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REGULAR ARTICLE

Effects of storage temperature and time on fecal progesterone concentration in camel (*Camelus dromedarius*)

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Abstract

This study aimed to evaluate the effect of temperature (-20°C, + 4°C, room temperature) and storage time (7, 15, 107 and 173 days) of feces on progesterone concentration in camel (*Camelus dromedarius*). Plasma and feces were collected from 10 pregnant and one non-pregnant camels. Extraction of fecal metabolites of progesterone was performed with methanol and petroleum ether. The analytical validation was provided by internal quality control (IQC) and the success of the validation tests (sensitivity, precision, recovery and parallelism). In comparison to the value found in the day of collection, the mean concentration of progesterone in feces which was frozen or dried at room temperature showed no significant difference after 7 and 15 days. A significant increase was found for fecal samples stored at + 4°C. After 107 and 173 days, freezing is inadequate condition of storage because the fecal progestagen concentrations vary significantly. However, drying feces at ambient temperature maintained stable progestagen concentrations. Therefore, results indicated that drying feces is a reliable method, independent from an electric power source and the freezing equipment.

Key words: Camel, Feces, Progesterone metabolites, RIA, Storage

Introduction

Sexual hormone measurements traditionally involve invasive techniques such as blood collecting. This technique is stressful for animal and requiring a strict conservation of samples. As an alternative, the measure of steroid metabolites in feces is widely suggested to monitor reproductive hormones in wildlife species (Graham, 2004; Freeman et al., 2010) as well as in domestic animals (Cebulj-Kadunc et al., 2000; Kornmatitsuk et al., 2007). It is a non-invasive method, since it avoids the stress effects related to blood sampling and fecal samples are easily collected and stored. However, the reliability of the results based on a rigorous analytical and physiological valiadation for each species is primordial (Capezzuto et al., 2008).

The storage of fecal samples is a critical concern because fecal bacteria metabolize fecal steroids within hours after deposit. For this reason, several studies were made to control for any variation due to storage procedures, which to avoid misinterpretation of the results.

In order to minimize degradation of the fecal steroid, many authors were recommended to store fecal samples at -20°C until analysis (Whitten et al., 1998; Mostl et al., 2005). Other studies showed that ethanol has been used as a preservative for short-term ambient temperature storage of fecal samples (Khan et al., 2002; Lynch et al., 2003). Furthermore, Terio et al. (2002) suggested that storage of fecal samples at room temperature in ethanol was the best alternative to freezing for subsequent analysis of steroid hormone concentrations because it stabilized the concentrations of metabolites for progestogen and estrogen in cheetah for a period of up to 2 weeks. Other ambient temperature field storage techniques included drying samples. In this case, Pettitt et al. (2007) showed that drying feces provides a reliable method for long-term preservation of fecal steroid concentrations and was optimal alternative when freezing was not a viable option.

The present study was designed to compare different methods of preserving (frozen at -20°C, stored at + 4°C, dried and stored at ambient temperature) fecal samples over several time periods (after 7, 15, 107, 173 days) in order to determine which method provided the most accurate and reliable technique for measuring fecal progesterone in camel.

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Materials and Methods

Animals and samples collection

Ten adults, fertile and pregnant camels were used. They weighed 502.4 ± 57.4 kg and were 13.2 ± 3.8 years old. Another non pregnant camel (#814) was used in the test of accuracy. They were moved during 6 to 7 hours grazing salty species. Animals had access to water once a day.

Blood and feces samples were collected in the morning for one day. The blood samples (5 ml) were withdrawn from the jugular vein into heparinized tubes, centrifuged immediately for 30 min, and the plasma were stored at -20°C until assayed. Fecal samples were collected directly from the rectum. They were taken in plastic bags clearly identified (number of camel, collection day).

Fecal sample processing

Storage conditions of feces

The initial concentration of metabolites of progesterone was determined by extraction immediately just after collection (day zero). In this study, we examined the effects of different storage treatments and periods on the fecal progestagen concentration. Each sample was thoroughly mixed. The aliquots were equally divided into three sub-samples and subjected to the various storage conditions described below (-20°C, 4°C, or room temperature) for a short (7 and 15 days) and medium (107 and 173 days) storage period. The extraction and radioactivity counting by a gamma counter were performed after each storage period.

