Oestradiol-17 β , progesterone, FSH and LH in prepubertal calves induced to superovulate

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Summary. Fluorogestone acetate (vaginal sponge for 4 days) and PMSG (i.m. injection at the time of sponge insertion) treatment was administered to seven 3-month-old calves to induce superovulation. Samples of peripheral plasma were taken every 4 h during treatment (4 days) and then every 2 h for 7 days. FSH, LH, oestradiol and progesterone were measured by radioimmunoassays. In all calves oestradiol concentrations increased 24 h after PMSG injection and reached the highest levels (41–502 pg/ml) during the preovulatory surge of both gonadotrophins. The surge of LH and FSH occurred from 12 to 22 h after cessation of treatment. The maximum levels of LH and FSH were 11–72 ng/ml and 23–40 ng/ml respectively and occurred within 4 h of each other. Between 40 and 68 h after the LH peak the concentrations of progesterone began to increase from basal values, reaching 24.0–101.7 ng/ml when the animals were killed. A quantitative relationship was found between plasma oestradiol concentration and the numbers of ovulating follicles. Progesterone levels seemed to be related to the numbers of corpora lutea and also to the numbers of unovulated follicles. Gonadotrophin output was not quantitatively related to ovarian activity or to steroid secretion.

Introduction

Even before birth, the ovary of the female calf contains many antral follicles (Erickson, 1966) which reach a maximum number before puberty (Henrickson & Rajakoski, 1959). It is possible to obtain final growth and ovulation of these follicles by means of gonadotrophin treatment (Marden, 1953; Howe, Black, Foley & Black, 1962; Jainudeen, Hafez & Lineweaver, 1966; Lineweaver & Hafez, 1970; Onuma, Hahn & Foote, 1970; Seidel, Larson & Foote, 1971; Bedirian & Baker, 1975). The production of eggs in the prepubertal female mammal is of interest from a physiological and a genetic viewpoint: the transfer of eggs obtained from young animals shortens the time between generations and also allows early testing of progeny. PMSG can stimulate the growth of numerous follicles, ovulation of which can be induced by LH or HCG, but the quality of the eggs obtained by such treatment is rarely satisfactory (Seidel, Larson, Spilman, Hahn & Foote, 1971). The spread in the time of ovulation may be the factor responsible for the defective quality of the eggs (Onuma *et al.*, 1970; Lineweaver & Hafez, 1970). The administration of a synthetic progestagen simultaneously with the gonadotrophic stimulation results in grouping of the ovulations (Testart, 1972), but the quality of the eggs obtained after artificial insemination is not yet satisfactory (Testart, 1974, 1975). The inadequate hormonal environment may be another cause of failure. The present work was therefore undertaken to examine the changes of concentrations of steroid and gonadotrophic hormones during a progestagen–PMSG treatment.

Materials and Methods

Seven female calves (French Friesian breed), aged about 3 months, each received 1800 i.u. PMSG (Intervet-France, Angers) by intramuscular injection. At the same time, a polyurethane sponge

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impregnated with 60 mg fluorogestone acetate (FGA, Searle, Montrouge, France) was inserted into the vagina; the sponge was withdrawn after 4 days. Jugular vein blood (10 ml) was collected every 4 h from the time of PMSG injection until sponge removal, then 5 ml blood were collected every 2 h during the following 7 days.

The calves were housed together and oestrus was checked at the time of blood collections by their mounting behaviour to one another. Eleven days after the beginning of treatment, the calves were killed and the ovaries examined.

Each plasma sample was assayed separately for LH, FSH and oestradiol-17 β ; for one animal (No. 521), FSH was not assayed. For progesterone measurements, equal volumes of plasma were pooled to obtain a mean value per day for the first 4 days, 3 mean values per day from Days 5–9, then 2 mean values over the last 2 days. All plasma samples were assayed in duplicate.

Gonadotrophins

LH was measured by using an homologous system already described (Kann, 1971). The hormone used for labelling and as standard was supplied by the CNRS (O-LH-M3); the sensitivity of the assay was 15 pg/tube, allowing the determination of 0.3 ng/ml, and 1 ng O-LH-M3 is equivalent to 2 ng NIH-O-LH-S1. The coefficient of variation for intra-assay precision was less than 10%.

