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Quantification of fecal estradiol and progesterone metabolites in Syrian hamsters (*Mesocricetus auratus*)

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Abstract

Alternative methods to the utilization of laboratory animal blood and its by-products are particularly attractive, especially regarding hamsters due to their small size and difficulties in obtaining serial blood samples. Steroid hormone metabolite quantification in feces, widely used in studies of free-ranging or intractable animals, is a non-invasive, non-stressor, economical, and animal saving technique which allows longitudinal studies by permitting frequent sampling of the same individual. The present study was undertaken to determine the suitability of this method for laboratory animals. Estradiol and progesterone metabolites were quantified by radioimmunoassay in feces of intact, sexually mature female Syrian hamsters during the estrous cycle (control) and in feces of superovulated females. Metabolites were extracted by fecal dilution in ethanol and quantified by solid phase radioimmunoassay. Median estrogen and progesterone concentrations were 9.703 and 180.74 ng/g feces in the control group, respectively. Peaks of estrogen (22.44 ± 4.54 ng/g feces) and progesterone (655.95 ± 129.93 ng/g feces) mean fecal concentrations respectively occurred 12 h before and immediately after ovulation, which is easily detected in this species by observation of a characteristic vaginal postovulatory discharge. Median estrogen and progesterone concentrations (28.159 and 586.57 ng/g feces, respectively) were significantly higher in superovulated animal feces ($P < 0.0001$). The present study demonstrated that it is possible to monitor ovarian activity in Syrian hamsters non-invasively by measuring fecal estradiol and progesterone metabolites. This technique appears to be a quite encouraging method for the development of new endocrinologic studies on laboratory animals.

Key words
- Endocrinology
- Fecal steroids
- Syrian hamster
- Reproduction
- Radioimmunoassay
- Estrogen

Introduction

Among conventional species of laboratory animals, Syrian hamster (*Mesocricetus auratus*) is an excellent subject for studies of reproductive biology due to its invariant four-day estrous cycle easily monitored by observation of the characteristic postovulatory vaginal discharge and its 16-day gestation, the shortest for any eutherian mammal (1). Because of such characteristics, Syrian hamsters are extensively used in physiological

and endocrinologic studies (e.g., Refs. 2-4) and in studies of the influence of stress on reproduction (e.g., Ref. 5-7) or on the relationship of nutrition to reproduction (e.g., Ref. 8). Most of these studies are based on the quantification of the hormones in blood. Though relatively small, the blood volume required to quantify serum hormones by radioimmunoassay (RIA) does not permit long-term longitudinal assessment of hormone levels (during an estrous cycle, for example) in very small animals like Syrian hamster. Generally in such species, to guarantee statistical precision, samples are collected from a large number of animals by invasive and highly stressing procedures such as cardiac puncture or decapitation. Alternative methods to monitor hormone fluctuations in these animals in conformity with ethical and human principles of animal experimentation (9) would be of great interest. The steroid metabolite quantification technique, widely used with free-ranging and intractable animals (10-15), appears to be an attractive non-invasive, cheap, alternative tool that avoids the stress effects related to blood sampling. The technique provides quite precise results by permitting frequent sampling of the same specimen (and thus longitudinal studies) and individuals can be used as their own controls. Such characteristics justify the use of this technique with laboratory and domestic animals. Billitti et al. (16) developed and validated a method for the assessment of testosterone levels by metabolite extraction and quantification in feces of two species of rodents, *Mus musculus* and *Peromyscus maniculatus*. Recently, similar techniques were employed to assess adrenal activity in the same species (17-19) and in Sprague Dawley rats (20). Muir et al. (21) used an enzyme immunoassay to measure estradiol, estrone and testosterone in mouse urine and feces.

The objective of the present study was to evaluate the validity of fecal estrogen and progesterone measurements in female Syrian hamster. For this purpose, estradiol and progesterone metabolites were quantified by solid phase RIA in feces of intact, sexually mature female hamsters during an estrous cycle. In order to determine if fecal steroid concentrations reflect changes in ovarian status, estrogen and progesterone metabolites were also quantified in superovulated female feces.

Material and Methods

Animals

Fourteen heterogenic, adult (10 weeks old), sexually mature female Syrian hamsters were supplied by the Laboratory Animal Facility of the Department of Pathology, Faculty of Veterinary Medicine, University of São Paulo, Brazil, whose Ethic Committee approved the experimental design. The animals were housed in standard individual propylene cages in the same animal facility under conventional conditions (12:12-h light/dark conditions, lights on at 6:00 am; room temperature: 22 ± 2ºC; 20 changes of air per hour; air pouch filters) according to the ILAR Guide for the Care and Use of Laboratory Animals (22). Specific pelleted food (Nuvilab CR1, Nuvital, Curitiba, PR, Brazil) and filtered bottled tap water were supplied *ad libitum*. In order to facilitate feces collection, absorbent paper pads were used in place of the traditional wood shaving bedding.

