

OVARIAN FUNCTION, FOLLICULAR OESTRADIOL- 17β , AND LUTEAL PROGESTERONE AND 20α -HYDROXY- PREGN-4-EN-3-ONE IN CYCLING AND PREGNANT EQUINES

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INTRODUCTION

The essentials of the involved sequence of endocrine changes associated with reproduction in the mare still present much scope for speculation. Thus some clinicians are inclined to regard these aspects of the equine endocrine system as obscurities and state that therapeutically hormones should be used sparingly, if at all (Sager, 1966). This view is certainly not due to apathy, as the fertility rate in these economically valuable animals seldom exceeds 50 per cent (Ensminger, 1966). Disturbances of endocrine function appear to be the major causes of this low fertility rate (Day 1939; Van Rensburg & Van Heerden, 1953).

Classical concepts of gonadal follicular quiescence during most of the sexual cycle have not been substantiated by work on several species during recent times. Therefore, in view of the many described effects of oestrogens on the functional corpus luteum, oestrogen synthesis in the presence of an active corpus luteum deserves further study. Equine gestation is also of particular interest because of the resemblance to primates in that the placenta actively produces steroids, and large amounts of gonadotrophins appear in the peripheral plasma. In the human, this gonadotrophin appears to be devoid of any action on the ovary, which in any event is dispensable at about the time of appearance of chorionic gonadotrophin. On the other hand pregnant mare's serum gonadotrophin (PMSG) is generally accepted as being responsible for marked ovarian activity, though some specious reasoning is necessary to justify this view.

The detailed studies of Short (1960, 1961b, 1964) on equine ovarian steroid biosynthesis, coupled with the size of the ovary, greatly facilitate the study of gonadal function in the mare. Short found that oestradiol- 17β was the major steroid in the fluid of follicles not undergoing luteinization, and that oestrone was consistently present at a concentration of about 20 times less. This we confirmed and therefore in this study only oestradiol- 17β was assayed. Progesterone and 20α -hydroxy-pregn-4-en-3-one appear to be the major steroids in the corpus luteum (Short, 1962) and both were assayed.

No systematic studies on luteal function in mares have been reported, but Short (1961a) and Knudsen & Velle (1961) have examined follicular fluid at various stages of the reproductive cycle. Short failed to find any significant difference in concentration in oestrous and luteal phase animals with respect to several steroids

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OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

measured, whereas Knudsen & Velle found high oestrogen values during oestrus and metoestrus. These results may be interpreted only to a limited extent, as the stage of the cycle was generally approximated. Our data suggest that changes occur rapidly and in a regular sequence, making accurate dating of material obligatory to obtain an improved understanding of physiological function. The nature of the material precludes a statistically valid experimental design, but we believe most of our interpretations are justified and may be a useful basis for further possible studies.

MATERIAL AND METHODS

Animals

Material for this study was obtained incidentally from normal horses slaughtered at the Institute's abattoir for other purposes. Suitable ovaries were collected from a total of 46 mares of the light farm type, and five mules (donkey male \times horse female). Sexual activity was studied in 40 of the mares for periods of several months or longer prior to slaughter. This group was kept on natural pasture but during winter supplementary feeding in the form of lucerne hay and concentrate mixtures was provided. All these mares were teased daily by vigorous stallions to detect oestrus, and were examined rectally every 1 to 3 days. During oestrus rectal palpation was performed daily or more frequently so as to establish precisely the time of ovulation. When the mares were served, a pregnancy diagnosis was made between the 17th and 20th day after ovulation as described by Van Niekerk (1965a).

Collection of material

Prior to slaughter the animals were weighed and stressful situations or starvation avoided. The genital tract was rapidly removed and the ovaries dissected free and weighed. The number and size of the follicles were recorded and the follicular fluid aspirated from all follicles greater than 3 mm in diameter. Corpora lutea were dissected, weighed, and incised longitudinally. When present, the cavities within the corpora lutea usually contained a sero-fibrinous mass which could not be removed to obtain accurate luteal tissue weights, as the material was required for a collateral morphological study. Follicular fluid and half of the corpus luteum were frozen on solid CO_2 within 40 minutes of stunning, then stored at -15°C until assay. Individuals were aged according to usual dentition criteria.

Reagents and apparatus

Glassware was washed with methanol and hydrochloric acid as described by France, Rivera, McNiven & Dorfman (1965).

"Riedel-De-Haen" or "Merck" A.R. quality reagents were used. Volatile chemicals were fractionally distilled and residues of the purified fractions examined by gas chromatography before use. If necessary, the solvents were purified further as described by Bush (1961). Progesterone and oestradiol- 17β were obtained from Steraloids Ltd., and 20α -hydroxypregn-4-en-3-one was kindly donated by the M.R.C. steroid reference collection. Whatman No. 20 chromatography paper sheets were laned into three 1.5 cm wide strips for the blank, sample and authentic reference steroid. Paper chromatography was performed in a thermostatically controlled room equipped with an air turbulence fan at 32°C . "Camag" thin layer chromatography apparatus was used at room temperature.

Gas chromatography was performed on a Beckman G.C. 4 fitted with a 6 ft glass column packed with 3 per cent S.E. 30 on Gaschrom Q. Operating conditions were as follows: on-column inlet 300°C ; column 210°C ; detector 270°C ; nitrogen carrier flow 50 ml/min.

Progesterone and 20 α -hydroxypregn-4-en-3-one assay

A portion of frozen luteal tissue weighing approximately 200 mg was taken from a point midway between the ovulation point and the embedded pole of the ovary and weighed to the nearest 0.1 mg. The sample was homogenized in alkali and extracted as described by Rowlands & Short (1959). Further purification was according to the method of Short (1958) but using 90 per cent aqueous methanol as the stationary phase and running the chromatograms for 3 hours. The eluates were measured in standard curvettes; our spectrophotometer could detect 0.1 μ g of Δ^4 3 ketosteroid in 3 ml ethanol.

