REPEATED SUPEROVULATION FOLLOWING ADMINISTRATION OF EXOGENOUS GONADOTROPHINS IN DUTCH-BELTED RABBITS

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Summary. Repeated superovulation and in-vivo collection of ova were carried out with thirty does divided into three groups of ten does each. One group received FSH–LH, a second group received PMSG–HCG and the controls received only LH. Injections of FSH–LH at three 16-week intervals followed by one 8-week interval resulted in 46·5, 35·4, 25·2 and 18·0 ovulation points/doe. This decrease with time was significant ($P < 0.05$). Corresponding values for the PMSG–HCG group which, however, received FSH–LH for the final superovulation were 13·6, 5·7, 6·2 and 20·3. The LH controls averaged 7·8, 7·0, 5·1 and 5·6 ovulation points. Overall treatment differences were highly significant ($P < 0.005$). From the 1920 ovulation points 1593 ova (83%) were recovered, of which 83·1% were cleaved. Young born from unrecovered ova accounted for 3·1%.

Control kindlings by the same does at regular periods resulted in normal litter size but in fewer does kindling as the experiment progressed. Results of two bio-assays for antihormones suggested that this decrease was due to hormonal refractoriness which was most pronounced in the PMSG–HCG group.

INTRODUCTION

Repeated superovulation of the same female mammal has not been studied extensively. Parkes (1943) reported that rabbits injected with gonadotrophins for more than 5 days responded poorly. Adams (1953) attempted to superovulate immature rabbits repeatedly every 28 days with horse anterior pituitary extract and found that ovulation did not occur after four successive treatments. Later studies (Adams, 1961) showed that a series of six injections of 20 i.u. HCG resulted in a decrease in fertility which he attributed to ovulation failure. Similar results have been obtained with cows (Willett, Buckner & McShan, 1953), ewes (Pålsson, 1962) and mice (Edwards & Fowler, 1958; Lin & Bailey, 1965).

Foote, Hafs, Staples, Gregoire & Bratton (1963) reported no decline in fertility in sixteen does which were injected only twice with 2·5 mg of pituitary luteinizing hormone (PLH, Armour). Kennelly & Foote (1965) obtained an excellent ovulatory response with Dutch-belted rabbits primed with follicle

stimulating hormone (FSH, Armour) and ovulated with PLH. Techniques to carry out these procedures repeatedly without harmful effects on the female would facilitate the study of environmental and genetic factors contributing to embryo mortality and abnormal development.

The object of this investigation was to superovulate does repeatedly and collect the maximum number of ova by an in-vivo procedure.

MATERIALS AND METHODS

Thirty 20-week-old female Dutch-belted rabbits and four mature Dutch-belted males were used. All animals were kept at 21° C with 12 hr of artificial light. Each animal was caged individually and was fed a high quality pelleted diet containing a minimum of 17% protein.

The thirty does were assigned randomly to three treatment groups, ten to each group. Two groups received hormones inducing superovulation and the third served as control. The superovulating and ovulating hormones were administered at three 16-week intervals (called periods) to produce ova for the in-vivo collections. At the mid-point of each 16-week period each doe was ovulated with the assigned ovulating hormones, inseminated and allowed to kindle to determine whether or not the treatments were interfering with normal fertility. The plan was as follows:

<i>Period</i>	<i>First 8-week segment</i>	<i>Second 8-week segment</i>
1, 2, 3	Superovulated and ova collected	Ovulated and allowed to kindle
4	Superovulated and ova collected	—

The same schedule was followed in all groups except the controls which were not superovulated. Half of the does in each group were treated in the reverse order, that is, allowed to kindle and then superovulated.

All hormones were dissolved in physiological saline. Ten does received 0.3 mg follicle stimulating hormone, FSH (Armour F.S.H.-P), subcutaneously (s.c.) twice daily for 3 days (Kennelly & Foote, 1965). On the 4th morning they received intravenously (i.v.) 2.5 mg pituitary luteinizing hormone, PLH (Armour PLH). The same does received 0.5 mg FSH s.c. twice daily for 3 days for their second, third and fourth superovulation to compensate for continued growth past 20 weeks of age. The fourth time, one-half of the does received 2.5 mg ovine NIH-LH-s8 and the rest received the usual PLH.