Syrian hamster females have a 4-day estrous cycle and ovulation occurs in the middle of the dark period of the 4th day. On the morning of the next day (day 1), there is a postovulatory discharge from the vagina that is readily recognizable because of its copious nature, tacky consistency and strong, pungent odor. This discharge occurs only when ovulation has taken place and is commonly used to date hamster cycle events (1). At least two consecutive 4-day cycles per female were monitored before the beginning of the trial.

Hormonal challenge

On day 1, the first day after ovulation, 7 females were injected *ip* with 35 IU pregnant mare serum gonadotropin to stimulate follicle development and induce superovulation. The animals were injected again 56 h later with 35 IU human chorionic gonadotropin (hCG) in order to trigger ovulation (see Ref. 23).

Sample collection

Since the estrous cycle of hamsters lasts 4 days (1), control females had their feces collected for five days from day 1 after ovulation (N = 202 samples). Superovulated female feces were collected for 3 days after the pregnant mare serum gonadotropin injection to euthanasia (N = 157 samples). Samples were collected at 4-h intervals, immediately identified and stored frozen at -20ºC to avoid the possible influence of environmental temperature and moisture on steroid concentrations (24).

Oocyte collection

Superovulated females were euthanized in a CO_2 chamber 16 h after the second hormone injection. Oviducts were immediately collected and dilacerated with a sterile needle. Oocytes were collected with a micropipette, separated and counted to determine the success of the superovulatory treatment. Control animals were euthanized just before ovulation during the subsequent cycle, in the afternoon of the fourth day after vaginal discharge. Mature follicles were collected from the ovaries and counted.

Fecal extraction

Steroids were extracted from feces using the ethanol-based procedure described by Thompson and Monfort (25). All samples were weighed before extraction. Sample weight ranged from 0.036 to 2.067 g, averaging 0.505 ± 0.321 g. In most cases, all the feces excreted during every 4-h period were used. Sometimes, at night, excreted feces weight exceeded one or even two grams. In these cases, an aleatory amount of feces was weighed and extracted. Since hamster feces are very dry, lyophilization was not necessary. Because they are very compact, the feces + aqueous ethanol (ethanol:distilled water, 9:1) was homogenized with a mixer (Polytron, Kinematica™ GmbH, Littau, Switzerland) in 10-ml glass tubes and then boiled for 20 min. All tubes were centrifuged at 500 *g* for 10 min. The supernatant was poured into another tube, and the pellet was resuspended in 5 ml 90% ethanol, vortexed for 1 min, and recentrifuged. Both ethanol supernatants were combined and completely dried, and the residue was dissolved in 1 ml methanol, vortexed for 1 min and kept frozen until the time for assay.

Extraction efficiency was checked by the addition of ^{3}H-estradiol to 8 independent fecal samples. These samples were treated as described above. An aliquot (100 µl) of each methanol-dissolved extract was transferred to a scintillation vial and, after the addition of 3 ml scintillation fluid, radioactivity was counted using a Packard liquid scintillation counter (Boston, MA, USA). The counts were compared to radioactivity added to determine the percentage of recovered radioactivity. Progesterone extraction efficiency was assessed in the same way.

The recoveries of added ^{3}H-estradiol and ^{3}H-progesterone were 68.68 ± 9.83 and 69.73 ± 7.35% (mean ± SD), respectively, for 8 measurements.

Radioimmunoassay

Estradiol and progesterone fecal metabolites were quantified using a commercial solid-phase RIA with antibody-coated tubes previously shown to produce reliable results for hamster blood (8) following the manu-

facturer's protocol (Count-A-Count Estradiol and Count-A-Count Progesterone, DPC®, Diagnostic Products Corporation, Los Angeles, CA, USA). The accuracy of the antibodies for hamster feces was checked, demonstrating parallelism between dilutions of pooled fecal extracts and the standard curve. The correlation coefficients were 0.996 (r-square = 0.987) for estradiol and 0.999 (r-square = 0.997) for progesterone.

Serial dilutions of a sample were assayed to define the best dilution to run the samples and the 1:40 dilution proved to be the most effective for both hormones. All samples were assayed in duplicate.

Assay quality control

The parameters used to check assay quality control were sensitivity (lowest detectable concentration), specificity (percent cross-reactivity) and inter- and intra-assay variation.

Mean sensitivity was 0.00272 ng/ml for the estradiol assay and 0.01 ng/ml for the progesterone assay. Respective cross-reactivity coefficients are provided by the manufacturer [http://www.dpcweb.com/package_inserts/ria/ria_int.html].

Intra- and interassay coefficients of variation were determined using a pool of 100-µl aliquots from 50 randomly selected samples and <10% for both hormones.