Oestradiol-17 β assay

The degree of hyperaemia of the follicle wall was usually proportional to the oestrogen content of the follicular fluid, thus according to expected concentration a maximum of 5 to 20 ml fluid was used for assay. The high concentrations in such a small sample made a simple method possible.

Follicular fluid aliquots were thawed and extracted once with 10 volumes diethyl ether. The follicular fluid was discarded and acidic contaminants removed by washing twice with $\frac{1}{4}$ th volume 1N NaOH saturated with NaCl (Mellin, Erb & Estergreen, 1965). After washing twice with 10 per cent volumes of water, the ether was dried on a Büchi vacuum evaporator and the residue transferred to small tubes for spotting with methanol. The laned paper sheets were placed in a descending chromatography tank using the system toluene: petroleum ether: methanol: water 5:5:8:2.

After one hour equilibration the chromatograms were run for 2.5 hours, air dried, and the reference strips dipped through a freshly mixed aqueous solution of 2 per cent ferric chloride and 2 per cent potassium ferricyanide. The oestradiol area on the sample strip was eluted with 4 ml methanol in a simplified Zander-Simmer type apparatus along with a blank of the same size and R_f value. The remainder of the sample strip was also dipped through the detecting reagent to estimate visually the amount of oestrone, and to ensure that the oestradiol spot had been fully removed.

Eluates were dried under nitrogen at 50°C, and the residue transferred to Kober tubes with 1.0 and 1.0 ml benzene to reduce paper carbohydrate impurities. The Kober reaction for oestradiol was carried out as described by Brown (1955) and the samples were read at 480, 515 and 550 m μ .

RESULTS

*Analytical methods**Progesterone and 20 α -hydroxypregn-4-en-3-one*

A series of 15 duplicate analyses was processed from different portions of the corpus luteum on different days; the mean values ranged from 1.4 to 83.2 μ g/gm. The standard deviation of the results from their means was 1.67 μ g/gm (S.E. 0.30 μ g/gm). This relatively low error of the method indicates that variation of the progesterone concentration in these large corpora lutea is low; furthermore in our hands the method of Short used yielded purer extracts from equine corpora lutea than when applied to bovine corpora lutea.

When 10.0 μ g progesterone was added to the alkaline tissue homogenate in five experiments, a mean of 74.4 per cent (S.E. 1.53) was recovered; consequently the results for both hormones were corrected for losses by multiplying with a factor of 1.33.

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

The characteristics of the isolated steroids were investigated in several thin layer chromatographic systems and by gas chromatography before and after acetylation. When the equine progesterone fraction was treated with 20 β -hydroxysteroid dehydrogenase as described by Heap (1964), the reaction product and its acetate exhibited the same chromatographic characteristics as authentic 20 β -hydroxypregn-4-en-3-one and 20 β -acetoxypregn-4-en-3-one respectively. These procedures revealed the absence of significant impurities and provided further evidence that the isolated steroids were identical to the authentic reference standards.

Oestradiol-17 β

When 5 to 10 μ g oestradiol-17 β was added to follicular fluid, a mean recovery of 80.7 per cent (S.D. 3.08; S.E. 1.26; six experiments) was obtained; the results were therefore corrected by multiplying by a factor of 1.25. Only two samples were assayed in duplicate; results of the one were identical and the other differed by 3.4 per cent.

Four samples were also assayed by the more elegant procedure of Short (1960). When corrected for the 15 per cent higher losses we experienced with this procedure the results were almost identical to those obtained in the more rapid assay described. Gas chromatography of the isolated phenolic steroid and its acetate revealed single major peaks with retention times identical to authentic oestradiol-17 β and its diacetate respectively. The very minor impurities present were also obtained from the benzene-soluble fraction of blank paper eluate residue.

The K \ddot{a} gi-Meisner reaction was carried out as described by Adlercreutz & Luukkainen (1965) on approximately 15 μ g of equine oestradiol; the characteristic pink colour produced by oestradiol-17 α was absent. Fractions were run in the silica gel thin layer system benzene:ethanol 22:3 along with authentic β and α oestradiol; the acetates were run in benzene:ethanol 19:1. In these systems oestradiol-17 α and its diacetate had a slightly greater R $_f$ value than oestradiol-17 β . The R $_f$ values of the equine fraction and the colours obtained with the phosphomolybdic acid and anisaldehyde-sulphuric acid reactions (Adlercreutz & Luukkainen, 1965) were identical to the results obtained with oestradiol-17 β .

Ovarian morphology

Ovarian weight in 16 cyclic mares showed no clear variations during the oestrous cycle (Table 1). In pregnant animals there was a tendency for the ovarian weight to increase to a maximum on the 27th day after ovulation, and then to decline progressively with advancing gestation (Table 2).

The total number of follicles larger than 3 mm in diameter also exhibited no distinct variations during the cycle. However, during early gestation numerous follicles were present. The number appeared to increase linearly from a total of six at 14 days, to 22 on the 25th day, followed by a rapid decrease during the 26 to 30 day period. Following ovulation, the corpus luteum remains functional for about 14 days; during this period 13 cycling mares had a mean of 9.8 (S.E. 1.1) follicles in the ovaries. Seven mares 16 to 28 days pregnant had significantly more ovarian follicles (mean 15.3; S.E. 1.5; $P < 0.01$).