The second group of ten does received 30 i.u. of PMSG (Equinex, Ayerst), s.c. once daily for 3 days. On the 5th morning they received i.v. 50 i.u. HCG (Fol-lutein, Squibb). The PMSG dosage was increased to 60 i.u./day for 3 days and the HCG was increased to 200 i.u. for the third superovulation in an attempt to overcome possible antihormone effects. For the fourth superovulation, these does received 0.5 mg FSH s.c. twice daily for 3 days and 2.5 mg PLH or ovine LH (NIH-LH-s8) i.v. on the 4th morning as in the previous group. The remaining ten control does received 2.5 mg PLH at the same time as the first two groups received their PLH or HCG, except that at the fourth ovulation, one-half of the control does also received LH (NIH-LH-s8), while the rest received PLH.

Immediately following injection of LH or HCG all does were artificially inseminated with 0.1 ml of semen obtained from males of proven fertility. The

same males were used throughout the experiment. Semen from one male was used to inseminate sets of three does, one from each treatment. Males were used in rotation so that semen was used to inseminate different sets of does in successive periods.

Ova were collected *in vivo* 26 to 32 hr after PLH or HCG injection. The does were anaesthetized with pentobarbital sodium, and the ovaries exposed by flank incisions. The oviduct and uterus were exteriorized and a 30-cm length of polyethylene tubing (Intramedic, PE 160) flanged slightly at the end was inserted into the fimbriated end of the oviduct and held in place by a miniature plastic clothes-pin covered with rubber. A blunted 20-gauge needle was inserted through the wall of the uterus and passed beyond the tubo-uterine junction into the isthmus of the oviduct. Ova were flushed toward the fimbriated end of the oviduct into a watchglass (50 mm diam.) using 3 ml of homologous serum (Chang, 1949). The ova were counted and examined for cleavage and abnormalities using a dissecting microscope. The number of ovulation points was recorded. A small sheet of absorbable gelatin (Gelfoam Sponge, Upjohn) was inserted before the incision was closed. Each doe received 0.25 ml of antibiotics (Combiotic, Pfizer) and was placed in a recovery box, heated to 37° C.

At the fourth superovulation, does were anaesthetized with ether and ova collected as previously described. The does were then killed with ether and the reproductive tracts removed and examined.

Blood samples were collected from the ear vein of six does at the end of the third period and before death. The serum was tested for an anti-HCG factor according to the procedure developed by Snook & Cole (1965). Six 24-day-old intact female rats (Carworth CFN strain) were used per group. The ventral prostate assay (Flux & Li, 1965) was used to test for an anti-PLH factor using six 23-day-old hypophysectomized male rats per group.

The data were analysed according to methods described in Steel & Torrie (1960).

RESULTS

Ovulation points and ova recovered

The number of ovulation points and ova recovered for all does are presented in Table 1 and those for the does responding in Text-fig. 1. Ovulation points and ova recovered differed ($P < 0.05$) among periods for the two superovulated groups, but not for the controls. This difference among periods was due to fewer does responding to exogenous hormones during the first three periods, especially in the PMSG-HCG group. However, the response per doe ovulating also declined in the FSH-LH group in the fourth period.

A highly significant treatment effect ($P < 0.005$) was observed during the first three periods. The fourth period was analysed separately because both superovulated groups received the same hormones. At this time, the superovulated groups did not differ from each other but did differ from the control ($P < 0.05$). No difference was detected between the equine PLH and the ovine NIH-LH-s8.

The percentage of ova recovered from in-vivo flushing for all treatments and periods was 82.7%. No significant differences were found among treatments or periods.

