Evaluation of extraction methods for progesterone determination in rabbit (*Oryctolagus cuniculus*) feces by radioimmunoassay

CORRESPONDENCE TO:
Cyro Ferreira Meirelles
Centro de Energia Nuclear na Agricultura
Av. Centenário, 303 – Caixa Postal 96
13400-970 – Piracicaba – SP
e-mail: cfmeirel@esalq.usp.br

1 - Seção de Ciências Animais do Centro de Energia Nuclear na Agricultura da USP, Piracicaba – SP

Métodos de extração para determinação de progesterona em fezes de coelho (*Oryctolagus cuniculus*) através de radioimunoensaio

Clotilde Maria KORNDÖRFER[1]; Cyro Ferreira MEIRELLES[1]; Ives Cláudio da Silva BUENO[1]; Adibe Luiz ABDALLA[1]

SUMMARY

The purpose of this study was to find a practical procedure for the extraction of progesterone (P4) from feces and to determine if the P4 plasma profiles during pregnancy were reflected in the total fecal P4 of pregnant rabbits. The rabbit was used as model for the techniques. Plasma and feces were collected from 11 rabbits during a period of 42 days. Three different methods of P4 extraction were used. The total P4 was measured by solid-phase radioimmunoassay (RIA) with ^{125}I-P4 as the tracer. Results suggested that it was possible to extract total P4 from rabbit feces with methanol and petroleum ether. Plasma and fecal P4 profiles were compared for both pregnant and ovariectomized rabbits. It was possible to differentiate total P4 extracted from day two through 28 after breeding ($p<0.01$).

UNITERMS: Progesterone; Feces; Radioimmunoassay; Rabbit.

INTRODUCTION

A technique for extraction of reproductive hormones from feces is an important tool for monitoring reproductive functions and also for animal ethologists. As a non-invasive process, it presents several advantages: permits the monitoring of reproductive physiology under diverse conditions, and samples are easily collected, transported, and stored.

Several reports show the use of non-invasive fecal estrogen and progesterone metabolite evaluations for monitoring reproductive function[8] but using a variety of extraction methods with different solvents. Bamberg; Schaerzenberger[1], described methods to detect progestins from feces of zebras (*Equus zebra*) and rhinoceroses (*Rhinceros spp*) and their methods seem to be practical and easy. Other authors have published methods to reduce labor and made limited use of organic solvents[9,11]. Three of those methods were used in this study in order to find a practical procedure for the extraction of progesterone (P4) from feces. In this study we describe methods for P4 extraction and assay from feces, using the rabbit as a model. We also observed P4 plasma profiles during pregnancy to determine if they reflected the total fecal P4 of pregnant rabbits as compared with ovariectomized females.

MATERIAL AND METHOD

Eleven sexually mature, 150 days old White New Zealand rabbits, weighing an average of 2.9 kg were used. Five rabbits were bilaterally ovariectomized. Thirty days later, six rabbits were mated. This was considered as Day 0 for the ovariectomized (Group I) and non-ovariectomized (Group II) rabbits. The animals were maintained in individual cages under standard laboratory conditions at the Animal Science Section of the Centro de Energia Nuclear na Agricultura of the Universidade de São Paulo, from January to April, 1992. Blood and feces' samples were obtained three times a week, during a period of 42 days. The blood was withdrawn from central and marginal veins into heparinized tubes, centrifuged immediately, and the plasma was stored at -15ºC. Fecal samples were collected in the morning. They were homogenized and stored at -15ºC. Three methods of P4 extraction were used: Bamberg; Schwarzenberger[1]; Möstl* and Desaulniers *et al.*[3] (referred to as methods A, B and C, respectively). Before removing the subsamples for extraction, the stored samples were well mixed to avoid steroid variations among individual fecal pellets.

Sample Preparation

Method A: Undried feces (0.5 g) were weighed in glass tubes. Distilled water (0.5 ml) and methanol (4.0 ml) were added.

* Möst, E. Wien: Institut Für Biochemie, Veterinarmedizinische Universität Wien. **Personal communication**. 1991.