Immediately following ovulation in cycling mares, the total volume of follicular fluid aspirated from the ovaries was variable, as only some mares developed additional large follicles which failed to ovulate at oestrus. The volume of fluid during the luteal phase was slightly but not significantly less than during the follicular phase of the cycle. There was some evidence of increased follicular growth in two of three

TABLE 1.—*The age, weight and reproductive data of anoestrous and cycling mares*

Mare		Reproductive status		Ovarian weight (gm)	Follicles			Corpora lutea			
No.	Age (years)	Weight (lb)			No. > 3mm	Volume of largest (ml)	Total fluid (ml)	Oestradiol (μ g/100 ml)	Weight (gm)	Progesterone (total, μ g)	20 α -hydroxy-pregn-4-en-3-one (μ g/gm)
1	1.8	698	Pubertal.....	82	19	4.5	23.8	3.4	—	—	—
2	4	816	Anoestrus.....	63	6	7.0	21.0	3.4	—	—	—
3	5	928	Anoestrus.....	110	15	9.0	66.0	4.1	—	—	—
4	4	584	Anoestrus.....	80	4	16.5	35.5	71.6	—	—	—
5	5	751	Anoestrus-oes-trus.....	98	13	37.0	79.5	88.5	—	—	—
6	11	1022	4 hours post ovulation.....	76	12	1.5	7.0	1.1	—	—	—
7	18	916	10 hours post ovulation.....	87	10	6.0	16.0	2.6	—	—	—
8	11	758	18 hours post ovulation.....	107	12	23.0	42.0	206.2	4.5	11	—
9	3	944	24 hours post ovulation.....	113	7	32.0	42.5	84.9	3.5	14	—
10	2	891	3 days post ovulation.....	74	7	1.0	5.8	<6.0	6.9	112	—
11	6	849	4 days post ovulation.....	134	5	40.3	43.0	210.9	11.4	230	5.3
12	3	701	6 days post ovulation.....	134	14	9.2	49.0	14.3	11.0	261	—
13	6	816	7 days post ovulation.....	56	—	—	3.4	11.5	10.8	270	—
14	6	977	8 days post ovulation.....	104	6	1.5	6.5	<5.0	8.3	361	8.8
15	11	818	8 days post ovulation.....	90	16	2.8	40.6	12.8	6.9	241	2.9
16	11	1030	10 days post ovulation.....	171	5	54	21.9	17.3	10.3	526	4.9
17	4	837	12 days post ovulation.....	106	11	12	64.7	104.4	5.7	363	13.4
18	9	1092	14 days post ovulation.....	142	15	34	42.0	97.9	5.0	355	40.0
19	3	967	16 days post ovulation.....	156	21	38	39.5	165.8	2.9	6	—
20	5	926	17 days post ovulation.....	103	15	20	74.0	351.0	2.0	3	—
21	5	704	19 days post ovulation.....	74	11	28.4	53.7	189.6	1.4	<1	—
22											

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

TABLE 2.—*The age weight and reproductive data of pregnant mares*

Mare		Days Gestation	Ovarian weight (gm)	Follicles			Corpora lutea			
No.	Age (years)			Weight (lb)	No. > 3mm	Volume of largest (ml)	Total fluid (ml)	Oestradiol (µg/100 ml)	Weight (gm)	Progesterone (total, µg)
23	6	1010	14	7	29	39.5	144.0	7.8	581	2.8
24	13	917	16	10	22	43.0	294.0	7.5	492	1.9
25	7	850	17	—	—	—	—	5.3	453	8.1
26	5	985	18	15	31	58.3	16.5	5.3	335	<0.5
27	11	882	18	12	49.0	49.0	14.1	6.1	326	<0.5
28	18	1228	21	40	65.0	72.0	80.9	7.3	383	—
29	10	147	21—Aborted	12	25	68.0	51.4	7.3	486	—
30	10	1015	23	22	33	64.5	117.5	7.6	570	<0.5
31	11	1419	25	21	21	68.0	114.0	6.9	764	<0.5
32	5	825	27—Resorbing	13	25	55.0	112.5	7.5	504	<0.5
33	5	795	28	14	14	45.0	10.0	7.5	571	<0.5
34	10	940	28	23	23	57.0	41.5	8.2	625	<0.5
35	14	930	30	4	33	57.0	40.5	7.5	418	—
36	16	980	30	6	17	41.5	96.6	8.9	—	—
37	8	950	36	5	5	9.3	3.1	10.2	500	<0.5
38	6	686	40—Lactating	3	5	—	—	*6.7	537	<0.5
38	18	1305	41—Resorbing	13	3.5	81.5	—	*9.3	584	<0.5
39	15	847	55	6	3.5	10.0	3.8	*10.9	528	<0.5
40	5	759	68	4	1.4	3.5	<15.0	*9.1	538	—
41	6	740	Approx. 90	0	—	—	—	*2.2	497	<0.5
42	6	1047	Approx. 130	0	—	—	—	*5.1	294	<0.5
43	4	724	Approx. 300	0	—	—	—	†11.2	1007	<0.5
44	6	1140	Approx. 315	0	—	—	—	Multiple small	±1600	—
45	10	1072	321	8	2.6	10.7	<2.5	C.L. Albi-cantia	±5	<0.5
								C.L. albi-cantia	<3.0	<0.3
								C.L. albi-cantia	<3.0	<0.3

† Secondary

* Primary

mares during the latter part of the luteal phase (12 to 14 days post ovulation), and in two pro-oestrous mares (16 and 17 days post ovulation). The total volume of follicular fluid from 4 to 10 days averaged 32.2 ml (S.E. 7.0), but was increased in the 12 to 19 day cycling mares (mean 54.4, S.E. 6.1, $P < 0.05$). The only oestrous mare examined had a relatively low volume of follicular fluid on the 2nd day of oestrus, possibly due to the fact that she was a young and small animal.

Growth of follicles at about the time of the first expected oestrus in pregnant mares is also clearly evident from a rise in the total volume of the follicular fluid from the 14th to the 23rd days post ovulation. This peak of about 70 ml on the 23rd day of pregnancy was followed by a linear decline to 25ml on the 30th day. A second rise was evident prior to the 40th day, at which time accessory ovulations could be expected.

By means of rectal palpation, individual follicles were sometimes found to exhibit considerable growth during the first three to four days after ovulation, thereafter they rapidly regressed as the corpus luteum developed. In the slaughter series one animal at four days post ovulation had a 40 ml follicle, but in the mid-luteal period the average volume of the largest follicle was less than 10ml. Pro-oestrous follicular growth, which appears to commence in the late luteal period, resulted in follicles containing about 35 ml fluid.

