PERIPHERAL PLASMA PROGESTERONE AND THE NUMBER OF FOETUSES IN NORMAL AND SUPEROVULATED RABBITS

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(Received 8th August 1969, revised 23rd October 1969)

Summary. Peripheral plasma progesterone levels in pregnant and superovulated pregnant rabbits at 10, 20 and 30 days *post coitum* were studied. No significant differences in levels of progesterone were noted throughout pregnancy in either the control or the superovulated rabbits. Although superovulation resulted in increased numbers of corpora lutea, normal foetuses and total implantations (normal and degenerate), no increase in peripheral plasma progesterone was indicated in the experimental animals.

INTRODUCTION

Superovulation in rabbits has been studied extensively since the original investigation by Pincus (1940). In all of these studies, a high degree of embryonic mortality resulting, in part, from overcrowding of the uterus has been noted (Parkes, 1942; Venge, 1950; Adams, 1960a, b, 1962; Hafez, 1964; Hafez & Sugawara, 1968). Although Adams (1962) found an increase in litter size, most workers have found that superovulation does not increase the number of young born.

Reports of the levels of progesterone during pregnancy in the rabbit are not in close agreement with one another; the discrepancy may result from differences in method or from use of different biological material, e.g. peripheral or ovarian vein blood. The earlier studies (Forbes, 1953; Zarrow & Neher, 1955) with the Hooker-Forbes assay, differ greatly from more recent investigations (Mikhail, Noall & Allen, 1961; Okano, Matsumoto, Kotoh, Endo & Seki 1966; Hilliard, Spies & Sawyer, 1968) because steroids other than progesterone were also influencing the assay.

In general, progesterone concentration increases to mid-pregnancy in the rabbit, declines during the second half, and is low before parturition (Mikhail *et al.*, 1961; Hilliard *et al.*, 1968). Similar findings, but with greater variation, were reported by Okano *et al.* (1966). Progesterone data are available for non-pregnant rabbits and those stimulated by gonadotrophin (Simmer, Hilliard & Archibald, 1963; Hafez, Tsutsumi & Kahn, 1965; Okano *et al.*, 1966) but little

is known about the progesterone levels in superovulated, pregnant rabbits. Hafez *et al.* (1965), using the Hooker-Forbes assay, reported ovarian tissue and effluent blood levels of progestins in superovulated rabbits. However, the data presented are sparse and were not correlated with the stage of pregnancy.

Although pre-natal embryonic mortality in superovulated rabbits has been attributed to overcrowding of the uterus, progesterone insufficiency might be a related factor. This study was undertaken to determine whether: (1) super-ovulation would increase litter size, (2) progesterone secretion in the super-ovulated animal was similar to that after normal ovulation, and (3) progesterone levels could be related to embryonic mortality.

MATERIALS AND METHODS

Sixty, virgin, Dutch-belted does (16 weeks or older) were used in this study; thirty were assigned to three control groups and thirty to three experimental groups. Each rabbit in the control group was mated once or twice by a male of known fertility. On the 10th, 20th or 30th day of gestation, the rabbits were anaesthetized with ethyl oxide and 30 to 40 ml of blood were withdrawn by heart puncture. The blood was placed in a centrifuge tube which contained ethylenediamine-tetra-acetic acid (EDTA) and centrifuged at 1800 rev/min (700 *g*) for 20 to 30 min. After centrifugation, the haematocrit was recorded and the plasma was removed and stored in the frozen state until the day of extraction. The numbers of corpora lutea, normal implantation sites and degenerate implantation sites were recorded; the rabbits were killed with an overdose of anaesthetic.

The experimental animals were superovulated according to the method of Kennelly & Foote (1965) which was slightly modified. Follicle stimulating hormone, 0.5 mg FSH (Nutritional Biochemical Corp.; NBC) was administered subcutaneously twice-daily for 3 days, followed by 50 i.u. of human chorionic gonadotrophin (HCG, NBC) injected intravenously immediately after mating on the 4th day. Laparotomy and blood sampling were performed the same way in control and treated animals.