TABLE 1
REPEATED SUPEROVULATORY RESPONSES AND OVA RECOVERED

Treatment	Criteria	Period			
		1	2	3	4 ^a
FSH-LH**	Aver. No. of ovulation points	46.5	35.4	25.2*	18.0*
	Aver. No. (and %) of ova recovered	39.8 (85.6)	32.3 (91.2)	19.0* (75.4)	12.7* (70.4)
PMSG-HCG	Aver. No. of ovulation points	13.6	5.7†	6.2†	20.3 ^b
	Aver. No. (and %) of ova recovered	11.0 (80.9)	2.9† (50.9)	5.6† (90.3)	16.8 (82.5)
LH (control)	Aver. No. of ovulation points	7.8	7.0	5.1	5.6
	Aver. No. (and %) of ova recovered	6.0 (76.9)	6.2 (88.6)	4.9 (96.1)	5.0 (90.0)

^a Only nine does per treatment were used in period 4 due to the death of one animal in each group.

^b The PMSG-HCG treatment was discontinued and FSH-LH was given to each doe.

* Differed ($P < 0.05$) from period 1.

† Differed ($P < 0.05$) from period 4.

** Differed ($P < 0.005$) in no. of ovulation points from PMSG-HCG and the control during the first three periods and only from the control during the fourth period.

TEXT-FIG. 1. Average number of ovulation points and ova recovered per doe responding for the FSH-LH, PMSG-HCG and LH control groups. Unshaded columns, number of ova recovered; cross-hatched columns, number of ovulation points in excess of ova recovered.

Percentage of ova cleaved

The percentage of ova cleaved is given in Table 2. No significant differences were found among treatments or periods. Of the 1593 ova recovered 83.1% were cleaved. Only five of ninety-five does which ovulated had no cleaved ova.

TABLE 2
PERCENTAGE OF CLEAVED OVA

Treatment	Percentage of cleaved ova			
	Period 1	Period 2	Period 3	Period 4
FSH-LH	81.5	86.0	66.5	74.0
PMSG-HCG	98.5	100.0	65.0	97.0*
LH (control)	97.5	68.5	84.5	78.0
Period means	92.5	84.8	72.0	83.0

* PMSG-HCG was replaced by FSH-LH.

Three of the five does appeared to be in a pseudopregnant condition, although they were caged separately throughout the experiment. The cleavage rate in the ninety does which ovulated and had cleaved ova was 85.5%.

Young born from ova missed during in-vivo flushing

At least 17.3% of the ovulation points were not accounted for by ova collected, but 3.1% of the 17.3% can be accounted for by young born after the in-vivo flushings (Table 3). More ova were missed in the FSH-LH treatment than in the

TABLE 3
AVERAGE NUMBER OF YOUNG KINDLED FROM OVA NOT RECOVERED
BY FLUSHING (TEN DOES/TREATMENT)

Treatment	Period	Aver. No. of young based on all does	No. of does kindling	Aver. No./doe kindling
FSH-LH*	1	1.7	6	2.8
	2	1.6	5	3.2
	3	0.2†	1	2.0
PMSG-HCG	1	1.3	3	4.3
	2	0.6	3	2.0
	3	0.0†	0	0.0
LH (control)	1	0.3	2	1.5
	2	0.2	2	1.0
	3	0.0†	0	0.0

* Differed ($P < 0.01$) from PMSG-HCG and LH (control) with respect to total No. of young born.

† Differed ($P < 0.01$) from periods one and two.

other two treatments ($P < 0.01$), but more ova were ovulated in this group. The PMSG-HCG group had the lowest ovum recovery rate (Table 1) and the highest percentage of ovulation points which resulted in young. The percentages of ovulation points resulting in young for the FSH-LH, PMSG-HCG and control does were 3.3, 7.4 and 2.5%, respectively. More young were born after the first two in-vivo flushings than after the third ($P < 0.01$). Six of the sixteen does which produced young after in-vivo flushings kindled twice.

A total of fifty-nine young were born with 13.6% stillborn. The average gestation for these missed embryos was 32.5 days.