The tubes were shaken on a vortex for 30 min. Petroleum ether (3.0 ml) was added. The tubes were again shaken for 15 sec. and centrifuged for 30 min. (1500g, 4ºC). To reduce temperature and to obtain clear separation of the two liquid phases, the tubes were frozen for 5 min. in a bath of methanol and dry ice. The methanolic phase (1.0 ml), which formed the lower layer was transferred to capped vials and stored at -15ºC until assayed by RIA.

Method B: Undried feces (0.5 g) were weighed in glass tubes, deionized water (2.5 ml) and methanol (1.0 ml) were added and mixed for 30 min. The tubes were centrifuged for 15 min (1500 g). The upper phase was then transferred to capped vials and stored at -15ºC for further RIA assay.

Method C: Ground dried feces (0.25g) were weighed in glass tubes. Deionized water (1.5 ml) and ethyl ether (3.0 ml) were added to the tubes. They were shaken on a vortex for 4 min. and centrifuged. The ether layer was transferred into another tube. The same procedure was repeated twice without adding deionized water. The pooled ether fraction (9 ml) was evaporated and reconstituted in ethanol (1.0 ml).

Fecal extracts were obtained from Group I and Group II. They were extracted by the three methods above. Concentrate and dilution serial (1:2; 1:4 and 1:10) were prepared in phosphate buffer (0.01M; pH 7.4; 0.01% BSA) and assayed. The final chosen dilution was 1:4 for methods A and B, and 1:10 for method C. After appropriate dilution and homogenization, 100 ml of the fecal samples were used for RIA.

Assays

Measurement of both plasma and fecal levels of P4 were performed by solid phase RIA, using a RIA-kit (FAO-IAEA, Seibersdorf, Austria) and ^{125}I-P4 as the tracer. As the RIA-kit was designed for use with plasma, the fecal standard curve was prepared adding crescent concentrations of P4 diluted in fecal extract of castrated male rabbits. The standard curve ranged from 0.25 ng/ml to 16.0 ng/ml. The curves were tested to verify their parallelism. All samples were tested in triplicate on the same RIA and were compared to the standard curve on the same assay. The interassay coefficient of variation for P4 fecal assays was obtained using three controls. They had high, medium and low levels of P4 as they were respectively obtained from a fecal pool of pregnant animals on days D1, D11 and D23 of gestation. The pools were divided equally and used in every assay. The biological control of RIA was obtained comparing plasma from ovariectomized and pregnant animals.

Statistics

A factorial design with two factors was used. Factor one was ovariectomized (n = 5) and non-ovariectomized (n = 6) animals. Factor two was method of P4 extraction of feces including blood plasma as a check. Regression analysis was used to find the more appropriate method of P4 extraction. Linear, quadratic and logarithmic models were tested to verify the relationship between P4 profiles from plasma and those from feces extracted by methods A, B and C. Normal parameters of correlation coefficient (r) and the F test were used. Hotelling's T^2 test was used to confirm parallelism between plasma and feces standard curves. Statistical analysis was performed using SAS[10].

RESULTS AND DISCUSSION

Quality control of RIA

Radioimmunoassay for P4 on plasma had an average maximum binding (B0/T) of 47.7%. The average of nonspecific binding (NSB) of the reagents was 5.1%. Twenty zero standard tubes were processed by the laboratory of origin: 100% for P4, 14% for 11-alfa-hydroxy-progesterone, 5.3% for in a single assay along with a set of non-zero standards and quality control samples. From a standard curve obtained using a logit-log plot and using the mean as the zero point, the apparent progesterone concentrations were determined at increasing standard deviations from the mean (0.1 ng/ml) as Midgeley *et al.*[7]. The intra- and inter-assay coefficient of variation for plasma was 6.39% and 8.82%, respectively. The antibody specificity, expressed by cross reactivity, was given corticosterone, 2.4% for alfa – hydroxy – progesterone, 3.5% for desoxycorticosterone, 0.02 % for testosterone, 0.02% for androstenedione, 0.01% for cortisol.