FSH was determined by the heterologous radioimmunoassay described by Kerdelhué, Kann & Jutisz (1972). Rat FSH supplied by NIAMDD was labelled with ^{125}I ; the antibody used was obtained by injection of highly purified FSH (O-FSH-M5), which was also used as the standard. The cross-reaction with LH and FSH was negligible. This assay detected 2 ng FSH/ml and 1 ng O-FSH-M5 is equivalent to 8 ng NIH-O-FSH-S3. Interassay coefficients of variation were sometimes higher than 20% but the intra-assay coefficients were less than 10%.

Steroids

Oestradiol-17 β . The method used was essentially that described by Thibier & Saumande (1975). However, modifications were introduced to allow measurement of the bound fraction in the same tube used for the immunological reaction: the bound and free fractions were separated by immunoprecipitation and the liaison between oestradiol-17 β and the antibody was destroyed by the addition of 2 drops 5 N-HCl. Liquid scintillator (1 ml) was added, and the tubes were shaken for 1 h on a mechanical shaker to allow the oestradiol to pass into the organic phase before counting the radioactivity in a liquid scintillation spectrometer. This method is practical and efficient; it allows counting efficiency to be increased and price of the assay to be decreased (Saumande & André, 1975). The coefficient of variation for intra-assay precision was 5% and the sensitivity was estimated as 2 pg/ml.

Progesterone was determined by radioimmunoassay with an antiserum raised in rabbits immunized against progesterone-11-hemisuccinate-BSA. The antiserum shows some cross-reactivity with 5 α - and 5 β -pregnane-3,20-dione (50 and 16% respectively). All other steroids tested (Tea, Castanier, Roger & Scholler, 1975) showed cross-reactions $\leq 2\%$. The assay procedure was as described by Thibier & Saumande (1975); it included a chromatography step on Sephadex LH-20. Bound and free steroids were separated directly in the scintillation fluid (toluene-PPO-POPOP) after freezing and decanting the organic phase containing free progesterone into small plastic tubes, which were then counted. The coefficient of variation for intra-assay precision was 9% and the limit of sensitivity was estimated as 0.11 ng/ml after consideration of the blank value and the sample aliquot volume.

Results

Oestrus and ovulation response

All the animals came into oestrus between 30 and 44 h after withdrawal of the vaginal sponges (i.e. Day 6) and all ovulated (Table 1). The magnitude of the response to the treatment varied greatly

Table 1. The effect, on Day 11, of treatment of 3-month-old calves with PMSG and fluorogestone acetate (FGA) on the ovaries

	Group A				Group B				
	Calf 516	Calf 519	Calf 520	Mean ($\pm$ S.D.)	Calf 517	Calf 518	Calf 521	Calf 522	Mean ($\pm$ S.D.)
Interval between FGA withdrawal and oestrus (h)	40	30	44	38.0	34	32	44	44	38.5
Ovarian weight (g)	30	34	18	27.8 ($\pm$ 8.3)	228	205	72	129	158.5 ($\pm$ 71.5)
No. of corpora lutea	3	8	6	5.7	40	5	22	14	20.3
No. of follicles > 6 mm in diam.	3	1	0	1.3	50	21	3	11	21.5
Total no. of CL + large follicles	6	9	6	7.0	90	26	25	25	41.5

between the calves. Calves 516, 519 and 520 (Group A) responded less well in terms of ovarian weight, large follicles and corpora lutea. The other 4 calves (Group B) had a high ovarian weight and many large follicles and corpora lutea.

Hormone levels

Gonadotrophins. After sponge removal, the plasma concentration of LH did not exceed 1 ng/ml in 4 calves, but varied between 5 and 10 ng/ml in 3 others (Nos 519, 520 and 522; Text-fig. 1). FSH concentrations were less variable than those of LH between and within individuals and were between 5 and 10 ng/ml. There was a discharge of both gonadotrophic hormones on Day 5 and the maximum plasma concentrations were obtained 12–22 h later (11–60 ng/ml for LH; 23–40 ng/ml for FSH). The time between the FSH and LH peaks did not differ by more than 4 h for any one animal. After the peaks, the concentrations of FSH and LH fell to levels similar to those observed during the first 4 days of treatment. There was no apparent correlation between the time of peak or the maximum values reached and the ovarian response.