Progesterone extraction

Frozen plasma was thawed at room temperature and the volume measured. The plasma was transferred to a 50-ml Erlenmeyer flask into which 50 λ ^{14}C -labelled progesterone (111,000 d/min; specific activity: 57.3 mc/m-mole, New England Nuclear) was added. Following equilibration (10 to 15 min), the lipids were extracted three times with 30 ml of ethyl ether and the ether fractions were dried in 40-ml centrifuge tubes on a water bath (25° C) under vacuum. Any protein which may have been present was precipitated by adding 8 ml of acetone to the dried residue. After centrifugation for 10 min at 1800 rev/min (700 *g*), the acetone fraction was removed and dried in a small sample vial under nitrogen. The centrifuge tube was then washed twice with 10 ml of ethyl ether and the ether fraction removed and dried as above.

Chromatography

Using benzene/petroleum ether (1/1), the extracts were chromatographed on

250 μ , thin-layer plates of silica gel GF 254 (Brinkmann Instruments) in the following systems; (1) heptane (70% methanol-saturated) for 4 hr (Luisi, Gambassi, Marescotti, Savi & Polvani, 1965), (2) hexane/ethyl acetate 3/1, (Armstrong, O'Brien & Greep, 1964), (3) benzene/ethyl acetate 2/1, two-dimensionally. The progesterone area was identified under ultraviolet light, eluted with 3 ml of chloroform/methanol (2/1) and 3 ml ether (twice). The progesterone eluates were further purified by chromatography (one dimension) on 200 μ thick plates in the systems hexane/ethyl acetate, 3/1, and isopropyl ether/ethyl acetate, 5/2. After eluting the progesterone area again, a final chromatographic purification was performed on either 150 μ thick silica gel plates or chromatograms (Eastman-Kodak), utilizing the hexane/ethyl acetate (3/1) and isopropyl ether/ethyl acetate (5/2) systems. Samples were eluted as before and, after drying, were stored frozen under a layer of benzene until final analysis.

Progesterone analysis

The progesterone samples were thawed and dried on a sand bath. Benzene (100 λ) was added to each vial after which 10 λ of each sample was transferred to a scintillation counting vial. Ten ml of POPOP-PPO (Packard Instrument Co.) in toluene was added to each scintillation vial and two 1-min counts were read in a TriCarb liquid scintillation counter (87% efficiency). The remaining sample (90%) was dried down and later taken up with 9 λ of 100% redistilled ethanol; 1 λ was injected into a Perkin-Elmer 881 gas chromatograph. The chromatograph columns were packed with SE-30/S (Perkin Elmer). Standard progesterone curves (0.1 to 0.5 μ g) were run each day for comparison with the unknowns.

RESULTS

The number of corpora lutea, normal fetuses and total implantations (normal and degenerate) at 10, 20 and 30 days *post coitum* (*p.c.*) are shown in Table 1. It is apparent that superovulation was successful and that the number of normal fetuses at 10 days was greater than that at 20 and 30 days. The number of normal fetuses in the experimental animals exceeded that of the controls at 30 days *p.c.*, indicating a significant increase in litter size ($P < 0.05$).

Peripheral plasma progesterone levels (μ g/ml) for pregnant and super-ovulated, pregnant rabbits at 10, 20 and 30 days *p.c.* are shown in Table 2. All values represent mean values of ten animals/group with the exception of the 10- and 20-days control groups which had seven and nine animals, respectively. These missing values were due to sample loss during extraction. Comparisons between 10-, 20- and 30-day animals within the control and experimental groups were not significantly different indicating that the peripheral progesterone levels did not change on these particular days of pregnancy in either the control or the treated groups. The relatively high standard errors in Table 2 were the result of animal variability. The mean recovery of progesterone in this experiment was 37.3%. The progesterone levels were not correlated with the number of corpora lutea or the number of implantations.

DISCUSSION

It appears that the high degree of pre-natal mortality which occurred between 10 and 20 days *p.c.* was the result of overcrowding in the uterus. Since implantations occurred in all the experimental animals and large numbers of foetuses were maintained until 30 days *p.c.*, a deficiency of progesterone as a causative factor is doubtful.

TABLE 1

NUMBER OF CORPORA LUTEA, NORMAL IMPLANTATIONS AND TOTAL NUMBER OF IMPLANTATIONS (NORMAL PLUS DEGENERATE) IN CONTROL AND SUPEROVULATED RABBITS