Statistical analysis

Average estrogen and progesterone concentrations were compared and their variation during the estrous cycle was analyzed. Because it is impossible to simultaneously monitor changes in hormone concentrations in the feces and in the blood of the same animal (obtaining enough blood for steroid quantification every 4 h for 5 days is not possible in Syrian hamsters) and collecting blood samples according to a similar schedule from intact and superovulated females would require the euthanasia of 250 animals, fecal data were compared with plasma profiles available in literature (1,3).

Results

Hormonal challenge

A mean number (± SD) of 23.29 ± 12.08 oocytes were collected from the ovaries of superovulated females and 7.00 ± 3.46 from control females. The statistically significant difference between these means (unpaired *t*-test with Welch correction, t = 3.429, P = 0.0140, d.f. = 6) indicates that the animals reacted well to the hormonal challenge.

Mean fecal estrogen concentration

Since fecal estrogen and progesterone concentrations in each group were not normally distributed, medians were calculated and compared instead of means. Median estrogen concentration was 9.703 ng/g feces in the control group (range: 0.880-50.138 ng/g feces) and 28.159 ng/g feces in superovulated females (range: 3.913-319.893 ng/g feces). The difference between these estrogen levels was statistically significant (Mann-Whitney test, U = 4953.0, P < 0.0001).

A slight daily increase between 9:00 and 13:00 h and a 22.44 ± 4.54 ng/g feces peak in mean fecal estrogen concentration at 13:00 h on the day before ovulation occurred in the control group. Mean fecal estrogen concentration also increased every day during the morning in superovulated females, which showed a mean peak of 71.42 ± 17.24 ng/g feces 8 h after hCG injection (Figure 1).

Median fecal progesterone concentration

Median fecal progesterone concentration was significantly higher in superovulated females (586.57 ng/g feces; range: 68.989-1890.900 ng/g feces) than in control group (180.74 ng/g feces; range: 26.462-969.040

ng/g feces; Mann-Whitney test, U = 4247.0, P < 0.0001).

Mean progesterone metabolite concentration in the feces of control females increased every day during the second half of the night. A mean peak of 655.95 ± 129.93 ng/g feces occurred at 5:00 h just after ovulation. Mean fecal progesterone concentration showed the same pattern of variation in superovulated females (Figure 2).

Discussion

Steroid metabolites are detected and can be measured in both urine and feces of many species, in different proportion according to their metabolism (26,27). We did not find a specific study of the Syrian hamster estradiol and progesterone metabolic pathways or excretion in the consulted literature. Gonadal or adrenal steroid metabolite quantification in feces has been validated in the rat and in the mouse by several investigators (16,19-21). Moreover, hamsters void very small amounts of extremely concentrated urine that can be collected only with difficulty.

Sampling of fecal pellets is an easy and reliable procedure. The present data clearly show that quantifying gonadal steroids in feces of Syrian hamsters is possible. We have also demonstrated that when female Syrian hamsters receive a hormonal treatment to stimulate ovarian activity they excrete more estrogen and progesterone in feces than intact females. These main results represent *per se* a physiological validation of the method. Using an extraction technique quite similar to the one we used but with higher efficiency (88.9 ± 8.1 *vs* 68.68 ± 9.83%), Muir et al. (21) measured fecal estradiol concentrations in both male and female mice. Estradiol levels in the feces of pregnant females ranged from 11.9 to 16.8 ng/g feces. These results are consistent with the median values obtained here for control females (9.703 ng/g feces). A reduced but perceptible amount of material is lost when homogenizing feces samples with the Polytron apparatus. This may explain our lower extraction efficiency and may be avoided using a household coffee grinder to grind dry feces before weighing, as done by Billitti et al. (16).

Kishi et al. (3) described the changes in

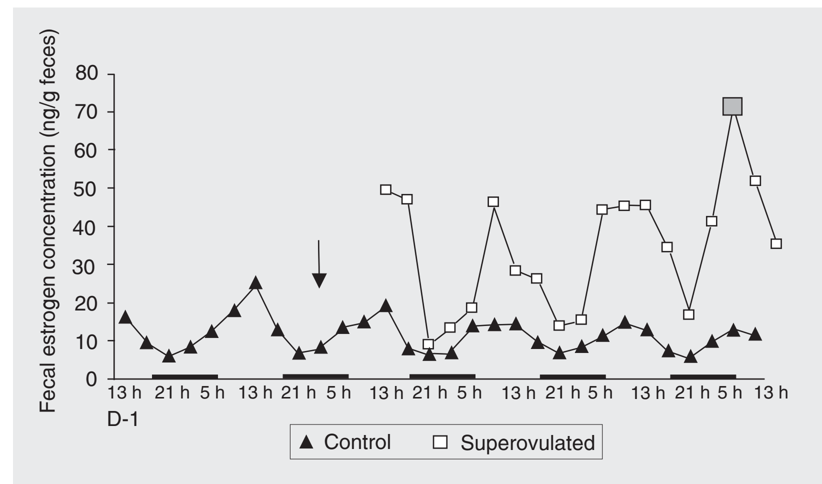

Figure 1. Changes in mean fecal estrogen concentration during the 4-day estrous cycle of two groups of adult female hamsters. Samples were collected at 4-h intervals throughout the cycle. The day after ovulation was designated as day 1 (D-1) of the cycle. The arrow indicates the estimated time of ovulation. The grey square indicates the time of hCG injection into superovulated females. Dark bars on the x-axis indicate “lights out”. Each value represents the mean of 7 observations (normally distributed).

