ACTH injection (30-60 mins after the second injection). The integrated plasma cortisol response to ACTH injection was 587.2 ng.h.ml $^{-1}$. Plasma cortisol concentrations had returned to basal levels at the end of the 6.5-h sampling session.

Basal UCC were 130 ± 9.9 ng/mg creatinine in the barn facility. Following ACTH administration, UCC showed a similar pattern of change to plasma cortisol, with a temporal lag of about 1 h (Figure 1). Urinary corticosteroid concentrations exhibited a 1.5- to 3.9-fold increase above basal concentrations at the first sample 1 h after ACTH (427 ± 75 ng/mg creatinine; $P<0.06$) and a 5.6- to 12.9-fold increase by the second sample at 2 h (1149 ± 159 ng/mg creatinine; $P<0.01$; Figure 1). The total urinary corticosteroid response to ACTH injection was 5325

FIGURE 1. Mean (\pm SEM) plasma cortisol and urinary corticosteroid concentrations in five lactating dairy cows following the administration of ACTH at time 0 and 120 (indicated by arrows).



ng.h.mg⁻¹creatinine. The UCC had declined by 6 h and returned to basal concentrations 12 h after the initial ACTH injection (Figure 1). The median number of urinations recorded for each cow in the 24-h period beginning at 0615 h on Day 4 was 9 (range 8-12; 9.6 \pm 0.8, mean \pm SEM).

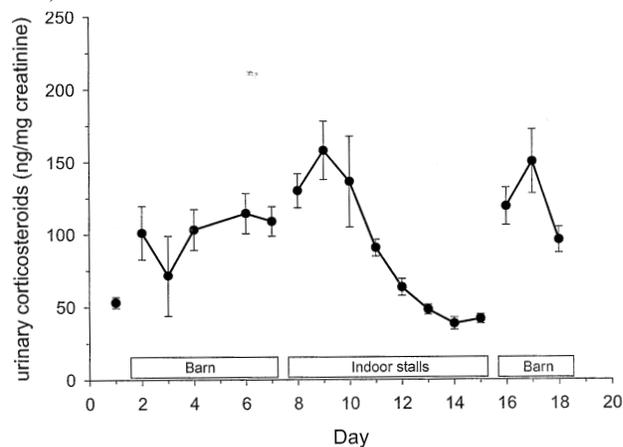
Expt 2: The mean (\pm SEM) UCC graph for four cows exposed to changes in housing environments is presented in Figure 2. Mean pre-treatment UCC was 53 \pm 3.8 ng/mg creatinine. On the day after the cows were moved to the barn facility, UCC had increased 1.1- to 2.4-fold (101 \pm 19 ng/mg creatinine; P <0.06). In general, UCC fluctuated but remained elevated during the first 5-d period in the barn. The mean UCC during this time was similar to that calculated for the same four cows in the barn during the ACTH study (105 \pm 9.3 vs. 122 \pm 8.7 ng/mg creatinine; P >0.1). The UCC increased 2.0- to 3.1-fold above pre-treatment values after the move to the indoor individual stalls on Day 7 (130 \pm 12 ng/mg creatinine; P <0.01). Urinary corticosteroid concentrations remained elevated for the first 3 d in the indoor stalls (141 \pm 19 ng/mg creatinine) and declined significantly by 32% to pre-treatment concentrations during the final 3 d indoors (43 \pm 2.7 ng/mg creatinine; P <0.05). The UCC exhibited a 1.6- to 3.5-fold increase as the cows were moved from the indoor stalls to the barn facility for the second period (P <0.05).

DISCUSSION

The study has established that the measurement of immunoreactive corticosteroids in urine is a valid method of monitoring adrenocortical activity in the dairy cow. Tests for parallelism and quantitative recovery of cortisol in cattle urine indicated that the cortisol EIA reliably measures corticosteroids in bovine urine. Elevated levels of urinary corticosteroids were detected after administration of ACTH and a psychological stressor (exposing the cows to changes in housing environment).

The range of plasma cortisol concentrations and the duration of the cortisol response following the ACTH i.v. injection in this study are in agreement with previously reported values in dairy (Verkerk *et al.*, 1994; Stelwagen *et al.*, 1998) and Brahman (Lay *et al.*, 1996) cattle. The biphasic response curve reflected the 2-h interval between ACTH injections. A parallel relationship between plasma

FIGURE 2. Mean (\pm SEM) urinary corticosteroid concentrations for four lactating dairy cows at pasture (Day 1) and following the move to an outdoor barn facility (Days 2-7, 16-18) or individual indoor stalls (Days 8-15).



cortisol and UCC in response to i.v. ACTH administration was evident with an estimated temporal lag of 1 h. The time lag represents the time taken for cortisol to be cleared from circulation, but is limited in this study by the intervals between urine sampling. Nevertheless, it is similar to lag times reported for bighorn sheep (2 h; Miller *et al.*, 1991a), cats (2 h; Carlstead *et al.*, 1992) and pigs (2-3 h; Hay *et al.*, 2000) following ACTH or CRH challenge. A higher frequency of urine collection via a urinary catheter would be necessary to precisely determine the temporal lag in dairy cattle.

Exposing cows to a change in environment consistently produced UCC 1.1- to 3.1-fold higher than pre-treatment values and elevated UCC for 3-4 d following each change, indicating an adrenocortical stress response. The decline in UCC observed over the final 3-4 d while in the indoor stalls is similar to results reported by Redbo (1993) for heifers transferred from pasture to indoor stanchions where UCC were high the day after tethering but had declined by 50% a week later. Similarly, confined lambs exposed to a daily cold shock (3 h at -5°C) exhibited increased UCC during the 3-6 h after cold exposure on Day 1, which declined by Day 3 (Berman *et al.*, 1980). The observed decline to normal UCC 3-7 d after an imposed stressor suggests a compensatory decrease in cortisol production as the animal adapted to the new situation. Similar declines in plasma cortisol in response to tethering and restraint have been reported for cattle previously (Ladewig and Smidt, 1980; Echterkamp, 1984).

Measuring immunoreactive corticosteroids excreted in the urine represents an alternative approach to studying and evaluating short- to medium-term duration stress in cattle. It offers several advantages, particularly the ease of collection and minimal requirement for sample preparation. Perhaps more importantly, it provides an integrated value of cortisol excretion over the period preceding sampling, which is not as susceptible to the immediate effects of handling and restraint.

Preferably, the assessment of stress would be based on a combination of biochemical, haematological and behavioural variables rather than a single measurement such as plasma or urinary cortisol. We have demonstrated that UCC assessment is a useful tool for monitoring adrenal

activity in dairy cattle. In future research, UCC will be used in conjunction with other indicators to quantify the responses of dairy cattle to management practices and to assess the effectiveness of remedial actions in welfare issues.

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