A total of 9 assays measured P4 from feces. The mean B0/T for fecal assays was 43.6% within a variation of 39.9% and 47.7%. For low, medium and high concentration of P4, the inter-assay coefficients of variation were respectively 8.7, 6.8 and 15.7. The intra-assay coefficient of variation was 6.7%. The heterogeneity of slopes test (SAS[10]) was applied to the dilution curves to verify relationship to the P4 standard curve. The results showed parallel curves ($p>0.234$) for method A. The analysis of RIA for method A was satisfactory, allowing use of the results for physiological studies of P4 on rabbit feces. The slopes tested for methods B and C did not show parallel results ($p<0.049$ and $p<0.012$, respectively) and the methods were inadequate for P4 studies on rabbit feces in this experiment.

P4 on plasma

In Fig. 1 the average profile of plasma P4 can be seen, determined in ovariectomized and non ovariectomized animals, within the periods before mating, during gestation and after birth. The levels of plasma P4 agrees with values in studies by others who used RIA[2,4,6]. The plasma P4 peak was between D9 and D15. Hormonal concentration obtained between days D25 and D28 exhibit a small increase but does not differ statistically from D21. The P4 increase in this period corresponds well with the peak found in other studies[4,6].

P4 on feces

Hormone levels were characterized exclusively by RIA. Therefore, the concentration obtained may be interpreted as the sum of both metabolites plus immunoreactive substances and will be referred herein as total fecal P4. Comparisons of results from mathematical models of P4 profiles in both plasma and feces are made in Tab. 1. The three methods, denoted as A, B and C were used. The logarithmic model, suggested by Wilcox *et al.*[12] gave the best results for method A. This relationship of plasma P4 and feces P4 was statistically significant.

The correlation coefficient for plasma and feces (Fig. 2) was r = 0.9257 ($p<0.01$). The calculations were made by accumulating amount of P4 in plasma and in feces. The biological control of RIA was evaluated using feces of pregnant and ovariectomized animals. The fecal P4 profile in method A, using petroleum ether and methanol as solvents, agreed with both physiological situations. Animals without ovaries should have basal values as seen herein. However, the distribution of P4 values in fecal samples obtained with methods B and C could not be explained by the tested methods.

Method A: The results obtained from method A reproduced what was happening in plasma (Fig. 1 and Tab. 2); probably, due to the better solubility of P4 in the organic solvents. Because of its high polarity, the methanol may have extracted other lipids. However, the use of centrifugation and low temperature probably reduced the interference caused by other lipids. Compounding this observation, the other solvent used to extract P4 was petroleum ether. This solvent specifically extracts P4, excluding some other steroids[5] and might allow the method to extract P4 better to reach a greater specificity for the RIA. The progestin 20-alfa-0HP is also largely secreted by the rabbit ovary and extracted by petroleum ether. However, the influence of 20-alfa-OHP could be restricted by the RIA specificity to P4. Fecal P4 extraction with this method was easy, did not require special equipment and was not labor intensive.

Method B: According to Tab. 3, the plasmatic P4 profile from pregnant animals was reflected on fecal P4 around D10 and D28, when using this method of extraction. However, none of the mathematical models tested were satisfactory to explain that observation. Methanol, the most polar solvent used on the three tested methods, was the only one used in method B. Probably, with this method other metabolics with similar solubility of P4 in this organic solvent were extracted and caused some interference on the RIA.

Method C: This method, using dried feces for P4 extraction, was the most labor intensive due to the different required steps. It was tested for the advantages it could offer in regard to transport and conservation of samples. The feces could be collected in distant locations, dried, and then sent to the laboratory for RIA assays. The biological evaluation (Tab. 3) and the parameters considered on the RIA quality control showed that the method was not valid for the conditions of this study. The high temperature used to dry the feces could be a possible explanation for the interference detected on RIA. The heat could facilitate the breakdown of some compounds into small fragments that could interfere with the assay as analogous compounds or metabolites. Under the conditions of this study, methods B and C did not extract P4 from feces which could reflect ovarian activity during rabbit pregnancy.

