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Further Observations on Hormonal Support of Pregnancy in the Ovariectomized Rabbit

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Abstract

While the necessity for progesterone administration throughout pregnancy in the ovariectomized rabbit is not questioned, the roles of 20α-dihydroprogesterone and oestradiol are still in doubt.

20α-dihydroprogesterone was shown to be a weak inducer of implantation with less than one-tenth the potency of progesterone. The significance of its high level of production on the day after mating remains obscure.

In combination with the earlier results of Kwun and Emmens (1974), further work with oestradiol suggests that at no stage is it clearly needed for successful maintenance of pregnancy. However, in low doses (0·125–0·2 μg/day prior to implantation, 0·2 rising to 1·6 μg, or remaining at 0·2 μg/day thereafter) it produced slight but sometimes significant improvements in implantation and foetal development percentages.

Birth processes were abnormal if progesterone injections were continued beyond day 29. Foetuses were most frequently retained *in utero* or born dead after a somewhat prolonged pregnancy. The cessation of injections on day 29, whether or not a low dosage of 0·2 μg of oestradiol per day were continued, resulted in 94–98% normal parturition, but the percentage of live births was still significantly below that of controls unless oestradiol was given.

Introduction

Kwun and Emmens (1974), in repeating and extending earlier work on the induction of implantation and maintenance of pregnancy in the ovariectomized rabbit, arrived at the following conclusions.

(1) In rabbits undergoing induced ovulation and artificial insemination on day 1, followed by ovariectomy on day 2, implantation could be brought about by progesterone alone. Best results were obtained by twice daily injection in oil of 1 mg/day on days 2, 3 and 4 followed by 2 or 3 mg/day on days 5–9 inclusive. The addition of up to 0·5 μg/day of oestradiol (oestra-3,17β-diol) did not consistently improve the results, and higher doses impaired them.

(2) The addition of oestradiol from days 10 to 30, if not in excess, appeared to improve the maintenance of pregnancy. However, progesterone alone maintained pregnancy in the majority of ovariectomized, adrenalectomized does, leaving any significant role for oestrogen in considerable doubt.

The experiments of Kwun and Emmens (1974), however, did not extend beyond killing and examination on day 30, just prior to expected birth, and the assertion that oestrogen might assist in the birth processes (Schofield 1957; Davies and Ryan 1972; Waynforth and Robertson 1972; Challis *et al.* 1973) was not examined. In addition, the suspicion that 20α-dihydroprogesterone (20α-hydroxypreg-4-en-3-one, formerly known as 20α-hydroxyprogesterone) might have a special function early in

pregnancy (Hilliard *et al.* 1967) was not explored. This substance is produced in approximately 10-fold greater amounts than progesterone between coitus and ovulation in the reflexly ovulating rabbit and again later in pregnancy (Hilliard *et al.* 1963), and has been shown to be capable of inducing implantation (Rennie and Davies 1965).

The potential role of 20α-dihydroprogesterone in early pregnancy has been explored, together with further variations of progesterone and oestrogen dosage. The effects of differences in anaesthetics, halothane (Fluothane, ICI) *v.* pentobarbital sodium (Nembutal, Abbott Laboratories), have been observed, in combination with differences in hormone treatment around the time of birth. The effects of a steady, low oestradiol dosage were studied, based on the observations of plasma oestrogen levels in normal pregnancy in the rabbit (Challis *et al.* 1973; Hilliard *et al.* 1973; Kwun, Shutt and Emmens, unpublished data). These authors all agreed that little or no rise in oestrogen concentration in peripheral plasma occurs during late pregnancy in the rabbit.

Materials and Methods

Young virgin albino rabbits (mean body weight ± S.D. 2·82±0·35 kg) aged 5–7 months were maintained and treated as by Kwun and Emmens (1974). When anaesthetized with pentobarbital sodium, they were injected via a marginal ear vein with a dosage of 30 mg/kg body weight. Sham-operated controls were used throughout. Details of variations in steroid treatment are given when describing the results of each experiment, but a schedule of twice daily administration of steroid in oil was adhered to in all tests. Controls received oil alone, and each steroid was given by separate intramuscular injection.