Fertility of does between in-vivo flushings

Table 4 shows the average number of young born during the kindling phase. A significant difference ($P < 0.05$) was found among treatments and between sequences, but no significant difference was found among periods. The LH controls produced more young than the PMSG-HCG treated does, primarily because more controls kindled. The animals which were allowed to kindle first produced more young ($P < 0.05$) than did the animals which were super-ovulated first. The average litter size for all does kindling was 5.4 young.

Autopsy

Upon completion of the experiment each ovary, oviduct and uterine horn was examined and placed in one of five groups according to its appearance and

functional state. Reproductive tracts from the three animals which died were examined and are included. The percentage of ovaries that were in good to excellent condition, slightly adherent but functional, moderately adherent but

TABLE 4

NUMBER OF YOUNG BORN BETWEEN IN-VIVO FLUSHINGS (FIVE DOES PER SEQUENCE AND TREATMENT SUBCLASS)

Treatment	Period 1		Period 2		Period 3		Sequence total and means		Grand total and means
	SO-K*	K-SO*	SO-K	K-SO	SO-K	K-SO	SO-K	K-SO	
FSH-LH									
No. does that kindled	3	3	4	4	3	4	10	11	21
No. young	18	19	15	13	17	24	50	56	106
Aver. young/doe that kindled	6.0	6.3	3.8	3.3	5.7	6.0	5.0	5.1	5.0
PMSG-HCG									
No. does that kindled	2	5	1	3	2	1	5	9	14
No. young	8	27	2	10	10	6	20	43	63
Aver. young/doe that kindled	4.0	5.4	2.0	3.3	5.0	6.0	4.0	4.8	4.5
LH (control)									
No. does that kindled	4	5	4	3	2	4	10	12	22
No. young	17	38	19	18	12	33	48	89	137
Aver. young/doe that kindled	4.3	7.6	4.8	6.0	6.0	8.3	4.8	7.4	6.2

* 'SO-K' Sequence = Superovulated and then kindled; 'K-SO' Sequence = Kindled and then superovulated.

TABLE 5

BIO-ASSAY OF RABBIT SERUM AGAINST PLH USING THE VENTRAL PROSTATE ASSAY (SIX RATS/TREATMENT)

Treatment	Aver. ventral prostate weight (mg)	Aver. testis weight (mg)
Control (Saline)	3.8	220.1
40 µg LH	6.8	237.7
40 µg LH + 1 ml control serum*	7.6	248.4
40 µg LH + 1 ml serum 215 ^b	5.9	255.8*
40 µg LH + 1 ml serum 181 ^c	5.4	216.3
40 µg LH + 1 ml serum 164 ^c	5.1	190.3†

* Serum from a doe which had never been injected with LH.

^b Serum from doe 215 which ovulated after exogenous hormones.

^c Serum from does 181 and 164, both of which failed to ovulate after exogenous hormones.

* Differed significantly ($P < 0.05$) from the control (saline).

† Differed significantly ($P < 0.05$) from the 40 µg LH treatment.

functional, extensively adherent and partially functional, and non-functional were 56, 25, 10, 2 and 7%, respectively. Corresponding values for the oviducts were 29, 45, 13, 5 and 8%, and for the uteri were 85, 13, 2, 0, 0%, respectively. The non-functional ovaries were embedded in the fat pad with no evidence of

having been stimulated by the exogenous hormones. Only five of sixty oviducts were cystic, one doe having two cystic oviducts.

Bio-assays

A bio-assay for anti-HCG in serum from a doe which failed to respond to HCG was conducted by injecting six groups of rats. In rats receiving saline (controls), 20 i.u. of HCG, 20 i.u. of HCG plus 1 ml of control serum or 20 i.u. of HCG and either 0.67, 0.83, or 1 ml of anti-HCG serum, ovarian weights averaged 16.3, 45.6, 39.2, 29.4, 28.3 and 20.9 mg, respectively. Corresponding uterine weights averaged 37.3, 110.9, 115.3, 97.9, 129.3 and 144.3 mg. At a dose level of 1 ml, anti-HCG serum significantly decreased ($P < 0.05$) ovarian weights and significantly increased ($P < 0.05$) uterine weights.