Fecal extraction

All fecal samples were extracted following Korndorfer et al. (1998) with some modifications. Briefly, each sample was weighed out to exactly 0.25 g and placed in glass tubes. Distilled water (0.25ml) and methanol (2.0 ml) were added. The tubes were shaken for 30 min (200 rpm). Petroleum ether (1.5 ml) was added. They were again shaken on a vortex for 15 sec, and centrifuged for 30 min (1500g, 4°C). The tubes were frozen for 5 min. The methanolic phase (1.0 ml) was transferred carefully into other tubes.

Radioimmunoassay

Fecal and plasma progestin concentrations from each sample were analyzed in duplicate by RIA using six Kits (Immunotech, France). The standards curve ranged from 0 to 49 ng/ml. Two internal controls (0.91 to 1.63 ng /ml) were run in every assay.

We validated the radioimmunoassay by serial dilutions from a fecal extract of pregnant camels (1, 1:4, 1:8, 1:16, 1:32 and 1:64) in phosphate buffer (0.01M; pH 7.4; 0.01% BSA). The intra- and inter-assays coefficients of variation for plasma and fecal was obtained using three levels of progesterone (low, medium, high). All samples were tested in triplicate in the same and other immunoassays, respectively. The recovery rate was determined by adding 250µl of I^{125}- Progesterone (approximately 47452 cpm) to 9 samples before extraction.

Linearity was estimated by a serial dilution (1:32, 1:64, 1:128) in phosphate buffer for a fecal sample #9810 rich in progesterone metabolites (2885 ± 381 ng/g DM fecal).

Statistical analysis

We calculated the concentration of plasma hormones (ng/ml) or fecal metabolites (ng/g MD of feces) in every sample. The concentrations of fecal progesterone metabolites were assessed using the GLM-Procedure of SAS (version 9.0). We used Dunnett multiple comparison test ($P<0.05$) to compare the mean concentrations of metabolites of progesterone after each storage period with the control. Results are expressed using mean ± standard error.

Results and Discussion

Analytical validation

For fecal assays, the average maximum binding (B0/T) was 52.4%. The average of nonspecific binding (NSB) of the reagents was 1.37%, and a total count (TC) of about 23957 cpm/500 µl. A standard curve is characterized by a concentration of progesterone corresponding to 20, 50 and 80% were 6.7, 1.12 and 0.2 ng/ml.

The intra and inter-assays coefficients of variation for internal quality controls were 6.5% and 6.1%, respectively. In pregnant camels, B/B0 of non-diluted samples was lower than 5%. Therefore, the methanol phase was diluted 1:64 in phosphate buffer prior to radioimmunoassay with B/B0 values ranging between 37.8 and 46.2%. In our study, the dilution ratio was higher than that found by Capezzuto et al. (2008) in the goat, which was 1:40. In addition, Ben Mohammed et al. (2011) used a dilution which was equal to 1:50 for cycling females and 1:160 for pregnant gazelles (Gazella gazella). To assay the metabolites of progesterone in the Cape ground squirrel (*Xerus inauris*), Pettitt et al. (2007) used a dilution that ranged from 1:51 - 1:150.

The intra-assay coefficient variation for a sample having high fecal P4 concentration was equal to 11.9%. This value was similar to that reported by Cebulj-Kadunc et al. (2000) in sheep (11.4%). It was slightly higher than that published by Isobe et al. (2005) in bovine, which was about

10.9%. For this same sample, the inter-assay coefficient variation was equal to 17.3%. This coefficient was similar to that found by Dantzer et al. (2010) for cortisol (17.9%). However, this coefficient was higher than that obtained by Korndörfer et al. (1998) in rabbit (15.7%) and Cebulj-Kadunc et al. (2000) in sheep (13.8%) for progesterone. The intra and inter-assay coefficient variation for a sample having high concentration plasmatic of P4 were, respectively, equal to 17.3% and 17.6%.

The recovery rate of progesterone metabolite from feces was 70.4 ± 0.9%. It was lower than that found by Kornmatitsuk et al. (2007) in cows (89.7%). The parallelism between the theoretical and observed concentrations of metabolites of progesterone in the feces was estimated by the linear regression analysis which gave the following equation with a correlation coefficient of 0.991: $y = 0.743\ x + 0.046$, where x = the theorical concentration and y = the observed concentration.