The following statistics were used: implantation percentage = 10^2 × total no. of implants on day 10 ÷ total corpus luteum count; foetal development percentage = 10^2 × total no. of foetuses (dead or alive) at birth or laparotomy on day 39 ÷ total no. of implants on day 10. This latter parameter replaces the foetal survival percentage of Kwun and Emmens (1974). All χ^2 tests of significance were conducted where necessary with correction for small numbers and further corrected for, or viewed in the light of, heterogeneity between animals where such existed. However, if χ^2 for a particular aspect of an experiment was not significant it was not usually considered necessary to explore the statistical situation further.

Results

Experiment 1

In experiment 1, the plan of which is shown in the first part of Table 1, the effects were examined of substitution of progesterone by 20α-dihydroprogesterone in part or whole on days 2, 3 and 4. It was fully factorial, with four rabbits per group in a 3 × 3 design plus controls. Progesterone was given at levels of 1, 0·25 or 0 mg/day, and 20α-dihydroprogesterone at 10, 2·5 or 0 mg/day. On days 5–9 inclusive all experimental groups received 2 mg of progesterone in addition to 0·25 μg of oestradiol per day.

Inspection of Table 1 is sufficient to show that 20α-dihydroprogesterone is weakly active with less than one-tenth of the potency of progesterone in this test. However, while not significantly affecting the results when progesterone was present, 20α-dihydroprogesterone did induce a low percentage of implantation when given on its own. Chi-square tests for main effects were as follows: for progesterone, $\chi^2_{(2)} = 37{\cdot}21$, $P < 0{\cdot}01$; for 20α-dihydroprogesterone, $\chi^2_{(2)} = 2{\cdot}73$, $P > 0{\cdot}20$; and for interaction,

$\chi^2_{(4)} = 23{\cdot}48$, $P < 0{\cdot}01$. The effect of 20α-dihydrogesterone was thus so weak that it did not show up as statistically significant.

A major role cannot therefore be postulated for 20α-dihydroprogesterone either in the implantation process, or probably thereafter.

Table 1. Effects of progesterone, 20α-dihydroprogesterone and oestradiol on implantation in rabbits ovariectomized on day 2 after insemination on day 1

Animals received two injections per day, with four per group. P, progesterone (mg/day); 20α-P, 20α-dihydroprogesterone (mg/day); E_2, oestradiol (μg/day)

Injections and doses				No. of animals pregnant	Total no. of corpora lutea	No. of implants on day 10	Percentage implant-ation	No. of implants per pregnancy
Days 2–4		Days 5–9						
P	20α-P	P	E_2					
				Experiment 1				
1	10	2	0·25	4/4	35	17	48·6	4·3
1	2·5	2	0·25	3/4	36	15	41·7	5·0
1	0	2	0·25	3/4	32	20	62·5	6·7
0·25	10	2	0·25	3/4	22	12	54·6	4·0
0·25	2·5	2	0·25	4/4	37	27	73·0	6·8
0·25	0	2	0·25	2/4	29	14	48·0	7·0
0	10	2	0·25	2/4	26	6	23·1	3·0
0	2·5	2	0·25	2/4	29	8	27·6	4·0
0	0	2	0·25	0/4	30	0	0	0
Controls (sham-operated)				8/10	71	53	74·7	6·6
				Experiment 2				
1	—	2	0·25	2/4	27	16	59	8·0
1	—	2	0·125	4/4	31	22	71	5·5
1	—	2	0	4/4	38	33	87	8·3
1	—	1	0·25	4/4	26	18	69	4·5
1	—	1	0·125	4/4	27	22	81	5·5
1	—	1	0	2/4	31	6	19	3·0
0·5	—	2	0·25	2/4	26	8	31	4·0
0·5	—	2	0·125	3/4	26	19	73	6·3
0·5	—	2	0	3/4	29	17	59	5·7
0·5	—	1	0·25	3/4	28	16	57	5·3
0·5	—	1	0·125	3/4	35	19	54	6·3
0·5	—	1	0	3/4	28	18	64	6·0

Experiment 2

Experiment 1 showed an unexpectedly high response to 0·25 mg of progesterone on days 2–4, the best single cell being a combination of 0·25 mg of progesterone plus 2·5 mg of 20α-dihydroprogesterone.