Physiological analysis

Fig. 1 shows a continuous increase of P4 on plasma and feces, extracted by Method A, until reaching a peak between D11 and D14. Plasmatic and fecal profiles of P4 then decreased gradually to D21. Between days D23 and D25 a second outstanding peak of P4 in feces occurred. It was greater than D14 and fell sharply after delivery. There was a suggestion of a secondary peak of P4 in plasma present between D25 and D26, but the values did not differ statistically from plasmatic P4 at D21 ($p>0.05$). Graphically, when the data were pooled within experimental groups, it was observed that the second peak of P4 on feces happened before the elevation of P4 on plasma. The anticipation of the second P4 peak in feces may be explained by the individuality of the animals with some females exhibiting earlier and higher P4 fecal concentration than others. Steroids were possibly transferred from plasma and detected on feces through RIA and can be noticed in Fig. 1 when the P4 profiles from plasma and feces of pregnant animals were compared. The results support that there is a discrimination of P4 fecal between days D4 and D28 for the two studied groups.

Table 1

Mathematical models tested between plasma (independent variable) and the P4 extraction methods from feces (dependent variable). LANA/CENA/USP, São Paulo, March 1992.

Methods	Models	F test	Corr. Coef (r)
A	Linear	14.21**	0.41
Petroleum ether	quadratic	7.54^{ns}	0.42
+ methanol	logarithmic	17.98**	0.45
B	Linear	0.55^{ns}	0.09
Methanol	quadratic	2.02^{ns}	0.29
	logarithmic	4.87^{ns}	0.26
C	Linear	0.16^{ns}	0.04
Ethyl ether	quadratic	2.46^{ns}	0.26
	logarithmic	1.04^{ns}	0.12

ns = non significative; ** = $p<0.01$.

Table 2

Values of T test comparing plasma and fecal P4 amounts, extracted with petroleum ether and methanol using ovariectomized and pregnant animals. LANA/CENA/USP, São Paulo, March 1992.

Physiological day	Plasma	Feces
-5	1.09^{ns}	1.42^{ns}
-2	0.81^{ns}	2.34^{ns}
0	0.90^{ns}	1.25^{ns}
2	1.35^{ns}	4.95**
4	4.6**	6.37**
7	2.79 *	4.98**
9	2.97 *	7.57**
11	5.53**	10.53**
14	5.07**	3.13 *
16	5.76**	5.96**
18	12.38**	9.80**
21	2.64 *	15.78**
23	2.32^{ns}	62.70**
25	4.23**	18.44**
28	5.13**	6.30**
30	3.85 *	2.45^{ns}
32	2.92 *	2.35^{ns}
35	0.42^{ns}	3.17 *
37	2.82 *	0.86^{ns}

ns = non significative;
* = $p<0.05$; ** = $p<0.01$; D0 = mating day.

Table 3

Mean concentration and standard deviation (SD) of plasma and fecal P4 observed with different extraction methods, in pregnant animals considering physiological days. LANA/CENA/USP, São Paulo, March 1992.

Days	Plasma (ng/ml)	SDPl	Feces (ng/g)					
			Method A	SDA	Method B	SDB	Method C	SDC
-8	0.12	0.11	9.27	2.78	5.66	0.86	96.83	40.07
-5	0.26	0.25	12.60	7.45	6.05	2.23	94.94	60.67
-2	0.25	0.13	10.16	2.68	5.28	2.82	88.95	72.20
0	0.19	0.14	10.84	4.25	4.52	3.19	54.29	27.70
2	1.07	0.47	11.15	3.38	9.78	5.38	56.28	31.45
4	2.00	0.72	11.74	2.55	3.98	1.86	48.50	31.75
7	4.20	2.01	16.35	6.21	5.59	2.62	55.74	36.80
9	4.47	1.41	18.86	7.89	7.47	2.94	61.94	21.93
11	5.72	1.30	19.47	4.52	8.22	4.77	70.80	43.56
14	4.77	3.63	26.61	8.38	6.68	2.02	72.20	42.96
16	4.26	1.70	21.36	5.13	7.95	1.73	81.37	41.20
18	3.51	0.84	20.19	2.16	8.85	3.03	78.81	36.61
21	3.32	0.50	21.80	11.61	9.70	3.87	81.02	36.44
23	3.23	0.12	31.68	23.63	9.89	6.39	87.74	40.37
25	3.37	0.43	31.11	12.63	12.28	6.77	146.31	88.85
28	3.78	1.47	23.47	6.77	9.84	3.34	131.61	73.50
30	2.24	0.64	23.14	8.81	8.92	4.42	108.81	72.73
32	0.46	0.35	16.71	6.23	5.53	2.31	66.69	29.81
35	0.50	0.38	10.07	4.63	5.84	2.67	59.22	37.72
37	0.52	0.35	11.97	2.34	4.91	2.58	57.37	36.34

Extraction methods: A (petroleum ether + methanol); B (methanol): C (ethyl ether). D0: mate: D31: birth; D-8: P4 mean value of ovariectomized animals (n = 5) during experimental period; D-5 to D37: mean daily value of pregnant animals (n = 6).