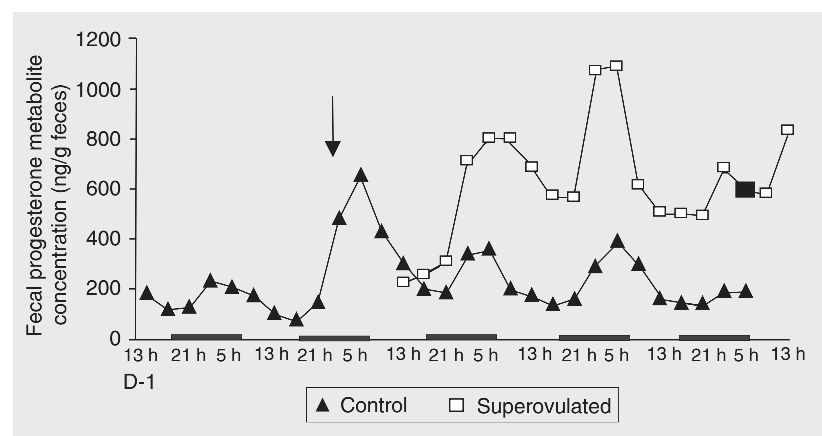

Figure 2. Changes in mean fecal progesterone metabolite concentration during the 4-day estrous cycle of two groups of adult female hamsters. Samples were collected at 4-h intervals throughout the cycle. The day after ovulation was designated as day 1 (D-1) of the cycle. The arrow indicates the estimated time of ovulation. The black square indicates the time of hCG injection into superovulated females. Dark bars on the x-axis indicate “lights out”. Each value represents the mean of 7 observations (normally distributed).

plasma estradiol and progesterone concentrations during the estrous cycle. They conducted a cross-sectional study by decapitating 5 of 200 female hamsters every 3 h to collect enough blood for steroid quantification and observed 2 four-fold increases in the concentration of plasma estradiol. The first peak occurred in the afternoon of day 3 of the cycle, when estradiol plasma concentrations increased up to 20:00 h (about 28 h before ovulation) and then declined abruptly. The second peak was observed at 14:00 h on day 4, about 10 h before ovulation.

In agreement with Kishi et al. (3), by monitoring 7 females throughout the cycle, we noted a peak of fecal estrogen concentration before ovulation (at 13:00 h on day 4) and a marked increase on the next day (Figure 1). Could such increases reflect plasma peaks after respective delays of 16 and 25 h?

In the same way, Kishi et al. (3) and Siegel (1) reported a peak in plasma progesterone concentration (12 ng/ml over a baseline value of 1 to 2 ng/ml) almost simultaneous with ovulation (24:00 h on day 4). In the present study, a progesterone metabolite peak was detected in feces about 4 h later (Figure 2).

The route of steroid excretion varies among species, as well as among steroids within the same species but the lag time of fecal steroids is longer in hindgut fermenters (26). In the mouse (*Mus musculus*), the gut transit time has been reported to be approximately 9-10 h (19). However, Touma et al. (19) injected mice with ^{3}H-labeled corticosterone and observed that the delay time between injection and the appearance of the respective signal in the feces was significantly influenced by the hour of the day of administration and the animal activity rhythm. When mice were injected at night, when their main activity takes place, radioactive metabolites appeared earlier in feces than when the animals received the labeled hormone in the morning during the passive phase. Hamsters also are more active during the nighttime and excrete more feces during this period. This could explain the different lag times between plasma peaks and fecal increases since the first plasma peak concentration occurs at 20:00 and the second at 12:00 h.

It appears that concentration peaks of both hormones in feces correlate with the plasma peaks even though the kinetics of the metabolization and excretion of each hormone is different.

These results demonstrate that it is possible to monitor ovarian activity in Syrian hamsters non-invasively by measuring fecal estradiol and progesterone metabolites. This technique could be a very valuable tool for longitudinal endocrinologic studies, as it enables frequent sampling of individual animals. Moreover, it could greatly reduce the number of hamsters used for research, as animals do not need to be killed at different times during the experiment to obtain blood samples for hormone measurements.

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