Text-fig. 1. Plasma concentrations of FSH (○) and LH (●) in Calf 519 (Group A) and Calf 522 (Group B). The vertical line represents sponge removal.

Oestradiol-17 β . The results for all the animals are shown in Table 2, and the responses of one calf from each of Group A and B are shown in Text-fig. 2. Even before stimulation with PMSG, 4 calves (Nos 518, 519, 520 and 521) showed plasma oestradiol concentrations of > 1 pg/ml. The peak response to PMSG was greater when the oestradiol levels increased rapidly (e.g. the responses of Calves 519 and 520 in Table 2). However, the oestradiol peak occurred at the same time, on Day 5, between 10 and 18 h after sponge withdrawal in all the animals. The concentrations decreased rapidly within 24 h and at the expected time of ovulation (approximately 48 h after sponge withdrawal for animals treated in this way: Testart, 1975) they were relatively low. After ovulation, elevated concentrations of oestradiol (> 20 pg/ml) occurred sporadically.

Table 2. Plasma concentrations of oestradiol-17 β and progesterone in 3-month-old calves treated with PMSG and fluorogestone acetate

	Group A				Group B				
	Calf 516	Calf 519	Calf 520	Mean ($\pm$ S.D.)	Calf 517	Calf 518	Calf 521	Calf 522	Mean ($\pm$ S.D.)
Oestradiol									
Conc. before treatment (pg/ml)	0.6	1.5	1.3	1.1	0.5	2.4	4.2	0.4	1.9
Maximum conc. (pg/ml)	41	61	65	56 $\pm$ 13	502	105	235	145	274 $\pm$ 185
Time from PMSG to reach threshold conc.* (h)	102	80	64	82.0	24	68	36	48	44.0
Time from LH peak to maximum conc. (h)	-2	-6	-2		+4	-2	-2	-2	
Area under curve (arbitrary units)									
Before ovulation†	1688	2005	2472	2055 $\pm$ 394	13370	4258	12229	5886	8936 $\pm$ 4534
After ovulation‡	371	77	216	221 $\pm$ 147	1693	281	3221	284	1370 $\pm$ 1402
Progesterone									
Time from LH peak to threshold conc.§ (h)	62	68	58	62.7	50	52	52	42	46.5
Conc. 72 h ¶ after LH peak (ng/ml)	12.3	2.93	2.92	6.05 $\pm$ 5.41	43.7	6.15	19.9	16.2	21.5 $\pm$ 17.9
Mean conc. before slaughter (ng/ml)	43.1	25.4	24.0	30.8 $\pm$ 10.6	80.0	101.7	70.6	91.3	87.9 $\pm$ 13.5

* 20 pg/ml—the maximum measured before oestrus in the cyclic animal.

† From beginning of treatment until 48 h after sponge removal.

‡ From 48 h after sponge removal until slaughter (Day 11).

§ 1 ng/ml: in adult cows, progesterone concentrations > 1 ng/ml are considered to be indicative of an active CL.

¶ Cyclic cows have usually attained threshold concentrations by this time.

The area under the curve of oestradiol concentrations was measured to assess the secretion level of this oestrogen. The preovulatory value (from the beginning of treatment until 48 h after sponge removal) was strongly correlated with the number of ovulations counted at Day 11 ($r = 0.92$, $P < 0.01$) but was not correlated with the number of large unruptured follicles. The postovulatory value (from 48 h after sponge removal until Day 11) was not correlated with the number of ovulations or of unruptured follicles. The animals which showed the highest maximum values and preovulatory area values also had the highest postovulatory area values.