Oestradiol-17 β

Anoestrous mares

A group of five anoestrous animals with variable ovarian follicular growth has been arranged according to the size of the largest follicle in Table 1.

A young mare aged 1.8 years exhibiting considerable prepubertal follicular growth had a total of only 0.81 μg oestradiol in the follicular fluid. Two mature anoestrous mares with about the same degree of follicular growth had amounts of oestradiol similar to that found in the prepubertal filly. In the following mare with a 16.5 ml follicle, the follicular fluid was found to contain 25 μg oestradiol, while the mare showing the most marked follicular growth with a 37 ml follicle had a total value of 70 μg . This latter value was similar to that found in cycling mares at about the time of oestrus, and examination of her genital tract suggested that the first oestrous period of the season was imminent.

Cycling mares

Within a day of ovulation, total oestradiol values (Fig. 1) in four mares ranged from less than 0.1 μg to 86 μg . This variation is due to variable accessory large follicles at oestrus which fail to ovulate. From data obtained by rectal palpation, it seems that follicular growth is not always effectively suppressed during the first few days of the cycle. The follicular fluid of the animal assayed on day four revealed a high oestrogen content in the ovaries of such individuals.

In all four luteal phase animals examined six to ten days after ovulation only traces, and in one case no oestradiol, were detected. However, as early as 12 to 14 days post ovulation high levels were again found in two cycling and one pregnant mare.

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

Further marked elevations of total oestradiol values were observed in the pro-oestral period; a 16-day pregnant mare and two 16 and 17-day cycling animals had the highest total oestradiol values found in the series. Only one young cycling mare was examined during oestrus and on the second day her value was relatively low.

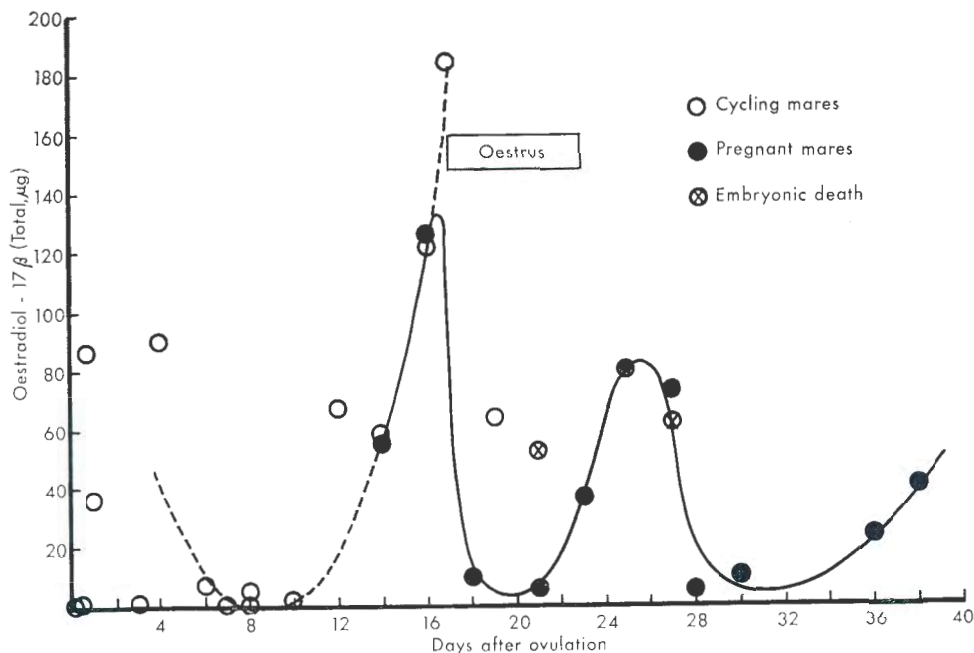


FIG. 1.—Total ovarian follicular fluid oestradiol-17 β in cycling (broken line) and pregnant (solid line) mares

Pregnant mares

As mentioned above, two pregnant mares exhibited oestradiol rises indistinguishable from cycling mares prior to their first expected oestrus. There was no reason to suspect that they were not normally pregnant as their corpora lutea were functional and embryos normal. This considerable rise is short-lived in pregnant mares, as is suggested by the depressed levels in Fig. 1 during the 17th to 22nd days of gestation.

The second suggested rise during pregnancy reached a peak at 25 days; this was unexpected as it occurred at a time when the follicular fluid volumes suggested linear regression. A third period of depressed oestradiol concentrations was terminated in a slow rise prior to the probable occurrence of accessory ovulations at about the 40th day of gestation.

By the 55th day of gestation, only 0.4 μg was detected in the ovaries of one mare, and none in the six mares in more advanced stages of gestation.

Follicle size and oestradiol concentration

From the data in Table 1, it may be seen that the concentration of oestradiol in eight samples from cycling mares, whose largest follicle contained less than 20 ml fluid, did not exceed 26 $\mu\text{g}/100$ ml. However, when the volume of the largest follicle exceeded 20 ml, the concentration was 98 to 351 $\mu\text{g}/100$ ml in eight such animals.

Though there appeared to be a slow linear rise in steroid concentration with increasing volume in the group with follicles less than 20 ml, the size above 20 ml was not related to the oestradiol concentration. Pregnant mares differed as in several animals with large follicles the concentration was low.

Individual large follicles were assayed separately from the remaining pooled fluid in 11 mares (Table 3). These results show that the largest follicle in cycling mares contains most of the oestradiol, while the amount in the smaller follicles is almost negligible. This did not apply to two of four pregnant mares; however, in the majority, the smaller follicles tended to have higher concentrations as the amount of oestradiol in the large follicle decreased. Further support for the possibility that the concentrations in smaller follicles is inhibited by an active large follicle is provided by mares 5 and 32. In both, the pooled fraction included a large follicle exceeding 20 ml, yet the concentration found was very low.

