EFFECT OF DIVERGENT SELECTION FOR UTERINE CAPACITY ON PROGESTERONE, ESTRADIOL AND CHOLESTEROL LEVELS AROUND IMPLANTATION TIME IN RABBITS

MOCÉ M.L., SANTACREU M.A., CLIMENT A.

Departamento de Ciencia Animal. Universidad Politécnica de Valencia.
P.O. Box 22012, 46071-VALENCIA, Spain.

ABSTRACT: The effects of selection for high (H) and low (L) uterine capacity on serum and uterine fluid levels of progesterone and serum levels of estradiol and cholesterol, near implantation (day 6 of gestation), were studied. Both lines had similar ovulation rates (15.2 *vs* 14.4), but different number of embryos (12.8 *vs* 11.0; $P<0.05$) for H and L, respectively. Progesterone, cholesterol and estradiol were similar in both lines (9.3 and 9.6 ng/ml serum progesterone; 15.6 and 13.7 ng/ml of uterine fluid progesterone; 28.1 and 27.6 pg/ml of estradiol; and 66.6 and 68.0 mg/dl of cholesterol for H and L, respectively). Lactation strongly affected the variables studied. Lactating does had higher ovulation rates than non-lactating (15.6 *vs* 14.0; $P<0.05$), but no differences were detected for the number of embryos recovered (12.2 and 11.6 for lactating and non-lactating, respectively) or for embryo survival (0.79 and 0.84 for lactating and non-lactating). L does had lower levels of serum progesterone (8.7 ng/ml), uterine fluid progesterone (13.0 ng/ml) and serum cholesterol (59.3 mg/dl) than non-lactating females (10.2 ng/ml, 16.3 ng/ml and 75.3 mg/dl respectively; $P<0.05$). Lactation did not affect serum levels of estradiol. Serum progesterone levels were slightly correlated with ovulation rate ($r=0.24$; $P<0.05$). Ovulation rate and number of embryos recovered were correlated with progesterone uterine fluid in lactating females ($r=0.55$ and 0.49 respectively; $P<0.05$). Embryo survival in lactating females was negatively correlated with the concentration of estradiol ($r=-0.39$; $P<0.05$).

Key words: Progesterone, estradiol, cholesterol, rabbit, uterine capacity.

RÉSUMÉ: Effet d'une sélection divergente pour la capacité utérine sur les niveaux de progestérone, d'oestradiol et de cholestérol, au moment de l'implantation chez la lapine.
Les effets de sélection pour une capacité utérine haute (H) et basse (L) ont été étudiés sur la concentration de progestérone dans le sérum et dans le fluide utérin, ainsi que sur les niveaux sériques d'oestradiol et de cholestérol, au moment de l'implantation (6ème jour de gestation). Les deux lignées ont un taux d'ovulation similaire (15,2 et 14,4 corps jaunes), mais un nombre différent d'embryons (12,8 *vs* 11,0; P<0,05, respectivement pour les H et les L). Les concentrations de progestérone, cholestérol et oestradiol sont similaires dans les deux lignées (9,3 et 9,6 ng/ml pour la progestérone circulante, 15,6 et 13,7 ng/ml pour la progestérone dans le fluide utérin, 28,1 et 27,6 pg/ml pour l'oestradiol, 66,6 et 68,3 mg/dl pour le cholestérol, respectivement pour les H et les L. La lactation influence de manière importante toutes les variables étudiées. Les lapines allaitantes ont un taux d'ovulation supérieur à celui des non-allaitantes (15,6 *vs* 14,0; P<0,05), cependant aucune différence significative n'est détectée pour le nombre d'embryons retrouvés (12,2 et 11,6, respectivement pour allaitantes et non-allaitantes) ou pour la survie embryonnaire (0,79 et 0,84 pour allaitantes et non-allaitantes). Les lapines allaitantes ont des concentrations plus faible de progestérone dans le sérum (8,7 ng/ml) et dans le fluide utérin (13,0 ng/ml), de cholestérol sérique (59,3 mg/ml) que les lapines non-allaitantes (respectivement, 10,2 ng/ml, 16,3 ng/ml et 75,3 mg/dl, P<0,05). La lactation n'influence pas la concentration d'oestradiol. Le niveau de progestérone sérique est faiblement corrélé au taux d'ovulation (r=0,24; P<0,05). Le taux d'ovulation et le nombre d'embryons retrouvés sont corrélés avec la concentration de progestérone dans le fluide utérin chez les lapines allaitantes (respectivement r=0,55 et 0,49; P<0,05). La survie embryonnaire chez les lapines allaitantes est corrélée négativement à la concentration en oestradiol (r=-0,39; P<0,05).