Progesterone. The plasma concentrations during the first 6 days after PMSG injection were very low (0.04–0.43 ng/ml). In the Group B animals, from the afternoon of Day 7 (i.e. a mean of 46 h after the LH peak) progesterone levels were > 1 ng/ml. By contrast, in Group A animals, this threshold was not attained until about 16 h later (Table 2). The progesterone levels in Group B animals rose rapidly (Text-fig. 2) and reached maximum values 6 days after the gonadotrophin peak. In Group A,

Text-fig. 2. Plasma concentrations of oestradiol (—) and progesterone (---) in (a) Calf 519, representative of Group A (small response), and (b) Calf 522 a representative of Group B (large response), after treatment with PMSG and fluorogestone acetate (horizontal bar).

the concentrations also rose after the gonadotrophin peak but they were lower than those in the Group B animals.

Discussion

The mean weight of the two ovaries in calves of identical breed and age is approximately 4 g before treatment (Testart, 1975). It is therefore increased 7-fold for the animals in Group A (weak response) and 40-fold for those in Group B (strong response) by Day 11 after injection of PMSG. All the calves superovulated, and the number of corpora lutea (3–40) in fact shows less variation than that reported for treatments not involving PMSG (Jainudeen *et al.*, 1971; Bedirian & Baker, 1975). The ovarian weight really reflects the number of unruptured follicles or the number of large follicles and corpora lutea, with which it is strongly correlated ($r = 0.89$, $P < 0.01$, and $r = 0.81$, $P < 0.05$ respectively) rather than with the corpora lutea only, for which there is no significant correlation (e.g. compare the follicles and corpora lutea of Calves 518 and 521, both in Group B, Table 1).

The significant relationship between ovarian weight and number of ovulations which we have been able to establish on Day 8 after the same treatment (Saumande & Testart, 1974) does not hold for Day 11, suggesting that there is follicular development between 4 and 7 days after sponge removal. It is unlikely that these follicles are from the same population as those which have ovulated, unless the atresia undergone by the entire follicle population of large follicles 2 days after the LH discharge (Arrau, 1974) is not associated with inhibition of the secretion of oestrogens. It is therefore probable that there is new follicular growth, which is not directly dependent on the injection of PMSG, but is a function of the loss of the larger follicles by ovulation. This new growth would primarily affect calves in Group B, because the increase in the number of follicles (i.e. > 6 mm) between Days 5–6 and 7–8 is 4 times greater (53% versus 13%) in animals showing numerous follicles (48) just before ovulation than in those with few follicles (11) at the same time (Testart, 1975). The greater postovulatory curve area of oestradiol secretion in those calves exhibiting the greater response to PMSG is in accordance with these ideas.

The basal levels of plasma LH and FSH in calves are generally less than 10 ng/ml. A peak of LH appears between 12 and 22 h after sponge removal and reaches 11–72 ng/ml. These concentrations

are comparable with those reported by Testart & Kann (1973). They confirm that, with the use of a progestagen, a discharge of LH can be provoked in the prepubertal animal which is comparable with that observed in the cyclic adult (Schams & Karg, 1969; Ectors, Henricks, Franchimont & Derivaux, 1974; Christensen, Hopwood & Wiltbank, 1974; Cummins, Blockey, de Brown & Goding, 1972; Lemon *et al.*, 1975). The LH discharge (5.5 ng/ml) reported by Spilman, Seidel, Larson, Vukman & Foote (1973) on the day after a PMSG injection was markedly lower than the values observed in our study and does not exceed the basal levels found for 4 of our 7 calves.

In all calves, a peak of FSH (23–40 ng/ml) accompanied the LH peak, as has been found for all species in which FSH has been measured at this time. Compared with the basal level, the peak value is relatively less than that for LH. It is difficult to compare this peak value with that of the cyclic animal because we have no data on this aspect, and the values reported in the literature (Akbar, Reichert, Dunn, Kaltenbach & Niswender, 1974; Derivaux, Ectors, Henrick & Franchimont, 1974; Schams & Schallenger, 1976) differ not only from our present findings but also from each other.