Plasmatic progesterone concentration in pregnant camels

In the third month of gestation, level of the plasmatic progesterone concentration varied from 3.0 ± 0.2 ng / ml to 5.2 ± 0.2 ng / ml, and averaged 4.4 ± 0.7 ng / ml (Table 1). The mean plasmatic progesterone concentration confirmed pregnancies.

Table 1. Mean concentrations of plasma progesterone in pregnant camels.

Camels	Mean ± SD (ng/ml)	Min (ng/ml)	Max (ng/ml)
#9703	4.6 ± 0.3	4.3	4.9
#0413	3.3 ± 0.3	3.1	3.6
#9606	4.3 ± 0.6	3.8	5.0
#9402	3.0 ± 0.2	2.8	3.3
#0013	5.2 ± 0.2	5.0	5.5
#9810	4.2 ± 0.4	3.7	4.6
#9408	4.6 ± 0.3	4.7	4.9
#9108	5.0 ± 0.3	4.6	5.4
#0019	5.2 ± 0.01	5.2	5.2
#9403	4.7 ± 0.2	4.5	4.9

In this study, plasmatic concentrations of progesterone were consistent with concentrations reported by Al Eknah (2000) in camels and by Adams (2007) in llamas and alpacas. Both authors proclaimed that the plasmatic concentration of progesterone was more than 2 ng/ml during pregnancy.

Effect of temperatures of short-term storage on the concentration of progesterone metabolites in feces

The initial concentration of metabolites of progesterone (day 0) varied from 313.3 to 3154.3 ng/g DM feces, and averaged 1441.9 ± 177.9 ng/g DM feces.

There were no significant differences in fecal progestagen concentrations between the control group (day 0) and after 7 to 15 days of storage at -20°C (Figure 1). The mean concentration of progesterone decreased slightly. It was equal to 1054.7 ± 122.2 ng/g DM feces and 1169.1 ± 135.5 ng/g DM feces, respectively, after 7 and 15 days of freezing. Our result were consistent with those obtained in baboons (*Papio cynocephalus*) fecal samples stored in 95% at -20°C for two weeks prior to lyophilization, as described by Lynch et al. (2003). In addition, this study showed that freezing immediately after defecation did not affect the concentration of progestagens. This result was an agreement with those reported by Galama et al. (2004) and Pettitt et al. (2007). Furthermore, a study conducted by Khan et al. (2002) showed that storing fecal samples at sub-zero temperatures, alcohol may prevent deterioration of steroid metabolites.

Following 7 and 15 days of storage at 4°C, the mean concentrations of metabolites of progesterone (±SE) showed a significant increase ($P<0.05$). It became equal to 3119.2 ± 325.6 ng /g DM feces at day 7 and 2126.6 ± 278.3 ng /g DM feces at day 15. Our results were in agreement with that found by Lynch et al. (2003). These authors reported that storage of feces in 95% ethanol for two weeks in a charcoal refrigerator caused a significant increase in fecal progestogen concentrations. However these samples still maintained the ability to distinguish reproductive states.

The significant increase in concentration of progesterone was due to the transformation of steroid metabolites of the conjugate form to the unconjugated form through the activity's effect of fecal bacteria and microorganisms (Ziegler et al., 1996; Khan et al., 2002). Thus, the increase of the steroid concentration could be the result of the antibody cross-reactivity with other hormonal metabolites created by microbial transformation during storage (Terio et al., 2002).