Mots clés : Progestérone, oestradiol, cholestérol, lapin, capacité utérine.

INTRODUCTION

Selection for litter size leads to modification of ovulation rate and prenatal survival (BLASCO *et al.*, 1993), but little is known about the correlated responses in factors regulating ovulation rate or prenatal survival. Progesterone plays a central role in regulation of prenatal survival. In rabbits, this steroid is produced exclusively by the corpora lutea, and its presence is necessary to maintain pregnancy. Progesterone level increases slowly from day 1, to reach a maximum level on days 10-12 of gestation. This high level declines slightly after day 12, and then remains constant until birth (CHALLIS *et al.*, 1973; BROWNING *et al.*, 1980). These changes in the serum levels of progesterone are related to changes in the

Correspondence: M.L. Mocé. E-mail: mamocer@dca.upv.es

protein composition of the uterine secretion (review by BEIER 2000 in rabbits; VALLET *et al.*, 1998 in pigs).

Initial hormone levels can have an effect on survival through gestation. For example WILSON and FORD, (2000), working with pigs, observed that the amount of estradiol-17β at the time of elongation had an effect on placental size at term. Estradiol regulates progesterone secretion, being considered the principal luteotropic hormone. The effect of these steroids on uterine secretion depends on their individual concentrations and on their ratio (BEIER, 1976; JÄNNE, 1981). MILLER and KEYES, (1975, 1978) observed that rabbit corpora lutea were independent from secretion of estradiol for a period of approximately 5 days after ovulation, but thereafter the corpora lutea required estradiol to continue their development and secretion of progesterone. MILLER and MCLEAN, (1987) suggested that, in rabbits, estradiol regulates the secretion of progesterone by regulating the uptake and storage of cholesterol and its metabolism to progesterone (see review by HOLT 1989).

Very little is known about the effect of selection for litter size on hormonal levels in rabbits and pigs. BLASCO *et al.*, (2000) performed a divergent selection experiment on uterine capacity (UC). Uterine capacity has been defined as the maximum number of foetus that the dam is able to support at birth when ovulation rate is not a limiting factor (CHRISTENSON *et al.*, 1987). In rabbits, BLASCO *et al.*, (1994) proposed litter size in unilaterally ovariectomized females as an estimator of UC. The two lines were divergently selected for 10 generations, and selection was relaxed in the 11th and 12th generations (BLASCO *et al.*, 2000). The resultant lines have similar ovulation rates (ARGENTE *et al.*, 1997; SANTACREU *et al.*, 2000) and similar fertilization rate (GALLEGO, 1997) but different number of implanted embryos (ARGENTE *et al.*, 1997; SANTACREU *et al.*, 2000). The aim of this work is to study the embryo mortality at day six of gestation in these two lines and to examine the serum and uterine fluid levels of progesterone, serum levels of estradiol and cholesterol and their relationships.

MATERIAL AND METHODS

Animals: A total of 141 females from the 11th and 12th generations of the divergent UC selection experiment were used. Seventy-eight females came from the line selected for high uterine capacity (H) and 63 from the line selected for low uterine capacity (L). All data were taken in the third, fourth or fifth parities. Females were housed in individual metal cages and kept under controlled 16L:8D photoperiod. Natural matings were to males from the same line as the females.

Characters measured: Females were weighed and then slaughtered six days after mating. Blood samples were obtained from 112 females (Table 1). Blood was centrifuged at 3000 rpm for 10 min. The serum was stored at –20° C until progesterone, estradiol and cholesterol were measured. A ratio was calculated by dividing serum estradiol by serum progesterone.

The entire reproductive tract was removed after

Table 1: Number of samples in each assay.