Observations	Days <i>p.c.</i>					
	10 days		20 days		30 days	
No. corpora lutea (Mean $\pm$ S.E.)						
Left ovary, control	3.7	(0.73)	2.2	(0.49)	3.6	(0.79)
Right ovary, control	4.2	(0.63)	4.8	(0.74)	4.7	(0.71)
Left ovary, superovulated	21.9	(5.34)	23.8	(4.71)	18.2	(1.95)
Right ovary, superovulated	20.7	(4.88)	21.3	(4.34)	19.7	(2.57)
No. normal implantations (Mean $\pm$ S.E.)						
Left uterine horn, control	3.6	(0.64)	1.5	(0.45)	3.0	(0.69)
Right uterine horn, control	4.0	(0.65)	4.1	(0.78)	3.6	(0.70)
Left uterine horn, superovulated	10.5	(0.82)	4.6	(0.93)	5.1	(0.55)
Right uterine horn, superovulated	8.3	(1.20)	4.4	(1.20)	4.5	(0.67)
No. implantations, normal plus degenerate (Mean $\pm$ S.E.)						
Left uterine horn, control	3.6	(0.64)	1.7	(0.42)	3.1	(0.70)
Right uterine horn, control	4.0	(0.65)	4.4	(0.65)	3.7	(0.68)
Left uterine horn, superovulated	10.5	(0.82)	10.1	(1.60)	10.1	(1.00)
Right uterine horn, superovulated	8.3	(1.20)	9.2	(1.60)	10.7	(1.30)

Peripheral plasma progesterone levels during pregnancy in the rabbit do not vary significantly at 10, 20 and 30 days *p.c.* These data differ from the ovarian venous progesterone levels as reported by Mikhail *et al.* (1961) and Hilliard *et al.* (1968) in that no increase in progesterone was observed at mid-pregnancy in the pregnant control rabbits. Even more interesting was the fact that progesterone levels did not increase during pregnancy in the superovulated rabbits, indicating that varying the number of corpora lutea does not affect the level of circulating peripheral progesterone. Because there was no significant dif-

TABLE 2

PERIPHERAL PLASMA PROGESTERONE ($\mu\text{G}/\text{ML}$) IN CONTROL AND SUPEROVULATED RABBITS

Days <i>p.c.</i>	Plasma progesterone ($\mu\text{g}/\text{ml}$) Mean $\pm$ S.E.	
	Pregnant	Superovulated, pregnant
10	0.200 $\pm$ 0.334 (7)*	0.151 $\pm$ 0.185 (10)
20	0.081 $\pm$ 0.095 (9)	0.127 $\pm$ 0.161 (10)
30	0.160 $\pm$ 0.186 (10)	0.130 $\pm$ 0.168 (10)

* No. of animals/group.

ference in the peripheral progesterone levels of the controls and the treated rabbits, it may be indicative that the amount of progesterone produced by each corpus luteum is less when many are present. It is also possible that ovarian progesterone secretion is greater in superovulated rabbits but that peripheral levels are similar to the controls because of differences in the rate of turnover.

Although other data indicate that progesterone levels increase sharply after mating or LH administration (Hilliard, Endröczy & Sawyer, 1961; Hilliard, Hayward & Sawyer, 1964), it was impossible to observe this effect in our study since the first levels were determined on Day 10. Decline in ovarian venous blood progesterone level at parturition has been noted by Mikhail *et al.* (1961) and Hilliard *et al.* (1968). The data of Okano *et al.* (1966) show a level of 0.18 µg/ml on Day 28, followed by a sharp drop on Day 31 to non-detectable levels. Since our data at 30 days *p.c.* are similar to their 28-day level, this may indicate that progesterone decreases sharply on Day 31.

Halliday (1959) reports that corpora lutea atretica occur frequently in rabbits and suggests that their occurrence may be related to ovum loss before implantation. Whether this affects the levels of progesterone and is related to embryonic mortality after implantation is uncertain. It appears that the 'physiological' level, noted in both pregnant and superovulated pregnant rabbits in this study, is at a high enough titre since normal implantations occurred in all of the experimental animals. Whether these levels after 10 days *p.c.* were sufficient to maintain pregnancy in all the experimental rabbits cannot be determined in this study. Because many experimental animals did contain a large number of normal fetuses at laparotomy on Day 30, the hormonal requirements for progesterone must have been fulfilled in some of the rabbits.

ACKNOWLEDGMENTS

The authors wish to thank Dr R. T. Duby, Mr D. C. Sharp, III, and Dr R. A. Damon, Jr, for their assistance in this study. A special word of thanks is extended to Mr F. J. Auletta, III, and Dr G. N. Currie for their advice and consultation throughout the extent of the progesterone analysis. This work was supported by the Northeastern Cooperative Regional Project NE-41, Endocrine Factors Affecting Reproduction in the Bovine Female.

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