Table 5 gives the bio-assays for an anti-PLH factor. No significant differences were found among the ventral prostate weights, but the testis weights were significantly ($P < 0.05$) decreased with serum from does which failed to ovulate following injection with PLH.

DISCUSSION

Ovulation points and ova recovered

The results indicate that rabbits can be superovulated numerous times following administration of exogenous gonadotrophins, but there was a significant decrease ($P < 0.05$) following the fourth series of injections. By this time, the does in the FSH-LH group had received PLH singly or in combination with FSH seven times within 56 weeks. The does receiving the PMSG-HCG treatment appeared to develop an immediate refractoriness to the hormone treatment as only four out of ten and five out of ten does ovulated following treatment in periods 2 and 3 (Text-fig. 1). The ovaries showed a moderate development of unruptured follicles, indicating that an anti-HCG response was the primary cause of ovulation failure. When these same does were given FSH and LH at their fourth induced superovulation seven out of nine does produced an average of twenty-six ova.

The results of the two bio-assays are not conclusive but do suggest anti-hormone production. Does which apparently developed a refractoriness to the HCG responded to FSH and PLH. This would indicate that the capacity of the ovaries to respond was not affected, but that the exogenous ovulating hormone was inhibited.

The decrease found in the PMSG-HCG treatment, and to a lesser extent in the FSH-LH treatment, agrees with the work of Parkes (1943) and Adams (1953) who also found rabbits refractory to exogenous hormones. This type of decreasing response which tends to follow an asymptotic curve was observed in the cow (Willett *et al.*, 1953). Lin & Bailly (1965) found the mouse developed refractoriness toward HCG rather than PMSG and that animals which developed refractoriness to exogenous hormones ovulated when mated.

Manipulation of the oviduct did not interfere with ovum pick up or collection as indicated by the high rate of recovery (average 82.7%). This compares favourably with in-vitro methods used by Kennelly & Foote (1965) and Adams

(1954) who collected 80% and 78.5%, respectively, of the ova from excised tubes. It is a higher rate than has been reported previously for in-vivo recovery of rabbit ova (Adams, 1953; Hafez, 1961).

Percentage of cleaved ova

The 83% cleavage rate for all does lies between the 75% cleavage rate for superovulated ova reported by Kennelly & Foote (1965) and the 96.1% reported by Adams (1960). The number of fertilized ova may have been underestimated in the present study because ova were collected 26 to 32 hr post-PLH injection. Varian, Maurer & Foote (1967) found that not all fertilized superovulated rabbit ova have started to cleave 26 hr after PLH injection.

Young born from ova missed during in-vivo flushing

The does in the FSH-LH treatment produced more ova but more ova were missed which resulted in young. However, the percentage of ovulation points resulting in young was greatest for the PMSG-HCG group. Fewer young were born after the third flushing. This reduction can be accounted for in three ways: (1) the technique for in-vivo flushing improved, (2) fewer ova were ovulated, so reducing the possibility of not recovering an ovum, and (3) a higher rate of embryonic mortality might have occurred. The latter seems to be unlikely as the litter size/doe kindling did not decrease during the third period (Table 4).

The gestation was 1.5 days longer than the normal colony gestation of 31.0 days, but it is similar to the gestation length associated with small litters.

Fertility of does between in-vivo flushings

Does which were allowed to kindle first produced more young than did those which were superovulated first. This may have been due to the type of exogenous hormone and number of injections previously given (Table 4). The does kindling first had one series of hormone injections and surgery less than those of the group treated in the reverse sequence. The significant reduction in the number of does kindling (Table 4) in the PMSG-HCG group is explicable on the basis of the apparent refractoriness to HCG which developed rapidly in this group. Subsequently this group responded to LH injections. It is worth noting that the litter size after three periods of superovulation and ovum collection were similar to litter size at the beginning of the experiment.

Autopsies

The autopsies indicated that in-vivo flushing technique and manipulation of the reproductive organs produced few gross lesions. With proper handling, ova can be collected many times by this in-vivo technique leaving the reproductive system of the treated female rabbit unimpaired.

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