Before PMSG injection, the concentrations of oestradiol are between 0.4 and 4.2 pg/ml. A plasma oestradiol peak appears 10–20 h after sponge removal, i.e. 6 h before to 4 h after the LH peak; this peak always exceeds 40 pg/ml and can reach 0.5 ng/ml. In a previous study (Saumande & Testart, 1974) we found, in one animal, a peak greater than 2 ng oestradiol/ml. These concentrations are very high for cattle, because in the cyclic cow the preovulatory peak is 15–30 pg/ml (Henricks, Dickey & Hill, 1971; Glencross, Munro, Senior & Pope, 1973; Dobson & Dean, 1974; Lemon & Saumande, 1974; Thibier & Saumande, 1975) and reaches only to 140 pg/ml when the cow is induced to super-ovulate (Saumande & Pelletier, 1975). For an equal number of induced ovulations, therefore, there is a difference in peak oestradiol concentrations between prepubertal and adult animals. This can be explained by the greater blood volume in the adult ($\times 5$) and also by the fact that for the same number of ovulations there are many more large follicles still present in the calf than in adults (Saumande & Testart, 1974).

The plasma concentrations of progesterone are always less than 0.5 ng/ml until the LH peak, as in the adult at a similar stage (Lemon & Saumande, 1974; Thibier & Saumande, 1975). Progesterone increased very rapidly in Group B calves, reaching 1 ng/ml between 42 and 68 h after the LH peak. This increase occurs sooner than in the adult in which the threshold of 1 ng/ml is reached 3 or 4 days after the LH peak (Stabenfeldt, Ewing & McDonald, 1969; Donaldson, Bassett & Thorburn, 1970). For the same number of corpora lutea, the values reached (19–89 ng/ml during the luteal phase) are always greater than those found in the superovulating adult (M. Lemon, personal communication), but comparable with those found in the calf treated with PMSG and LH (Spilman *et al.*, 1973). As in the adult cow (Spilman *et al.*, 1973), the activity of the corpus luteum can be followed by the change of secretion of progesterone and 20 β -dihydroprogesterone. The same correlation occurs with progesterone and 17 α -hydroxyprogesterone (cited by Testart, 1975) and is identical to that found in the cyclic animal (Thibier, Castanier, Tea & Scholler, 1973).

Testart (1975) has shown that in similarly treated calves, ovulation takes place between 38 and 58 h after withdrawal of the vaginal sponge. The peak of the gonadotrophic hormones therefore occurs about 24 h before the start of ovulation, which corresponds exactly with the timing of these events in the cyclic adult (Henricks, Dickey & Niswender, 1970; Christenson, Echternkamp & Laster, 1975; Lemon *et al.*, 1975). In the present study oestrous behaviour was observed 16–30 h after the LH discharge, i.e. when the oestradiol levels had already fallen (Text-fig. 2).

The growth of the follicles stimulated by PMSG does not seem to be modified by FGA (Testart & Arrau, 1972), as indicated by the increase in oestradiol secretion which appears in spite of the presence of progestagen: in all the animals the threshold of 20 pg oestradiol/ml was reached by 102 h after PMSG injection. The maximum values of oestradiol coincide with the peak of gonadotrophic hormones, as in the cyclic or superovulating adult (Chenault, Thatcher, Kalba, Abrams & Wilcox, 1975; Lemon *et al.*, 1975). The decrease of oestradiol secretion after the gonadotrophin peak corresponds with results *in vitro* which have shown that the follicle loses its ability to synthesize oestradiol after exposure to the preovulatory discharge of LH (Hori, Ide, Kato & Miyake, 1970; Moor, 1974; Lieberman *et al.*, 1975).

It is important to stress the relationship of the hormonal profiles with the ovarian response. Those calves with a marked response (large number of follicles > 6 mm + corpora lutea) are those in which the levels of progesterone and oestradiol increase sooner and attain the highest values. This confirms that oestrogen secretion is proportional to the follicular population before ovulation. The observations on Calf 521 are interesting: there were 22 follicles in the ovaries 7 days after the LH discharge and only 3 corpora lutea, but the plasma progesterone concentrations were comparable with those of the other animals in Group B. This observation suggests that unovulated follicles might secrete progesterone, probably because they have luteinized, as was observed in adult cows induced to superovulate by gonadotrophins (Booth, Newcomb, Strange, Rowson & Sacher, 1975). The steroid levels observed in the treated calves are clearly much higher than those in adults, and it is possible that they may have adverse consequences not only on oocyte maturation, but also on the conditions necessary for impregnation and fertilization and the preparation of the genital tract for the reception of eggs.

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