TABLE 3.—*Oestradiol concentration in the largest follicle in comparison to remaining pooled follicles*

Mare No.	Reproductive status	Largest follicles		Pooled follicles		
		Volume (ml)	Oestradiol ($\mu\text{g}/100\text{ ml}$)	Number	Total volume (ml)	Oestradiol ($\mu\text{g}/100\text{ ml}$)
5	Anoestrus-oestrus...	37	186.2	12	42.5	3.4
22	2nd day of oestrus..	28	225.0	10	5.3	< 10.0
9	1 day post ovulation	32	106.2	6	10.5	20.0
11	4 days post ovulation	40	225.0	4	3.0	< 6.0
12	6 days post ovulation	9*, 9*, 8*	< 1.0	11	22.9	23.7
15	8 days post ovulation	18*, 17*	12.8	5	5.6	< 3.2
20	16 days post ovulation	38	320.0	20	36.0	3.1
26	18 days pregnant....	31	12.5	14	27.3	21.0
29	23 days pregnant....	25	2.8	11	47.0	77.3
32	27 days resorbing...	25*	240.0	3	5.0	3.1
		25	6.9			
35	36 days pregnant....	33	63.5	5	24.0	9.1

* Regression of these follicles was established by rectal palpation

Luteal function

Progesterone

As soon as eight hours after ovulation progesterone was detected in the follicle wall, which at this time showed no macroscopic signs of luteinization. Thereafter the concentration in luteal tissue continued to increase remarkably linearly throughout the 14 day luteal phase (Fig. 2). From this data it seems that regression is rapid and occurs on the 14th or 15th day following ovulation. The material occupying the central cavity of the corpus luteum during the mid-luteal period was found to contain from four to eight times less progesterone than the luteal tissue itself.

In pregnant animals maximal cycling luteal values are maintained, though there is some suggestion of a reduced concentration at about the time the next oestrus would have occurred. In the 25 to 30 day gestational period, all five mares

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

had higher luteal progesterone concentrations; those of the two animals on the 27th day were the highest in this study and their values were confirmed by duplicate analyses. From this time to the 40th day decreased values were again encountered.

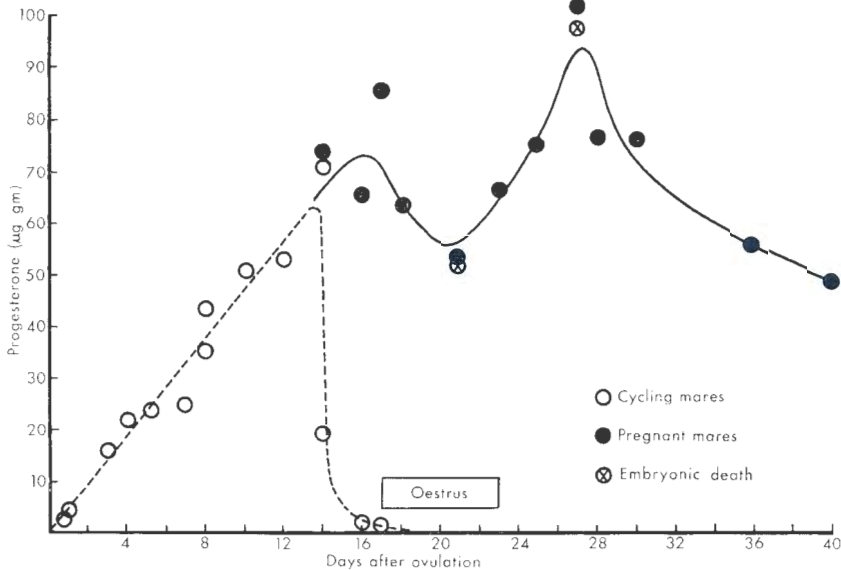


FIG. 2.—Progesterone concentration in corpora lutea of cycling (broken line) and pregnant (solid line) mares

After the 40th day of gestation many mares ovulate without any disturbance of pregnancy, and the original corpus luteum is generally believed to regress (Amoroso, Hancock & Rowlands, 1948; Berliner, 1963). In the four mares examined between 41 to 90 days gestation, an ovulation at about this time was obvious. The primary corpus luteum was still large and easily distinguished from the secondary corpus luteum. Contrary to general belief, assays revealed that the primary corpus luteum of pregnancy remained active in all four mares, the progesterone concentration being at least equal to maximal luteal phase values (Fig. 3). Furthermore, a marked rise in the concentration of progesterone in the primary corpus luteum may be associated with the accessory ovulation as suggested by the high values of the mares examined at 41 and 55 days gestation.

The young accessory corpus luteum initially had progesterone values similar to late luteal phase mares, but the concentration seemed to increase markedly between 60 to 90 days gestation. Concurrently, the primary corpus luteum seems to become less active. By 130 days, numerous luteinized follicles were present and the primary and secondary corpora lutea could not be distinguished. Though the concentration of progesterone in this luteal tissue was only about half that encountered in late luteal phase mares, the amount secreted was probably large due to the abundance of this tissue. Only small corpora albicantia were present after about the 300th day, and progesterone was detected in only one of three mares approaching term.