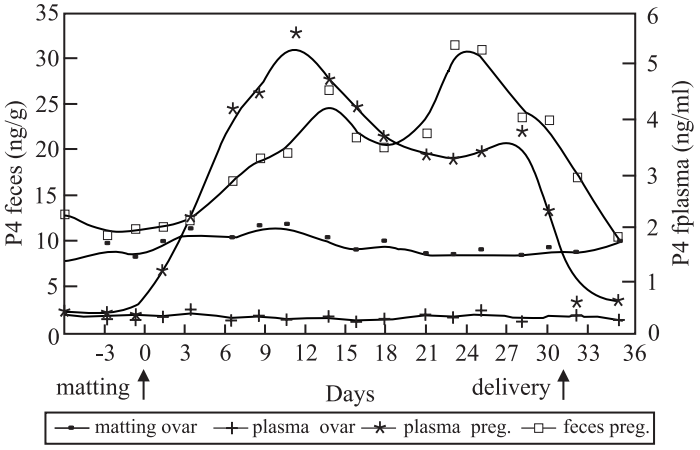

Figure 1

Mean P4 concentration in plasma (n = 6) and P4 in feces (n = 6) extracted with petroleum ether and methanol (method A), considering physiological days in pregnant (preg.) and ovariectomized (ovar.) rabbits.

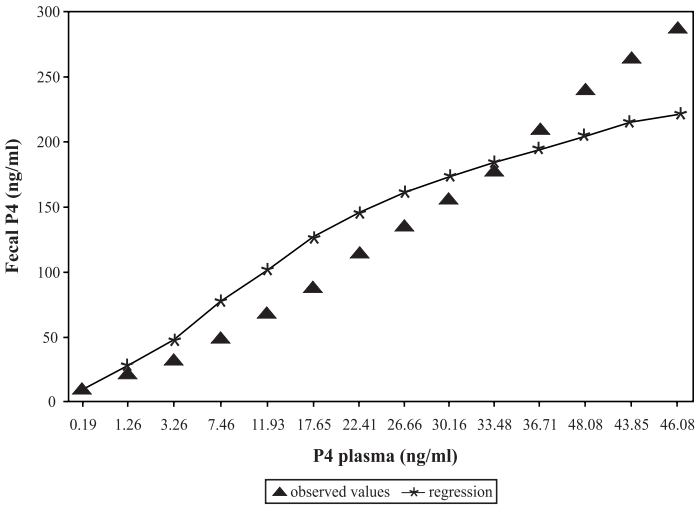

Figure 2

Correlation of P4 in plasma and feces using mean values for P4 concentration in plasma (n = 6) and feces (n = 6), and using petroleum ether and methanol as solvents (method A).
Feces = $e^{3,196+0.276 x \log(plasma)}$, r = 0.926; p<0.01.

CONCLUSION

Determination of P4 from rabbit feces was possible using methanol and petroleum ether as extraction solvents. Modification of the profile of plasma P4 was reflected by alterations of P4 fecal concentration occurring during the discrimination of P4 profiles in feces excreted by pregnant and ovariectomized rabbits from the second to the 28th day after breeding.

KORNDÖRFER, C.M.; MEIRELLES, C.F.; BUENO, I.C.S.; ABDALLA, A.L. Evaluation of extraction methods for progesterone determination in rabbit (*Oryctolagus cuniculus*) feces by radioimmunoassay. **Braz. J. vet. Res. anim. Sci.**, São Paulo, v. 35, n. 3, p. 112-116, 1998.