	11th generation		12th generation	
	H	L	H	L
Serum progesterone (ELISA)	24	29	-	-
Serum progesterone (RIA)	-	-	29	23
Uterine fluid progesterone (RIA)	-	-	27	22
Serum cholesterol (CHOD-PAP)	28	32	-	-
Serum estradiol (ELISA)	27	30	-	-

H: high uterine capacity line, L: low uterine capacity line.

slaughter and the number of corpora lutea was taken as an estimate of the ovulation rate (OR). The uteri were excised and flushed once with 8 ml of saline solution to recover and count the number of embryos (RE). The uterine flushings obtained from 49 females (Table 1) were centrifuged at 3000 r.p.m. for 10 min to eliminate uterine cells and debris. The supernatant was stored at –20 °C until progesterone assay. Embryo survival at day six of gestation (ES) was estimated as the ratio between RE and OR.

Assays: Two procedures were used for progesterone measurement (Table 1). In 48 females progesterone levels were measured both in uterine flushings and serum by RIA (PROG-CTRIA, CIS BIO INTERNATIONAL, Filiale de Schering S.A., B.P. 32-F91192 Gif-Sur-Yvette Cedex/France). RIA was carried out using ^{125}I labelled progesterone. The sensitivity for the assay was 0.05 ng/ml. The intra- and inter-assay variation coefficients were 3.5% and 4.5% respectively. The cross-reactions were:

6.2% with deoxycorticosterone,
2.2% with 20-α-dihydroprogesterone,
2.1% with 6-β-dihydroprogesterone
and less than 2% with other steroids.

In 53 females, progesterone levels were only measured in serum by ELISA using an automatic analyser (Technicon Immuno 1, BAYER, Tarrytown, NY, USA). The sensitivity of the assay was 0.1 ng/ml. The inter-assay variation was 3.7%. The cross-reactions were 1.3% with 17-α-hydroxyprogesterone for 1 μg/ml, and 0.8% with pregnenolone for 1 μg/ml.

Serum estradiol was measured by using an ELISA kit (EIA-2693, DRG INSTRUMENTS GmbH, Frauenbergstr. 59, D-35039 Marburg, Germany). The sensitivity for the assay was 4.6 pg/ml. The inter-assay variation was 1.99%. The cross- reactions were less than 0.2% with the other steroids.

Serum cholesterol was measured by using an automatic analyser (COBAS MIRA, ROCHE) that used an enzymatic procedure (CHOD-PAP, ROCHE BM).

Statistical analysis: A chi-squared test was performed to determine whether there were differences between the H and L lines in the percentage of females that ovulated. Those females that did not ovulate were excluded for the posterior analysis. Least squares analyses were performed using the GLM procedure of SAS (SAS, 1997). Table 2 shows the effects that were used in each analysis.

To assess the relationships between variables, the following model was fitted:

$$Y = Line + A + Lac + Line{*}A + S + bX + (b{*}Lac)X + e$$

where Y and X are the variables of interest, the Line effect, A the assay effect, Lac the lactation effect, Line*A the interaction between the line effect and the assay effect, S the season effect, b a coefficient of regression, b*Lac the interaction between lactation and the regression coefficient and e the error. When the b* Lac interaction was significant, we estimated the correlation between the residuals of lactating and non-lactating females separately. When data come from only one of the assays, the effect A was removed. When season effect was not significant, it was removed from the analyses.

RESULTS AND DISCUSSION

Twenty females did not ovulate: 15 of 78 (19.2%) from the H line and 5 of 63 (7.9%) from the L line. This difference, although not significant, was relevant, thus more data are needed to assess whether there are real differences between the two lines. Table 3 shows the differences between both lines for the characters studied. Both lines had similar ovulation rates, but the number of embryos recovered at day six of gestation

Table 2: Models fitted for OR: ovulation rate, RE: number of embryos, ES: embryo survival, SPr: serum levels of progesterone, SCh: serum levels of cholesterol, UPr: levels of progesterone in uterine fluid, SEs: serum levels of estradiol and SR: serum ratio of estradiol/progesterone.

