Quantification of cortisol, cortisol immunoreactive metabolites, and immunoglobulin A in serum, saliva, urine, and feces for noninvasive assessment of stress in reindeer

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Abstract

This study was designed to develop reliable methods for quantification of cortisol and cortisol immunoreactive metabolites (C-CIM) and immunoglobulin A (IgA) in reindeer serum, saliva, urine, and feces as tools for the objective noninvasive assessment of well-being and immunocompetence in reindeer. Although C-CIM was readily quantifiable by radioimmunoassay in serum, urine, and feces, the levels in saliva samples were low, rendering quantification unreliable. Whereas IgA concentrations were high in feces samples, they were much lower, albeit quantifiable, in serum and urine; the levels in saliva samples were too low for quantification with the use of an enzyme-linked immunosorbent assay that we developed. Further studies are in progress to validate the usefulness of fecal levels of C-CIM and IgA in the assessment of welfare in reindeer.

Résumé

Le but de la présente étude était de développer des méthodes fiables pour quantifier le cortisol et les métabolites immunoréactifs du cortisol (C-CIM) ainsi que les immunoglobulines A (IgA) dans le sérum, la salive, l'urine et les fèces des rennes afin d'avoir des outils permettant une évaluation objective et non-invasive du bien-être et de l'immunocompétence des rennes. Bien que les niveaux de C-CIM dans le sérum, l'urine et les fèces étaient facilement quantifiables par radio-immunoessais ceux dans la salive étaient faibles, rendant la quantification non fiable. Alors que les IgA étaient présentes à des concentrations élevées dans les fèces, elles étaient présentes en quantités beaucoup plus faibles, bien que quantifiables, dans le sérum et l'urine, alors que dans la salive les niveaux étaient trop faibles pour être quantifiables à l'aide du test immuno-enzymatique développé. Des études supplémentaires sont en cours pour valider l'utilité de déterminer les niveaux de C-CIM et IgA dans les fèces pour évaluer le bien-être des rennes.

(Traduit par Docteur Serge Messier)

Herding of reindeer (*Rangifer tarandus tarandus* L.) is a socioeconomically important activity for people living in the northern part of the Nordic countries. During the past 40 years, the traditional intensive methods of herding relatively tame animals have been replaced by extensive methods involving motor vehicles such as snowmobiles and motorcycles, as well as helicopters for herding almost wild animals (1). New directives concerning hygienic standards in Sweden have resulted in the closing of small local abattoirs and transport of the reindeer by truck over long distances (sometimes more than 1000 km) for slaughter.

The quality of reindeer meat deteriorates after stress (2). Some handling methods, such as the traditional lasso technique for sorting, cause considerable physical and mental stress, leading to depletion of muscle glycogen (3). The increasing stress associated with herding, corralling, and physical restraint of less and less tame animals results in lesions and elevated blood cortisol concentrations (4). With increased concern about compromised well-being of the reindeer (5), there is a need for objective measures of stress and animal welfare.

Blood samples from reindeer fitted with automatic blood sampling devices have demonstrated that restraint causes a significant increase in levels of circulating cortisol (6). Because blood sampling in a herd is difficult and stressful to the animals, measurement of the fecal glucocorticosteroid concentration may be useful in the noninvasive assessment of recent stress (4 to 12 h before collection of the fecal sample). Assays for quantification of fecal corticosteroids have been developed for a number of species, including bighorn sheep (7) and roe deer (8).

Several studies in humans have suggested a correlation between stress and below-normal levels of secretory immunoglobulin A (IgA) in saliva (9,10). In the dog, a negative correlation was observed between salivary cortisol and IgA levels (11). We developed an enzyme-linked immunosorbent assay (ELISA) for quantification of IgA in rat feces (12) and found significant correlations between levels of corticosterone and IgA in individual samples and the respective total amounts secreted by the animal per time unit per kilogram of body weight (13), as well as a negative correlation between fecal corticosterone excretion and fecal IgA excretion (14).

The purpose of the present study was to develop reliable methods for quantification of cortisol and cortisol immunoreactive metabolites (C-CIM) and IgA in reindeer feces, urine, and saliva, with the aim of

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Received July 26, 2004. Accepted June 21, 2005.