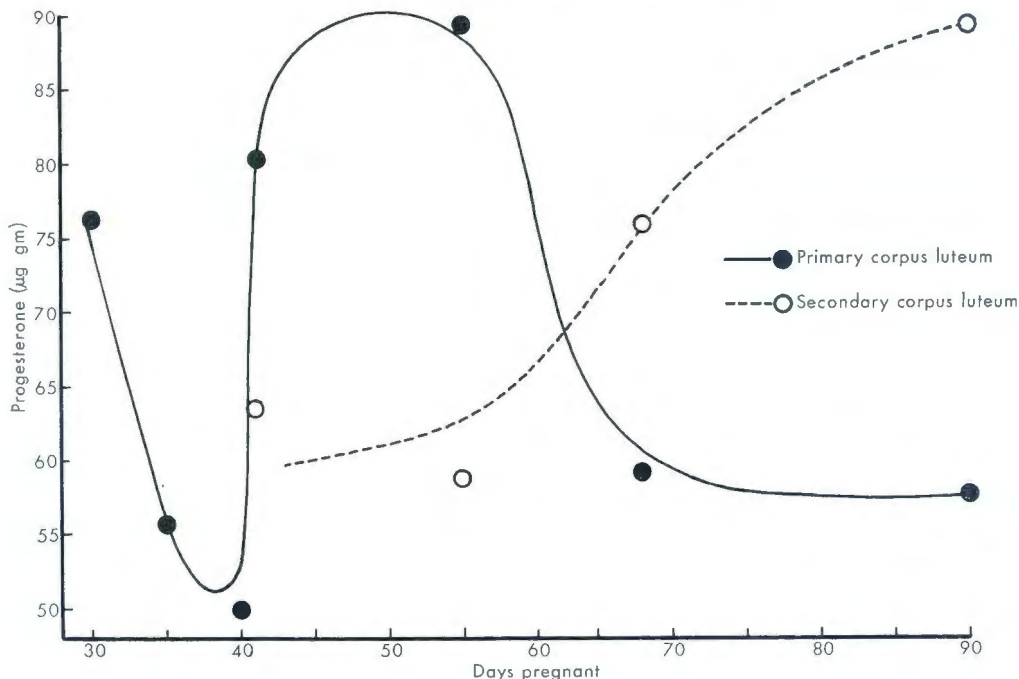


FIG. 3.—Possible sequence of variation in progesterone concentration in the primary and secondary corpora lutea during the second and third months of gestation

20 α -Hydroxypregn-4-en-3-one

This steroid was detected in all six cycling mares examined, at an average concentration of 7.0 (2.9 to 13.4) $\mu\text{g/gm}$. This does not include a mare whose corpus luteum was thought to be regressing and which was found to contain 40.0 $\mu\text{g/gm}$.

Pregnant mares at 14, 16 and 17 days post ovulation also contained amounts similar to that found in cycling animals. However, no traces were detected in any of 17 mares examined between 18 to 321 days gestation.

Mules

The reproductive tracts of nine adult 800 to 1,000 lb female mules were examined. In four instances the ovaries appeared inactive; they weighed from 5 to 40 gm and a few very small follicles and corpora albicantia were occasionally present.

One of the remaining five resembled a mare in oestrus; a 47 ml follicle contained 288.1 $\mu\text{g}/100$ ml oestradiol and the concentration in fluid from five smaller pooled follicles was 20.3 $\mu\text{g}/100$ ml. Another mule which had ovulated about two days previously had, in addition, a large follicle containing 115 ml haemorrhagic fluid; the concentration of oestradiol in this fluid was 103.1 $\mu\text{g}/100$ ml.

Three animals had active corpora lutea. One resembled that of a horse about 5 days post ovulation and it contained 15.9 and 6.2 $\mu\text{g/gm}$ progesterone and 20 α -hydroxypregn-4-en-3-one respectively. In the other two mules the concentration of progesterone was fairly high, yet both had recently ovulated follicles. Progesterone was present in these corpora lutea at a concentration of 53.2 and 38.7 $\mu\text{g/gm}$ and 20 α -hydroxypregn-4-en-3-one was less than 0.5 and 9.9 $\mu\text{g/gm}$ respectively.

DISCUSSION

Sequence of ovarian events

Immediately following ovulation, the number of follicles and the level of total oestradiol were unpredictable, due to variability in the number of accessory follicles at oestrus. In the course of the first four days following ovulation, we noted considerable follicular growth by means of rectal palpation in a few mares; this phenomenon was confirmed in one mare slaughtered on the fourth day, which also had a high oestradiol value. Continued follicular growth after ovulation has also been reported by Van Rensburg & Van Heerden (1953); high oestrogen values in "metoestrous" mares were reported by Knudsen & Velle (1961). In conformity with the one value reported by Short (1962), we found very low luteal progesterone values in this period. If therefore seems that there may be considerable pituitary gonadotrophin release at about this time.

With rapidly increasing luteal progesterone in the mid-luteal phase, oestradiol values were found to be exceedingly low at this time. The number of follicles present and the volume of follicular fluid were not reduced, but the size of the largest follicle appeared to be markedly suppressed. In the late luteal period, however, both cycling and pregnant mares with active corpora lutea show similar and considerable development of active follicles between the 12th and 16th days, indicating gonadotrophin release in both groups commencing about the 12th day. While the corpora lutea of cycling mares regress rapidly on the 14th day, those of pregnant mares become even more active up to 17 days. At this time, immediately before the first oestrus would be expected, the oestradiol content of pregnant mares' follicles drops precipitously, while those of cycling individuals attain maximal values.

In our series, the one oestrous animal had considerably less follicular oestradiol than was encountered in the pro-oestrous animals; the values given by Short (1961a) and Knudsen & Velle (1961) for oestrous mares were all considerably lower than our pro-oestrous values. It therefore seems probable that the follicular fluid oestrogen content decreases from the commencement of oestrus. These observations could explain Short's (1961a) failure to find significantly increased oestradiol values in oestrous mares.

Marked suppression of follicular steroidogenesis in 18 to 21-day pregnant mares is possibly accompanied by some concurrent depression in luteal activity. Thereafter there is a rapid increase in both follicular activity and luteal function reaching a peak at 25 to 27 days. Oestradiol peak values at 16 and 25 days precede peaks of luteal activity by a day or two. The apparent consistent follicular growth in animals approximately 25 days pregnant has been observed by others (Van Rensburg & Van Heerden, 1953; Van Niekerk, 1965a; Bain, 1967). Regression of these follicles was again rapid, and by 30 days very few follicles were found in the ovaries. Progesterone concentrations similarly declined progressively from maximal values at 27 days post ovulation, until the 40th day.