	Line	G	A	FW	Lac	Line*G	Line*A	S
OR, RE, ES	+	+		+	+	+		
SPr	+		+	+	+		+	
SCh, UPr	+			+	+			
SEs, SR	+			+	+			+

Line: high and low, G: generation 11th, 12th, A: assay (RIA or ELISA), FW: covariate weight of the female, Lac: lactation status at slaughtering (lactating or non-lactating), Line*G: line*generation interaction, Line*A: line*assay interaction, S: season effect (spring and summer).

was lower ($P<0.05$) in the L line. SANTACREU *et al.*, (2000) reported similar results, with a difference of 2.1 implanted embryos between lines. The difference between lines was relevant for embryo survival but it was not significant, thus we need more data to assess whether there are differences between the lines. SANTACREU *et al.*, (2000) found an embryo survival of 0.89 for H and 0.77 for L at day 7 of gestation. These differences were significant, and rather similar to ours.

The lines had similar serum levels for cholesterol, progesterone, estradiol, and the ratio of estradiol to progesterone. Furthermore, the progesterone levels in uterine fluid were unaffected by line. Thus, the difference in implanted embryos between the lines cannot be explained by differences in cholesterol, progesterone or estradiol at day six of gestation.

The effect of selection on hormone levels has been studied by several authors, but the results do not show a clear pattern. BARKLEY *et al.*, (1979) studied peripheral levels of estradiol and progesterone during pregnancy in selected strains of mice, and observed that the line with high prenatal survival (selected for large litters) had higher levels of progesterone than the lines with low prenatal survival (selected for rapid post-weaning gain and small litters, respectively) especially in the second half of gestation. They also found that the line selected for large litters had higher levels of estradiol than the line selected for rapid post-weaning gain. In mice, RIBEIRO *et al.*, (1994) observed that selection for litter size increased the levels of cholesterol. In pigs, KELLY *et al.*, (1988) did not find any difference in progesterone and estradiol levels during preovulatory and postovulatory phases of the estrous cycle between a control line and in a line selected for high ovulation rate. Thus, selection for litter size or components of litter size brings about variable results and the same is observed when studying the underlying hormonal changes.

Table 3: Least square means (±S.E.) for the high (H) and low (L) uterine capacity lines.

	H	L
Ovulation rate	15.2 ± 0.3	14.4 ± 0.3
Number of embryos	12.8 ± 0.5^{a}	11.0 ± 0.5^{b}
Embryo survival	0.86 ± 0.03	0.78 ± 0.03
Serum progesterone (ng/ml)	9.3 ± 0.3	9.6 ± 0.3
Serum estradiol (pg/ml)	28.1 ± 1.1	27.6 ± 1.0
Serum cholesterol (mg/dl)	66.6 ± 3.5	68.0 ± 3.2
Uterine flushing progesterone (ng/ml)	15.6 ± 1.0	13.7 ± 1.2
Estradiol/progesterone ratio	3.2 ± 0.3	3.6 ± 0.2

Means within a row with different superscripts differ ($P<0.05$).

The mean value for progesterone in serum at day six of gestation was 9.4 ng/ml, in

Table 4: Least square means (±S.E.) for lactating and non-lactating does.

	Lactating	Non-lactating
Ovulation rate	15.6 ± 0.3[a]	14.0 ± 0.3[b]
Number of embryos	12.2 ± 0.5	11.6 ± 0.6
Embryo survival	0.79 ± 0.03	0.84 ± 0.03
Serum progesterone (ng/ml)	8.7 ± 0.3[a]	10.2 ± 0.3[b]
Serum estradiol (pg/ml)	28.0 ± 0.8	27.7 ± 1.2
Serum cholesterol (mg/dl)	59.3 ± 2.7[a]	75.3 ± 3.9[b]
Uterine flushing progesterone (ng/ml)	13.0 ± 1.2[a]	16.3 ± 1.0[b]
Estradiol/progesterone ratio	3.7 ± 0.2	3.1 ± 0.3

Means within a row with different superscript differ ($P<0.05$).

agreement with other authors (COWAN *et al.*, 1976; NOWAK and BAHR, 1983 and LOPEZ *et al.*, 1993), but almost twice the values reported by HARRINGTON and ROTHERMEL, (1977) and KHADR *et al.*, (1994).