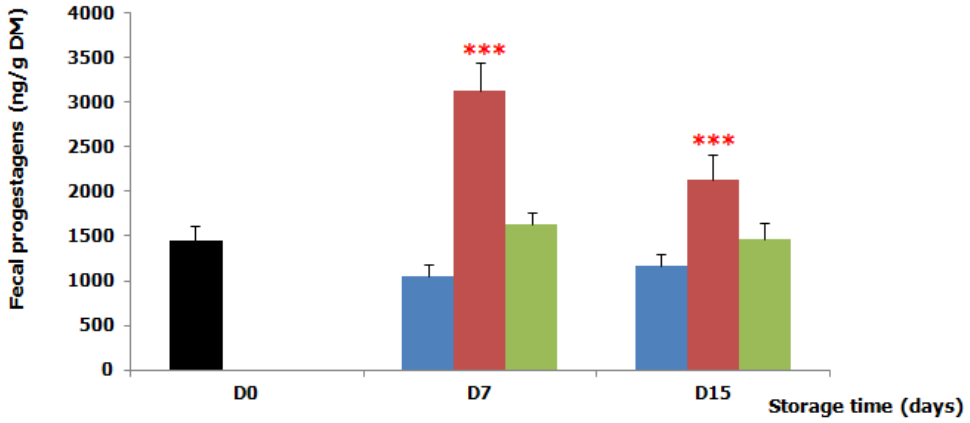

Figure 1. Effect of temperature of short-term storage on the mean fecal progestagen concentrations ±SE for a pregnant female (*Camelus dromedarius*).
Fecal samples stored at -20°C; fecal samples stored at 4°C; and fecal samples dried and stored in ambient temperature. The *** indicates statistical significance (P<0.05) with the initial concentration.

However, samples that were dried and stored in room temperature did not have significantly different fecal progestagen from initial concentration at 7 and 15 days. In fact, the level of metabolites of progesterone varied from 449.0 ng/g DM feces to 2759.8 ng/g DM feces and averaged 1626.3 ± 144.8 ng/g DM feces after 7 days of storage at room temperature. It becomes equal to 1467.4 ± 188.2 ng/g DM feces with a range from 492.3 ng/g DM to 3683.7 ng/g DM feces after 15 dayes. This study showed that storing fecal samples in room temperature did not affect progesterone metabolites concentration for 15 days. Hence, our result was similar with that published by Pappano et al. (2010) for both glucocorticoid and testosterone metabolite levels until 4 weeks of storage. Furthermore, this study was consistent with that reported by Brockman and Whitten (1996) in a primate (*Propithecus verreauxi*) for which the concentration of fecal progesterone remained stable after 3 weeks of drying and storing at room temperature. Also, Galama et al. (2004) showed that stability in concentration of fecal progesterone in rhinoceros was observed even after one month of storage at room temperature. Then, Khan et al. (2002) suggested that storing of fecal samples at room temperature for no longer than 30 days for glucocorticoids and for a shorter period for estrogen was preferable, if the freezing was not feasible.

In the present study, stability in the concentration of fecal progesterone may be explained by a high level of dry matter (45%) in comparison with feces in cows (15% DM) or a lack of moisture which can slow down bacteria growth and may prevent the transformation or the deterioration of steroids. The form of feces (pellets) which are easier to dry should be also considered.

Effect of temperatures of medium-term storage on the concentration of progesterone metabolites in feces

In comparison with the initial concentration (day 0), the mean concentration of metabolites progestagen decreased significantly (P< 0.05) after 107 and 173 days of freezing (Fig 2). It was respectively equal to 932.8 ± 108.4 ng/g DM feces and 949.7 ± 121.1 ng/g DM feces.

Our finding was comparable to that published by Khan et al. (2002) who reported that the concentration of some fecal steroid metabolites varies after months of storage at -20 °C. Similarly, Neumann et al. (2002) showed that keeping samples after one and three months at -20°C caused a significant decrease in fecal progesterone concentration of giraffe in comparison with the initial concentration. In contrast, a study by Pettitt et al. (2007) concluded that the level of fecal progesterone remained stable during three months of freezing. Neumann et al. (2002) showed also no significant changes of progesterone concentration, when samples were stored at -20°C after one and three months, for both rhinoceros and gazelle. In order to have a strong conclusion, it was interesting to validate the effect of freezing on fecal progesterone concentration after one month of storage.