The mares we examined at later stages of pregnancy all appeared to have ovulated a single follicle at about the 40th day. Though it is generally believed that the primary corpus luteum of pregnancy regresses at about this time, our results suggest that its activity is transiently potentiated and that it remains quite active at least up to the end of the third month of pregnancy. Such accessory ovulations are therefore essentially a supplementary source of progesterone, rather than acting as a "replacement" source. At this stage Short (1959) found that the concentration of progesterone in peripheral blood was approximately doubled, though the presence of

accessory corpora lutea in the mares studied by him was not established. In our series, mares with more than one corpus luteum had very little, if any, concurrent follicular development or oestradiol in the follicular fluid.

Gonadotrophic function

Unquestionably pituitary gonadotrophins must be actively secreted during early gestation, as mares pregnant 16 to 28 days have significantly more ovarian follicles than cycling luteal phase animals. Bain (1967) has also drawn attention to consistent follicular growth before the appearance of serum gonadotrophin.

Our data strongly suggest that waves of follicular growth and oestradiol synthesis commence at about 2, 12, 22 and 32 days post ovulation. This indicates a cyclical pituitary release of gonadotrophin every 10 days, irrespective of whether a functional corpus luteum is present or not. The resulting follicles virtually reach maturity as far as size and oestradiol synthesis are concerned before regressing.

The majority of pregnant mares ovulate only after the appearance of serum gonadotrophin at about the 40th day. It is generally assumed that such ovulations are solely due to the action of gonadotrophin secreted by the endometrial cups. Objections to such a supposition are the fact that ovarian activity actually diminishes with increasing levels of serum gonadotrophin (Rowlands, 1949; Bain, 1967), and the usual failure to simulate the equine ovary with even massive doses of PMS. Undoubtedly PMSG has intrinsic FSH and LH properties, but the ovarian response to gonadotrophins produced during pregnancy differs in even closely related species (Van Rensburg, 1964). Whereas human chorionic gonadotrophin has a powerful follicle stimulating action in the intact ewe, it appears to be devoid of any such action on the human ovary.

Many ova are frequently found in the Fallopian tubes of pregnant mares, but their presence may now be explained by the extraordinary tubal persistence of unfertilized ova in this species (Van Niekerk & Gerneke, 1966). In our experience, actual ovulation in pregnant mares was only encountered between approximately the 40th to 50th day, soon after the expected appearance of serum gonadotrophin. At later stages, particularly when serum gonadotrophin activity was declining, numerous corpora lutea, formed by luteinization of follicles, were present in the ovaries. The evidence therefore suggests that ovulation during pregnancy may be dependent on the synergistic effect of pituitary gonadotrophins and low levels of PMSG; after about 50 days gestation, increased gonadal and placental steroid inhibition of pituitary gonadotrophins would exclude further ovulations.

Large follicles containing 20 ml or more fluid consistently contained a high concentration of oestradiol in cycling mares, but not in pregnant mares. This difference in steroidogenesis is probably due to suppression of pituitary LH by steroids produced in the ovary and placenta; and exemplifies the fact that different gonadotrophic mechanisms are necessary for follicular growth and oestrogen synthesis.

There is some evidence that oestradiol produced in one follicle may affect the concentration in others. When a low concentration was found in a large follicle, the concentration found in the smaller follicles tended to be higher; and when two large follicles were present, only one contained large amounts of oestradiol. These observations raise the possibility of a local intra-ovarian feedback system, which is related to a selective follicular growth mechanism.

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

Qualitatively, corpora lutea from mares more than 17 days pregnant differed from cyclical corpora lutea in that 20α -hydroxypregn-4-en-3-one was not detected. The presence of this steroid cannot be related to the age of the corpus luteum as it was not detected in fresh accessory corpora lutea in pregnant animals. Highest levels were found on day 14 in two cycling mares, suggesting that an increase is associated with luteal regression. In rats there is considerable evidence that prolactin inhibits the conversion of progesterone to 20α -hydroxypregn-4-en-3-one, and it has been suggested that this mechanism may be basically related to the luteotrophic action of prolactin (Hansel, 1966). The facts in mares seem to support such a theory, the corpus luteum of pregnancy being under maximal prolactin influence.

Infertility considerations

Experienced clinicians have not infrequently reported oestrus in mares with small ovaries devoid of any palpable follicles. Our studies on individual follicles indicate that appreciable amounts of oestrogens are not produced by a follicle before it contains about 20 ml of fluid. It is therefore unlikely that such oestrous behaviour results from the secretions of very small follicles. If such behaviour is not simply a psychological aberration then steroids produced by the ovarian stroma or adrenal may be responsible.

Waves of follicular growth and atresia without ovulation are commonly encountered in some mares, particularly at the commencement of the breeding season. From our deductions concerning an inherent cyclical pituitary gonadotrophin release about every 10 days, peaks of follicular growth may be recognized at about this interval. A short course of progesterone therapy sometimes appears to restore ovulatory cycles; such treatment should be timed to end 2 or 3 days before the next expected peak of follicular growth. This type of infertility is undoubtedly associated with domestication and psychological factors (Van Rensburg & Van Heerden, 1953); endocrinologically the main defect is an inadequate LH release. In view of the known stimulation of LH secretion following heterosexual association in many species, a high recovery rate when these mares are turned out with stallions is not surprising.

The uterine horns of oestrous mares are thin and flaccid, but there is a considerable increase in wall thickness and tone, imparting a turgid tubular quality on palpation, during the luteal phase (Van Niekerk, 1965a). The degree of these changes is closely related to the concentration of progesterone in the corpora lutea, and makes it possible to anticipate pregnancy 14 to 15 days after ovulation. The marked increase in uterine wall thickness between the 12th and 18th days with a gradual increase up to 25 days described by Van Niekerk indicates functional persistence of the corpus luteum due to pregnancy.