Table I. Concentrations of cortisol and cortisol immunoreactive metabolites (C-CIM) and immunoglobulin A (IgA) in samples from reindeer

				C-CIM				IgA			
				Feces (nmol/kg dry wt)	Urine (nmol/L)	Serum (nmol/L)	Saliva (nmol/L)	Feces (mg/kg dry wt)	Urine (mg/L)	Serum (mg/L)	Saliva (mg/L)
1	M	0.5	21.6	ND	48	66	< 10	191	26	< std.	543
2	M	0.5	ND	2.84	ND	116	< 10	72	98	65	< std.
3	M	0.5	24.0	0	136	100	< 10	41	1785	< std.	< std.
4	M	0.5	23.3	ND	75	46	< 10	8	250	81	< std.
5	M	0.5	20.7	0	ND	73	< 10	16	< std.	8	< std.
6	M	0.5	22.6	0	96	115	< 10	14	61	120	< std.
7	F	Older	32.2	0.28	178	131	< 10	294	1744	30	< std.
8	M	3.5	48.6	0.82	95	33	< 10	4	3550	< std.	< std.
9	F	Older	29.6	1.89	78	159	< 10	1	984	171	< std.
10	M	2.5	38.1	0.75	97	101	< 10	1	529	44	< std.
11	M	2.5	35.8	1.68	93	128	< 10	1	206	30	< std.
12	M	2.5	ND	1.12	86	71	< 10	6	492	167	< std.

ND — not determined; < 10 — value uncertain but between the 2 lowest standards in the enzyme-linked immunosorbent assay; < std. — less than the lowest standard

developing tools that would allow objective noninvasive assessment of reindeer well-being.

Biologic materials were obtained from 12 reindeer (6 males aged 6 mo, 3 males aged 2.5 y, 1 male aged 3.5 y, and 2 older females immediately after slaughter at the abattoir in Övre Soppero, Sweden. Samples of feces were collected from the rectum, urine from the bladder, and saliva from the oral cavity. Blood was collected from the heart; the serum was separated by centrifugation. All samples were frozen immediately at −20°C and later transported to the laboratory in a polystyrene box with dry ice for immediate processing.

The frozen feces samples were thawed and dried at 30°C for up to 5 h, then placed in a mortar and ground into a powder. The powder was diluted 1:4 (w:w) with distilled water and the mixture left to swell for 1 h, after which it was homogenized with a homogenizer. A mixture of 5 g of the homogenate and CH_2Cl_2 (1:3.5 [w:v]) was vortexed for 30 s in 5-s periods and then centrifuged for 20 min (1690 × *g*) at 4°C; the tubes were kept at this temperature until the extract was subjected to evaporation. The water phase at the top of the tube was removed, and a hole was made in the layer of feces covering the CH_2Cl_2 phase. The CH_2Cl_2 phase was transferred to a clean glass tube. The extract was washed once with 0.1 M NaCl (1 mL per 5-mL extract) and then twice with similar amounts of distilled water. After each washing, the tube was vortexed for 10 s and then centrifuged for 5 min (1690 × *g*) at 4°C, and then the aqueous phase was removed. After the washing process, the extract (organic solvent phase) was transferred to glass tubes in 1.5-mL aliquots and subjected to evaporation under N_2 gas. The material remaining after complete evaporation of the CH_2Cl_2 was a greenish algae-like powder. The powder was diluted in Tris-HCl buffer and the cortisol concentration measured by a quantitative commercial radioimmunoassay (RIA) (Spectria batch CM 133; Orion Diagnostica, Espoo, Finland) according to the manufacturer's instructions. Radioactivity was measured in a gamma counter (LKB Wallace, Uppsala, Sweden) for 3 min per sample. Deltasoft 3 software (BioMetallics, Princeton, New Jersey, USA) was used to calculate the C-CIM concentration in the samples and standards. The linear range in the RIA standard curve was 30 to 500 nmol/L. The cortisol concentration in the urine, serum, and saliva samples was also measured with this assay. All samples and standards were tested in duplicate. The intra-assay coefficient of variation was less than 5% and the interassay coefficient of variation less than 8%.