In agreement with other authors (COWAN *et al.*, 1976; KHAN-DAWOOD and YUSOFF-DAWOOD, 1984), the mean value for the concentration of progesterone in uterine fluid (15 ng/ml) is higher than the concentrations previously reported in serum.

In the early literature there are reports of substantial differences in the concentration of estradiol at day six of gestation (80 pg/ml *versus* 4 pg/ml *vs* 200 pg/ml as reported by CHALLIS *et al.*, 1973; BROWNING *et al.*, 1980 and KHAN-DAWOOD and YUSOFF-DAWOOD, 1984, respectively). The mean value obtained in the present experiment (27.8 pg/ml) is similar to those found in more recent experiments (DUGRÉ *et al.*, 1989; FORTUN *et al.*, 1993). The variability reported in the results from 1973 to the mid eighties could be due to the lack of sensitivity of the assays used to detect the very low concentrations of estradiol.

The mean value for cholesterol (64.7 mg/dl) is lower than the concentration reported by CHIERICATO *et al.*, (2000) (82 mg/dl) and very different to the results presented by FAVARATO and ZATTA, (1990) (30 mg/dl).

Several explanations may account for the variability of the results found in the literature. In this experiment, it was observed that for serum progesterone, the values obtained using ELISA were lower than those obtained using RIA (8.3 ng/ml *vs* 10.4 ng/ml respectively). Although RIA and ELISA were applied to different sets of samples (Table 1), the females belonged to the same lines and were maintained in similar experimental conditions, thus these differences are likely due to the methodology applied. Moreover, in some of the previous experiments (FOWLER *et al.*, 1977) the number of animals used was small thus affecting the results obtained, which are highly dependent on sample size because these characters are highly variable. This variability can also be due to the lactation state and age of the females (VIRAD DROUET *et al.*, 1984; FORTUN *et al.*, 1993) as was observed in the present experiment when comparing lactating and non-lactating females.

Ovulation rate was higher for lactating females than for non-lactating females, but no differences were found in the number of implanted embryos or in embryo survival (Table 4). FORTUN *el al.*, (1993) and

Table 5: Correlation coefficients between residuals estimated with all females, with only lactating females between "[ ]", or with only non-lactating females between "( )".

	SEs	SCh	UPr	OR	RE	ES
SPr	0.21	-0.01	0.30*	0.24*	0.16	[0.09] (-0.19)
SEs		-0.07		0.17	-0.25	[-0.39*] (-0.42)
SCh				0.02	0.19	0.20
UPr				[0.55*] (-0.01)	[0.49] (-0.01)	0.06
SR				-0.06	-0.25	-0.21

Different from zero *$P<0.05$.
Serum progesterone (SPr), serum estradiol (SEs), serum colesterol (SCh), uterine flushing progesterone (UPr), SR: ratio SEs/SPr, ovulation rate (OR), number of embryos (RE) and embryo survival (ES).

FORTUN and LEBAS, (1994) did not find differences in the ovulation rate between lactating and non-lactating females. The non-lactating females had higher progesterone concentrations in both serum and uterine fluid than the lactating females. These results corroborate the results obtained by FORTUN *et al.*, (1993), who found higher serum progesterone levels in non-lactating females at 7 and 17 days of gestation but no differences in embryo survival at day 7 of gestation. They suggested that lactation has a negative effect on foetal survival, but not on embryo survival. Rabbits are able to lactate and gestate at the same time. However, as the pregnancy progresses the nutritional requirements of foetus become much higher. FORTUN *et al.*, (1994) suggest that the competition between the lactating mother and the foetus brings about smaller foetuses, but this does not explain the higher foetal mortality. They proposed that the higher foetal mortality detected in lactating females was more dependent on the hormonal background associated with lactation than on the metabolic balance of the mother. Prolactin seems to be responsible in part for the higher foetal mortality in lactating females. Progesterone levels do not show a clear pattern in relation to foetal mortality (FORTUN *et al.*, 1999).