Early diagnosis of pregnancy may be important as 10 per cent or more mares show oestrus and stand for the stallion one cycle length after having become pregnant (Van Niekerk, 1965a). This phenomenon is not surprising as our findings indicate that pregnant mares usually produce large amounts of oestrogen immediately before the first expected oestrus.

The numerous cases of early foetal death studied by Van Niekerk (1965b) all commenced resorption on about the 25th day of pregnancy. This time coincides exactly with a major peak of follicular growth and oestrogen synthesis, but precedes maximal progesterone values by two days. It is quite possible that an unfavourable oestrogen:progesterone ratio at this time predisposes to foetal death, thus at times

it may be beneficial to supplement progesterone during the critical 20 to 30 day period. The fact that uterine wall thickness and the increase in progesterone concentration closely parallel each other during the first month of pregnancy, suggests that a large excess of progesterone is not normally produced, as is probably the case in bovines.

Van Niekerk's clinical evidence suggests that after foetal resorption at 25 to 35 days, the corpus luteum usually persists for more than a month; such animals examined by us had virtually normal luteal progesterone values. The success of uterine saline irrigations in initiating oestrus in these animals is possibly due to distension of the uterus causing regression of the corpus luteum, as such an action has been demonstrated in other species. It would be of interest to compare the efficacy of this procedure in anoestrous animals with and without functional corpora lutea.

Some mares lose their foals between the 40th and 60th day of pregnancy, and it is suggested that this is caused by a lack of proper implantation (Lieux, 1963). Van Rensburg & Van Heerden (1953) reported loss of the foetus in mares which failed to exhibit secondary ovulations at about this time, and deduced that an inadequate supply of progesterone was responsible. Our work demonstrates that these secondary ovulations do not merely act as a replacement source of progesterone, but supplement the supply from the existing corpus luteum which does not regress. We have some evidence that secondary ovulations may be suppressed in lactating mares bred at foal heat, and such a mechanism may account for the increased foetal wastage reported by some workers in these mares.

Though luteal insufficiency has not been proved to be a cause of abortion in mares, its occurrence is widely assumed by clinicians. When evaluating the results of treatment, a distinction should be made between probable maternal (ovarian) deficiency, and placental insufficiency during advanced stages of gestation. Actual rates of progesterone release from the luteinized equine ovary have not been measured, but are probably at least in the region of 100 mg per day. Only substantial amounts of supplementary progesterone may therefore be expected to be of clinical advantage during early gestation.

The placenta is the sole source of progesterone after about the fifth month of gestation. High concentrations are found in the foetal circulation, but progesterone as such is not released into the maternal circulation. Supplementation of progesterone at this time is therefore speculative; theoretically, in the absence of circulating maternal progesterone, even the small amounts commonly administered may be of value. However, lowered progesterone production by the placenta may only be one manifestation of general placental insufficiency. This supposition is supported by the experience of Lieux (1963) who states that foals born of mares given progesterone because of impending abortion in late pregnancy were unhealthy and not economical to raise.

SUMMARY

Reproductive patterns were studied in the majority of 46 mares before obtaining the ovaries for examination at slaughter. Steroids identified and determined were progesterone and 20α -hydroxypregn-4-en-3-one in the corpora lutea and oestradiol- 17β in the follicular fluid.

A study of ovarian morphology revealed no significant cyclical variations of the ovarian weight and number of follicles, but the total volume of follicular fluid increased in the latter half of the cycle. Consistent and marked follicular growth was present at about the 25th day of gestation. Mares 16 to 28 days pregnant had significantly

OVARIAN FUNCTION IN CYCLING AND PREGNANT EQUINES

more follicles than cycling mares, and the ovarian weight and volume of follicular fluid were also increased at this time. Peak total oestradiol values at 25 days and maximal progesterone concentrations at 27 days preceded implantation on the 28th day.

Oestradiol was present before puberty and the amount in anoestrous animals seems to increase gradually as the breeding season approaches. In cycling mares, total follicular fluid oestradiol values are variable for up to five days after ovulation, but oestradiol is virtually absent during the mid-luteal period. From the 12th day there is a rapid increase to maximal values immediately prior to oestrus. Limited evidence suggests that ovarian oestradiol decreases from the commencement of oestrus. Pregnant animals exhibit an indistinguishable pro-oestrous rise, but drop suddenly when oestrus would be expected on the 17th day. When more than one corpus luteum was present and after two months gestation, no oestradiol was detected.

Waves of follicular growth and oestradiol synthesis commence at 2, 12, 22 and 32 days after ovulation, suggesting a cyclical release of pituitary gonadotrophin every 10 days, irrespective of whether an active corpus luteum is present or not. Maximal follicular growth during pregnancy was encountered at 25 days, before the appearance of PMSG. Actual ovulation was only encountered soon after the expected appearance of PMSG, suggesting a synergistic effect between PMSG and pituitary gonadotrophin. When steroidal inhibition of pituitary gonadotrophin increased and PMSG levels were maximal, the ovaries were relatively quiescent. Many active luteinized follicles were encountered as PMSG levels waned.

Luteal progesterone concentrations rose linearly for 14 days; in cycling mares, regression took place on the 14th or 15th day. In pregnant animals the concentration continued to rise, reaching a peak at 27 days. Secondary corpora lutea, resulting from ovulations during the second month of gestation, had an initial concentration resembling cycling animals, but marked increases were found during the third month of gestation. The primary corpus luteum does not regress with the occurrence of secondary ovulations as is generally believed, but remains active for at least three months.

20 α -Hydroxypregn-4-en-3-one was always present in the corpora lutea of cycling animals and increases were associated with luteal regression. This steroid was never detected in animals more than 18 days pregnant, including fresh secondary corpora lutea.

The ovaries of mules contained similar amounts of steroids as found in horses. However, the occurrence of ovulation in the presence of functional corpora lutea suggests failure of the luteal regression mechanism, or alternatively an inability of the hypothalamus to regulate gonadotrophin secretion in the usual manner.

The theoretical implications of these findings on sterility work are discussed.

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