It was therefore decided to make a further check of tolerance to variation in progesterone and oestradiol dosage at the pre-implantation and implantation stages. The structure and results of a 2 × 2 × 3 factorial with four rabbits per cell are shown in the second part of Table 1. Sham-operated controls were not included because of limitations of time and animals available and because such a test does not demand independent controls. Progesterone was given at two levels, 1 and 0·5 mg/day on days 2–4 and 1 and 2 mg/day on days 5–9, with 0·25, 0·125 or 0 μg/day of oestradiol.

The results once more show good tolerance to twofold variations in progesterone level, and a barely significant effect of oestradiol, 0·125 μg/day being the best. Chi-square tests were as follows: for progesterone given on days 2–4, $\chi^2_{(1)} = 2{\cdot}7$, $P > 0{\cdot}05$; for progesterone given on days 5–9 inclusive, $\chi^2_{(1)} = 2{\cdot}7$, $P > 0{\cdot}05$; for oestradiol given on days 5–9 inclusive, $\chi^2_{(2)} = 7{\cdot}20$, $0{\cdot}02 < P < 0{\cdot}05$. This slight effect of oestradiol was due to a maximal overall response (69% implantation) at 0·125 μg/day, the other two responses being 59% for 0·25 μg/day and 54% with no oestradiol. With such weak main effects, no further partitioning of χ^2 was attempted, as it could only lower the significance of an already dubious effect.

Table 2. Design of experiment 3

Each group was further subdivided (for anaesthesia with either halothane or pentobarbital sodium) with 4–6 animals per subgroup and two injections per day. P, progesterone; E_2, oestradiol

Treatment group and dosages	Days after insemination 2–4	5–9	10–14	15–19	20–24	25–29	30–term or day 38
1. Ovariectomized							
P (mg/day)	1	3	6	6	3	1	1
2. Ovariectomized							
P (mg/day)	1	3	6	6	3	1	1
E_2 (μg/day)	0	0·1	0·2	0·4	0·8	1·6	1·6
3. Controls							
Peanut oil (ml/day)	1	1	1	1	1	1	1

Experiment 3

In this experiment the injection plan, shown in Table 2, was similar to that used by Kwun and Emmens (1974), but with lower oestradiol doses throughout. In addition, the animals were not killed on day 30, but continued to receive injections until birth occurred, or to day 38 of pregnancy. On days 2 and 10 each group of 9–12 animals per replicate was split into two subgroups, one anaesthetized with halothane, the other with pentobarbital sodium.

Recent evidence suggests that both halothane and barbiturates interfere with ovulation by reducing the synaptic activation of neurones involved in the production of gonadotrophin releasing hormone (Richards 1972; Whitehead and Ruf 1973), while pentobarbital sodium is believed not to interfere with implantation in rats (Zeilmaker 1963), the effects of halothane not being known.

The results are shown in Table 3, for implantation percentages and foetal development percentages. Overall, progesterone alone gave 64% implantation and with oestradiol it gave 65%, as against 70% in controls ($\chi^2_{(2)} = 1{\cdot}65$, $P > 0{\cdot}05$). Halothane anaesthesia was accompanied by 61% implantation, as against 73% with pentobarbital sodium, and this was a highly significant difference ($\chi^2_{(1)} = 7{\cdot}7$, $P < 0{\cdot}01$). Although replication averages did not differ significantly ($\chi^2_{(1)} = 0{\cdot}82$, $P > 0{\cdot}05$), there was highly significant interaction ($\chi^2_{(7)} = 18{\cdot}9$, $P < 0{\cdot}01$) which was seen on inspection to be due to irregular effects of halothane *v.* pentobarbital sodium in the two replicates. In all, therefore, it cannot be asserted from this test that the two anaesthetics differed consistently in their influence on implantation.