RESUMO

O propósito deste estudo foi determinar uma metodologia mais prática para a extração de progesterona (P4) das fezes e observar se os perfis plasmáticos de progesterona (P4) durante a gestação de coelhas era refletida na concentração total de P4 nas fezes. Esta espécie animal foi utilizada como modelo para as diferentes metodologias. O plasma e as fezes foram coletadas de 11 coelhas, durante período de 42 dias. Três métodos diferentes de extração de P4 foram testados. A P4 total foi medida com auxílio de radioimunoensaio de fase sólida (RIE) utilizando ^{125}I-P4 como traçador. Os resultados sugerem que foi possível extrair P4 total das fezes de coelhas com metanol e éter de petróleo. Os teores de P4 plasmática e fecal foram comparados entre animais gestantes e ovariectomizados. Foi possível a diferenciação ($p<0,01$) dos teores totais de P4 excretados nas fezes entre a 2º até 28º dia após cobertura.

UNITERMOS: Progesterona; Fezes; Radioimunoensaio; Coelhos.

REFERENCES

1- BAMBERG, E.; SCHWARZENBERGER, F. Fecal steroid assay for monitoring of oestrous cycle and pregnancy. *In*: CONGRESS OF THE INTERNATIONAL SOCIETY FOR ANIMAL CLINICAL BIOCHEMISTRY, 4., Davis, 1990. **Proceedings**. Davis: University of California, 1990. p.95-9.

2- BATTISTA, M.G.; POPE, W.F.; FOOTER, R.H. Plasma and uterine progesterone and embryo survival in rabbits following asynchronous transfer to unilaterally ovariectomized recepiens. **Theriogenology**, v.27. n.6, p.897-905, 1987.

3- DESAULNIERS, D.M.; GOFF, A.K.; BETTERIDGE, K.J.; ROWELL, J.; FLOOD, P.F Reproductive hormone concentrations in faeces during the oestrous cycle and pregnancy in cattle (*Bos taurus*) and muskoxen (*Ovibus moschatus*). **Canadian Journal of Zoology**, n.67, p.1148-54, 1989.

4- HARRINGTON, F.E.; ROTHERMEL, J.D. Daily changes in plasma progesterone concentrations in pregnant and pseudopregnant rabbits. **Life Science**, v.20, p.1333-40, 1977.

5- JOHANSSON, E.D. Plasma progesterone in pregnancy measurement by a rapid competitive protein binding technique. **Acta Endocrinologica**, v.61, n.4, p.607-17, 1969.

6- LEE, V.H.; FIELD, P.A. Rabbit relaxin: the influence of pregnancy and ovariectomy during pregnancy on the plasma profile. **Biology of Reproduction.**, v.45, p.209-14, 1991.

7- MIDGELEY, A.R.J.; NISWENDER, G.D.; REBAT, R.W. Principles for the assessment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). **Acta Endocrinologica**. p.142-63, 1969. Supplement.

8- SCHWARZENBERGER, F.; MÖSTL, E.; PALME, R.; BAMBERG, E. Fecal steroid analysis for non-invasive monitoring of reproductive status in farm, wild and zoo animals. **Animal Reproduction Science**, v.42, n.1-4, p.515-26, 1996.

9- SHIDELER, S.E.; ORTUÑO, A.M.; MORÁN, F.M.; MOORMAN, E.A.; LASLEY, B.L. Simple extraction and enzyme immunoassays for estrogen and progesteron metabolites in the feces of *Macaca fasciculares* during non-conceptive and ovarian cycles. **Biology of Reproduction**, v.48, p.1290-8, 1993.

10- SAS – STATISTICAL ANALYSIS SYSTEM. **SAS system for linear models**. Cary : SAS Institute, 211p, 1986.

11- WASSER, S.K.; MONFORT, S.L.; WILDT, D.E. Rapid extraction of fecal steroids for measuring reproductive cyclicity and early pregnancy in free-ranging yellow baboons (*Papio cynocephalus cynocephalus*). **Journal of Reproduction and Fertility**, v.92, p.415-23, 1991.

12- WILCOX, C.J.; THATCHER, W.W.; MARTIN, F.G. Statistical analysis of repeated measurements in physiology experiments. *In*: INTERNATIONAL ATOMIC ENERGY AGENCY. Vienna, 1990. **Proceedings**. Vienna : Livestock Reproduction in Latin America, 1990. p.141-55. (Panel proceeding series)

Received: 23/01/1996
Accepted: 02/09/1997