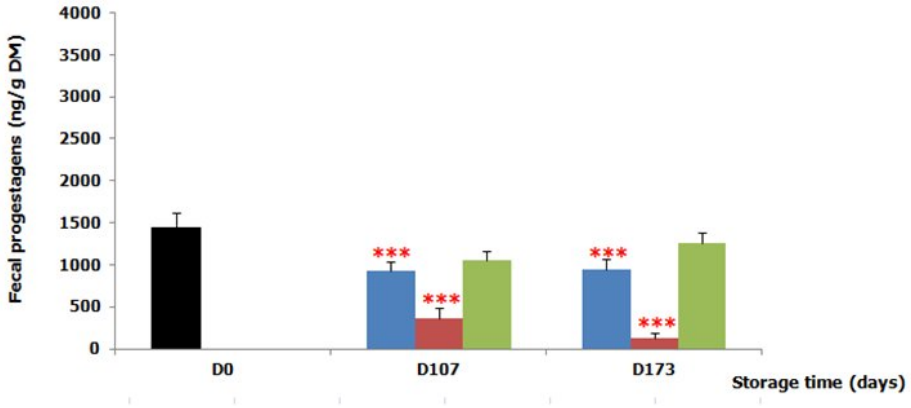

Figure 2. Effect of temperature of medium-term storage on the mean fecal progestagen concentrations ±SE for a pregnant female (*Camelus dromedarius*).
Fecal samples stored at -20°C; fecal samples stored at 4°C; and fecal samples dried and stored in ambient temperature. The *** indicates statistical significance (P<0.05) with the initial concentration.

After 107 and 173 days of storage at 4°C, the mean concentration of metabolites progestagen droped dramatically and significantly (Fig 2). It became respectively equal to 366.3 ± 125.2 ng/g DM feces (with a rate of reduction is equal to 75.5%) and 133.2 ± 48.9 ng/g DM feces (with a rate of reduction is equal to 90.8%).

In camel, the mean progesterone concentration decreased significantly, when fecal samples stored at 4°C, compared with those obtained in feces dried and stored at room temperature. Contrary, Schlenker et al. (1999) reported that concentrations of progesterone and estrogen in fecal cow samples decreased more rapidly after 13 weeks of storage at room temperature (30°C) than at 5°C.

This sudden and significant drop in progesterone concentration in fecal camel samples was explained by the presence of moisture in storage environment, which causes growth and multiplication of bacteria and detritus. Moreover, this study showed the presence of fungus on all fecal samples stored after 107 and 173 days at 4°C, which could be correlated with the level of the progestagen concentration.

The increases or decreases in fecal hormone concentrations during long-term storage may be influenced by a combination of variables including storage treatment, storage time and antibody specificity (Pettitt et al., 2007). Other Studies suggested that changes in hormone concentrations could be due to the influence of storage treatment and time on the activity of fecal bacteria and microorganisms (Khan et al., 2002).

After 107 and 173 day of keeping at room temperature, the mean concentration of metabolites progestagen decreased slightly (Figure 2). It averaged 1059.5 ± 108.3 ng/g DM feces and 1253.9 ± 136.0 ng/g DM feces, respectively, after 107 and 173 days. Consequently, drying and storage feces at room temperature did not affect the level of fecal progesterone for 6 months.

Our result was similar with that reported by Galama et al. (2004), who have showed that progesterone concentration remained stable for at least 180 days when fecal rhinoceros samples were stored at room temperature after drying in a solar box cooker (45°C) or mixing feces in methanol (80%). Moreover, Pettitt et al. (2007) showed that drying and storing feces at room temperature for 330 days was a best alternative of storage in field.

While Khan et al. (2002) showed that metabolites concentrations of estrogen (122%) and glucocorticoid (92%) increased after 90 and 120 days, respectively, when fecal samples stored in a 95% ethanol solution at room temperature. After 180 days, concentrations of both hormones metabolites declined to near initial concentrations. Similarly, Galama et al. (2004) reported that metabolites concentrations of progesterone increased significantly in feces stored at room temperature without heat or chemical treatment.

Conclusion

In our study the measure of metabolite progesterone concentrations in fecal camel by radioimmunoassay was validated. In addition, freezing samples immediately after collection was an optimal means of preventing bacterial metabolism of steroid metabolites and maintain the stability of the fecal progesterone concentration in camels for a short term of storage. Then, we proved

that storing fecal samples at 4°C causes significant changes in progesterone concentrations. Therefore, it was an inappropriate method for short and medium terms of storage. However, we showed that drying and storing camel fecal samples at room temperature for 180 days was advisable. Consequently, it is a field method of preservation fecal, independent of freezing equipment and it is a reliable method to monitoring the physiology and behavior of camels.

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