Foetal development was similarly affected. Replications, treatments and interaction were all statistically insignificant in their effects, while the two anaesthetics

had a marginal difference in influence, halothane being just significantly less effective than pentobarbital sodium (64% *v.* 75%; $\chi^2_{(1)} = 4{\cdot}2$, $P < 0{\cdot}05$). It must be concluded that halothane anaesthesia did not favour the overall process of implantation and foetal development compared with pentobarbital sodium.

Table 3. Effects of progesterone, progesterone plus oestradiol, and of halothane and pentobarbital sodium anaesthesia on implantation and foetal development percentages in rabbits ovariectomized on day 2 after insemination on day 1 (experiment 3)

See Table 2 for details of treatment schedules. P, progesterone; E_2, oestradiol; I/CL, total implants/corpora lutea; F/I, total foetuses (alive or dead)/implants. Anaesthetic treatments given in parenthesis: H, halothane; PS, pentobarbital sodium. Rep., replicate.

Treatment and dosage groups	Implantation				Foetal development			
	Index	Rep. 1	Rep. 2	Total	Index	Rep. 1	Rep. 2	Total
1(a). P(H)	I/CL	28/46	20/41	48/87	F/I	17/28	13/20	30/48
	%	61	49	55	%	61	65	63
1(b). P (PS)	I/CL	29/41	26/33	55/74	F/I	22/29	21/26	43/55
	%	71	79	74	%	76	81	78
2(a). P + E_2(H)	I/CL	26/48	29/42	55/90	F/I	16/26	21/29	37/55
	%	54	69	61	%	62	72	67
2(b). P + E_2(PS)	I/CL	33/43	21/35	54/78	F/I	25/33	16/21	41/54
	%	77	60	69	%	76	76	76
3(a). Controls (H)	I/CL	26/38	33/52	59/90	F/I	11/26	26/33	37/59
	%	68	64	66	%	42	79	63
3(b). Controls (PS)	I/CL	24/42	43/48	67/90	F/I	17/24	31/43	48/67
	%	57	90	74	%	71	72	72

Since hormone treatments did not differ from one another or from controls, on either implantation rates or foetal development, it must also be concluded that progesterone alone is adequate for the continued development of the foetus to term.

Table 4. Effects of hormone administration continued to term (experiment 3)

Pregnancy was terminated on day 39 where necessary. H, halothane; PS, pentobarbital sodium

Treatment	Progesterone		Progesterone + oestradiol		Controls	
	H	PS	H	PS	H	PS
No. pregnant at term	8/10	8/8	8/8	9/9	9/11	11/11
Range of gestation periods (days)	33–39	31–39	39	39	32–39	32–35
No. of foetuses alive	2	14	0	0	33	44
No. of foetuses dead	28	29	37	41	4	4
No. alive (%)	7	33	0	0	89	92
No. born naturally (%)	50	95	5	44	97	100

However, with injections of progesterone and oestradiol continued to term there were no live births and natural parturition occurred only in 2 out of 17 animals. In the group given progesterone alone, parturition was protracted for 2–3 days and many young were born dead or were dead *in utero* by day 39, when all does were laparotomized or killed. Details are shown in Table 4, and in view of the absence of

overall replication differences in Tables 3 or 4, these replications are combined in Table 4.

While the only failures to remain pregnant occurred in the halothane-treated groups, and halothane had seemingly a worse influence on parturition than pentobarbital sodium in the progesterone-treated group, $\chi^2_{(1)}$ is only 2·4 and not statistically significant. The only and clearly obvious differences are between treatment groups and the controls, from which it is apparent that continuation of replacement therapy to term, even at the low progesterone dosage of 1 mg/day, was a mistake, and that its cessation prior to the expected birth period had to be investigated.