The mechanisms explaining the reduced levels of progesterone in lactating females are still unclear. FORTUN *et al.*, (1999) reported that the high levels of prolactin associated with lactation could be responsible for the reduced concentration of progesterone, since females that were injected with prolactin from day 7 to day 21 of gestation had lower levels of progesterone that those females that were injected with vehicle only. Table 4 shows that cholesterol was lower in lactating than in non-lactating females. No differences were found in estradiol levels between lactating and non-lactating females in agreement with FORTUN *et al.*, (1993). Lactating females showed a higher value for the ratio of estradiol to progesterone than non-lactating females (3.7 *vs* 3.1, Table 4). This difference is not significant, but is high enough to be considered relevant because is higher than half of the standard deviation of trait. Thus, more does are needed to assess whether this difference really exists.

Table 5 shows the correlation between the residuals of the least squares analyses of the variables studied. Levels of progesterone in serum were correlated with levels of progesterone in uterine fluid (r=0.30; $P<0.05$), which corroborates the results obtained by FOWLER *et al.*, (1977), who reported that the concentration of progesterone in the uterine flushings depended on the plasma progesterone levels and the levels of uterine proteins. It has been postulated that the high levels of progesterone in uterine fluid can be due to the binding of progesterone to the uterine proteins which sequestrate the steroid from plasma (Borland *et al.*, 1977). Uteroglobin is one of the uterine fluid proteins that binds progesterone (Beato and Baier, 1975). The reason why uteroglobin binds progesterone remains unclear. Uteroglobin may carry progesterone from the uterine lumen to the blastocyst, or it may bind progesterone to prevent the toxic effect that high levels of progesterone may have on the embryo (Beier, 1976; Bochskanl and Kirchner, 1981).

Although cholesterol is the precursor of the steroids produced by steroidogenic tissues, no correlations were found between cholesterol and progesterone nor between cholesterol and estradiol levels. Progesterone and estradiol were not correlated. Kensinger *et al.*, (1986), working with pigs, did not find correlations between these steroids.

The correlation between ovulation rate and serum progesterone was low and positive. Kesinger *et al.*, (1986) found a higher correlation ($r=0.68$; $P<0.01$) in pigs. In rabbits, according to Mills and Stopper, (1989) plasma progesterone is closely related to the number of corpora lutea.

The correlation between ovulation rate and progesterone levels in uterine flushings was relatively high and positive ($r=0.55$; $P<0.05$) in lactating females but no correlation was observed in non-lactating females. The regulation of the hormonal mechanisms is complex, since the effect of a particular hormone depends on the quantity of hormone and on the number of receptors and their interaction with other hormones. Thus correlation coefficients can have higher or lower values due to a large number of different causes. Perhaps in this case the effect of prolactin on progesterone modified these correlations.

No correlations were observed between ovulation rate and serum levels of cholesterol or estradiol. These results were expected, since neither cholesterol nor estradiol are secreted by the corpora lutea.

In lactating females there was a correlation between progesterone in uterine fluid and the number of embryos recovered, but no correlation was found between progesterone in uterine fluid and embryo survival. The concentration of serum progesterone or estradiol at day six of gestation is not correlated with the number of embryos recovered, in agreement with Challis *et al.*, (1973) in rabbits. Kensinger *et al.*, (1986) did not find any relationship between the number of foetuses and these two steroids in pigs slaughtered at 110 days of gestation.

Embryo survival in lactating females was negatively correlated with the concentration of estradiol. Jänne, (1981) observed that low doses of estradiol increased the effect of progesterone on uteroglobin secretion, while higher doses reduced the progesterone action and brought about a reduction in the synthesis of uteroglobin. Reduction in uteroglobin secretion may lead to an asynchrony between embryo and uterine secretion that increases embryo mortality. It seems that the ratio of serum estradiol to serum progesterone and the serum levels of cholesterol are not correlated with embryo survival or with the number of embryos recovered.

In summary, it seems that the differences in number of embryos between H and L occur before day six of gestation and the differences are not due to differences in the concentrations of progesterone, estradiol or cholesterol. Moreover, it was observed that lactation has a strong effect on the concentration of progesterone and cholesterol, but not on embryo survival up to day six of gestation. Lactating and non-lactating females had different correlations between ovulation rate and progesterone in uterine flushings, showing the complexity of hormonal regulation patterns.

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