For quantification of fecal IgA, the frozen feces samples were thawed and dried at 35°C for 2 to 3 h, then were placed in a tube in which the material was split into small particles and then diluted 1:20 (w:v) with phosphate-buffered saline (0.1% Tween, pH 7.2). During the next hour, the mixture was vigorously vortexed 5 times and then centrifuged at 1600 × *g* for 25 min. The supernatant was transferred to a new tube and centrifuged at 5800 × *g* for 10 min. The final supernatant was used in the IgA ELISA as described previously (12), with goat antiserum to rat IgA (Fc)/7S (Nordic, Tilburg, The Netherlands) as the primary antibody. This antibody preparation reacts with reindeer IgA, and crossed tandem immunoelectrophoresis has demonstrated complete immunochemical cross-reaction between rat IgA and reindeer IgA (15). Antibodies against proteins of one species (the human) can be used to quantify homologous molecules in other species (16).

For standards, rat IgA purified from ascites induced by IR22 myeloma cells (ICN Biomedicals, Aurora, Ohio, USA) was diluted to 10 concentrations (2.7 to 84 ng/mL). For internal assay controls, we used 13 samples of reindeer urine diluted 1:30 000, 1:70 000, and 1:270 000 and 10 samples of normal rat serum diluted 1:1000, 1:5000, and 1:12 000. We quantified IgA in saliva, serum, and urine using the same ELISA. All samples and standards were tested in duplicate. The intra-assay coefficient of variation was 3.0% and the interassay coefficient of variation 5.8%.

In the reindeer feces, urine, and serum, C-CIM was readily quantifiable by RIA (Table I). The levels of C-CIM in the saliva samples, however, were low (between the 2 lowest standards); thus,

quantification was unreliable. Whereas IgA concentrations were high in the feces samples, they were much lower, albeit quantifiable, in serum and urine (Table I); the levels in saliva samples were too low for quantification.

The high serum cortisol levels, compared with those recorded in previous studies of stress in reindeer (5,6), indicate that the reindeer in this study were stressed at the time of slaughter.

There was no significant negative correlation between the C-CIM and IgA levels in the different biologic materials, which is perhaps not surprising considering that material from only 12 animals was used for development of the assays and that the animals had been subjected to transportation stress before slaughter. From studies in the rat, fecal IgA seems to be a marker of stress of longer duration, and a negative correlation between fecal corticosterone and IgA levels disappeared after acute surgical stress (14). In the reindeer studied, the parasite burden, which may affect the amount of IgA excreted in the feces, was not known. The samples were obtained during the winter, and since reindeer metabolism is basically catabolic during autumn and winter and anabolic during spring and summer (17), the fecal concentrations of cortisol and IgA may vary with the season, as demonstrated for cortisol and a number of hormones in the plasma of Alaskan reindeer (18).

Glucocorticoid levels in body fluids are often useful indicators of acute stress but not chronic stress, since elevated levels may return to normal or nearly normal when a stressor persists. Blood sampling usually necessitates capture and restraint of the reindeer, resulting in increased plasma levels of cortisol (5,6). Fecal sampling may represent an opportunity for nonstressful, noninvasive quantification of corticosteroids as well as IgA, both of which may be relevant for assessment of reindeer welfare.

During large parts of the year, reindeer live in a climate characterized by below-freezing temperatures, which means that fecal droppings will be frozen and preserved immediately. From a practical point of view, this might be very helpful, in that appropriate storage temperatures for samples are automatically provided in the animals' natural habitat. This may minimize the problem of bacterial metabolism of fecal steroids (19) and make comparisons between herds or within a herd over time feasible, because samples that are several days or weeks old could be collected and analyzed.

Recent studies in rats have demonstrated a linear correlation between the concentrations of corticosterone and IgA in feces and the amounts excreted in feces per kilogram of body weight per hour (13). This correlation may be important when single feces samples are used in future studies. However, in individual rats, the variation between fecal pellets in IgA concentration may be as high as 41% (13), indicating that minor concentration changes in a fecal marker may be of little biologic relevance.

In reindeer, how well levels of C-CIM and IgA in single feces samples correlate with the total amounts excreted per day remains to be studied. This information will be needed to validate the usefulness of single feces samples for reliable quantification of secreted amounts of C-CIM and IgA per unit of time. Further studies are in progress to validate the assistance of fecal levels of C-CIM and IgA in the assessment of reindeer welfare.

Acknowledgments

We appreciated the skillful laboratory assistance of Emma Andersson.

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