Table 5. Design of experiment 4

Animals (10 per treatment group in each replication) received twice daily injections. P, progesterone; E_2, oestradiol

Treatment group and dosages	Days after insemination 2–4	5–9	10–14	15–19	20–24	25–29	30–term or day 38
1. Ovariectomized							
P (mg/day)	1	3	6	6	3	1	1
2. Ovariectomized							
P (mg/day)	1	3	6	6	3	1	0
3. Ovariectomized							
P (mg/day)	1	3	6	6	3	1	0
E_2 (μg/day)	0	0·2	0·2	0·2	0·2	0·2	0·2
4. Controls							
Peanut oil (ml/day)	1	1	1	1	1	1	1

Table 6. Effects of stopping progesterone injections on day 29 of pregnancy and of a low constant dose of oestradiol on implantation and foetal development percentages in rabbits ovariectomized on day 2 after insemination on day 1 (experiment 4)

See Table 5 for details of treatment schedules. P, progesterone; E_2, oestradiol; I/CL, total implants/corpora lutea; F/I, total foetuses (alive or dead)/implants. Rep., replicate.

Treatment and dosage groups	Implantation Index	Rep. 1	Rep. 2	Total	Foetal development Index	Rep. 1	Rep. 2	Total
1. P to term or	I/CL	40/75	31/61	71/136	F/I	20/40	18/31	38/71
day 38	%	53	51	52	%	50	58	54
2. P to day 29	I/CL	34/67	39/81	73/148	F/I	14/34	21/39	35/73
	%	51	48	49	%	41	54	48
3. P to day 29,	I/CL	53/80	35/70	88/150	F/I	33/53	22/35	55/88
E_2 to term	%	66	50	59	%	62	63	63
4. Controls	I/CL	30/73	40/81	70/154	F/I	15/30	15/40	30/70
	%	41	49	45	%	50	38	43

Experiment 4

The plan of this experiment is shown in Table 5. The experiment was replicated, with 10 animals per group initially in each replication. It investigated the difference between ceasing progesterone injection on day 29, with or without a continuing low dose of oestradiol (0·2 μg/day), and carrying the injections to term or day 38 as before. Halothane anaesthesia was still used, despite its drawbacks, as it is easier

to manage and adequate controls receiving the same treatment were included. There was an added advantage that results obtained in this experiment could be compared with those obtained in earlier experiments.

Results are shown in Table 6. Fertility and performance levels in the whole experiment were somewhat poorer than usual, and reasons could not be found to account for this. Chi-square tests showed no overall effects of treatment: $\chi^2_{(3)}$ for implantation was 5·6 ($P > 0{\cdot}05$) and for foetal development it was 6·8 ($P > 0{\cdot}05$).

Table 7. Effects of progesterone treatment continued to term or day 38, or to day 29 with or without oestradiol (experiment 4)

Pregnancy was terminated on day 39 where necessary. P, progesterone; E_2, oestradiol

Treatment	P to term or day 38	P to day 29	P to day 29, E_2 to term	Controls
No. pregnant at term	15/17	10/12	16/17	9/15
Range of gestation periods (days)	33–39[A]	33–39[B]	33–39[C]	31–34
No. of foetuses alive	2	14	32	21
No. of foetuses dead	36	21	23	9
No. alive (%)	5	40	58	70
No. born naturally (%)	63	94	98	97

[A] Ten animals 39. [B] Two animals 39, remainder 33–37. [C] One animal 39, remainder 33–36.

Birth records are shown in Table 7. Progesterone continued to term or day 38 gave the same results as before, but when it was stopped at day 29 more foetuses survived, but still significantly fewer than in the control group ($\chi^2_{(1)} = 4{\cdot}7$, $P < 0{\cdot}05$). With oestradiol added, the percentage of live births rose to 58%, not significantly less than in controls ($\chi^2_{(1)} = 0{\cdot}71$, $P > 0{\cdot}05$), so one is left in the rather equivocal position that still more exploration is required to determine whether any oestrogen treatment consistently improves the number of births obtained with progesterone treatment alone. However, in both groups in which progesterone injections ceased on day 29, the same high percentage of natural parturition (94–98%) occurred as in controls (97%).

Discussion

In these experiments, we are essentially asking whether progesterone alone is sufficient for the support of normal implantation and pregnancy in the rabbit, including normal birth. Potential influences of 20α-dihydroprogesterone and particularly of oestradiol have been examined, with somewhat equivocal results.

The role of 20α-dihydroprogesterone, later than on day 1 of pregnancy, would seem questionable. It has a weak influence on implantation and would not appear to be produced in sufficient amounts to be physiologically effective. Although a recent report (Labhsetwar 1972) suggests that 20α-dihydroprogesterone may play a physiological role by potentiating or augmenting the effect of progesterone on decidualization in rats, in the present tests when it was given together with progesterone it did not show any additive effect on implantation rates. However, its role immediately prior to ovulation is still unknown, unless the suggestion of Hilliard

et al. (1967) that it may prolong and increase the release of luteinizing hormone proves correct.

The role of oestradiol at the time of implantation and at birth remains problematical, and would not appear to be very important. As long as obvious excess was not given, oestradiol was found to improve foetal maintenance in progesterone-treated ovariectomized rabbits (Kwun and Emmens 1974), but not in ovariectomized, adrenalectomized animals. In the present tests oestradiol had a barely significant effect in increasing the implantation percentage in experiment 2 (based on 48 rabbits), but no effect in experiment 3, either on implantation or foetal development. In experiment 4, while a very low dose of oestradiol appeared to increase the percentage of live births, the improvement was not statistically significant compared with progesterone alone. These contrasts were with totals of up to 20 animals per group, and so it appears that the role of oestradiol would not seem to be significant. The important step was to stop hormone injections before the birth processes commenced, otherwise most of the young were born dead or left unborn and dead *in utero*. The percentages of natural birth in experiment 4 were similar as long as progesterone was not administered to term (94–98%). One suspects that there may be a small influence of oestradiol in late pregnancy, but very large groups of animals would be required for unequivocal demonstration of this influence.

Several authors (Hilliard and Eaton 1971; Challis *et al.* 1973; Kwun, Shutt and Emmens, unpublished data) have noted a small rise in plasma oestradiol just before implantation in the rabbit, usually of borderline statistical significance, and it seems likely that it is a genuine phenomenon. It may well be that the rabbit has evolved from species which resembled the rat or mouse in needing oestrogen for implantation, and in exhibiting a rise in oestrogen levels during early pregnancy, and that we are seeing the vestige of this in the present-day animal, with hormonal effects of equally low-grade biological significance.

The percentage values for the control implantation and foetal development in this work have usually been low. Adams (1960) used does mated once naturally and then in addition artificially inseminated and injected with 25 i.u. of luteinizing hormone. He performed laparotomy on days 10–17, and found 79–95% implantation and 77–81% survival, using the same methods of calculation as Kwun and Emmens (1974). He used pentobarbital sodium as the anaesthetic, and would thus seem to have given maximal chances of success. O'Ferrall (1973) proceeded much as we have done (25 i.u. of luteinizing hormone followed by artificial insemination and laparotomy at days 10–13), with pentobarbital sodium anaesthesia, and found 80% implantation and 54% survival. Tesh (1969), using no anaesthetics, reported 75–87% implantation and 53–80% foetal survival whilst Battaglia and Meacham (1969), using does twice mated naturally found 95% implantation and 58% foetal survival (no other details given). It is worth considering why our own control results have been rather low, particularly for implantation, and overall in experiment 4. We have seen that the use of halothane may explain part of this deficiency—had pentobarbital sodium been used throughout the work, both implantation and foetal development percentages would have been expected to rise from 60–65% to 70–75%. However, a difference between natural mating and gonadotrophin injection followed by artificial insemination may also exist (see the results of O'Ferrall 1973), so that the overall satisfactory nature of the experimental work, from the point of view of embryonic

wastage, might well be raised by using natural mating. Even the temperature at which the rabbits were kept (21–27°C) may be too high for optimal results, although local rabbits might be expected to have acclimatized to this by now.

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