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ADMINISTRATION OF GnRH *IN VIVO* STIMULATES PROGESTERONE AND INHIBITS ANDROGEN ACCUMULATION BY OVARIAN FOLLICLES ISOLATED FROM PUBERTAL RABBITS

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Summary—A single i.v. injection of gonadotropin releasing hormone (GnRH) to pubertal female rabbits led to an ovulatory pulse of LH but no ovulations resulted. By contrast, 5 i.v. injections over 6 h led to 1–3 ovulations in 5 of 8 animals treated. Twenty-four hours after the initial injection animals were killed and follicles isolated. Large follicles >1 mm dia, from both GnRH treated groups released more progesterone during the control incubation period than those from saline treated. Small follicles <1 mm dia, from the same GnRH groups accumulated 3–6 times more progesterone than those from saline treated when stimulated with luteinizing hormone (LH). Testosterone accumulation by small and large follicles was not affected by one injection of GnRH but was depressed in follicles from rabbits treated with 5 injections of GnRH. A single injection of GnRH enhanced the ability of small and large follicles to release estradiol which was depressed 30% in the presence of LH. Multiple GnRH injections depressed estradiol accumulation by small and large follicles. These data suggest the administration of GnRH *in vivo* can have stimulatory as well as inhibitory effects on subsequent follicular steroid release and accumulation *in vitro*.

INTRODUCTION

In primates including man, the ovulatory dose of luteinizing hormone at midcycle occurs in a pulsatile manner [1,2]. Gonadotropin-releasing hormone (GnRH) if given in a pulsatile fashion to mimic physiological conditions can induce ovulation in the infertile patient with hypogonadotropic hypogonadism [3]. On the other hand, supraphysiological doses of GnRH or the potent agonist while stimulating pituitary release of gonadotropins, can have a major inhibitory influence on gonadal functions [4–6].

The rabbit is an induced ovulator and coitus results in a single surge of LH which does not appear to be secreted in a pulsatile fashion [7–10]. A single i.v. injection of GnRH to adult female rabbits results in an ovulation rate of 100% [11]. However, the effects of GnRH during puberty is not well documented. The aim of the present investigation was therefore to determine (1) whether a single injection of GnRH can induce an ovulatory surge of LH in pubertal rabbits, (2) whether abnormal ovulatory pulses of LH induced by multiple injections of GnRH can increase the ovulation rate and (3) whether follicles isolated from these GnRH treated rabbits would produce steroids in abnormal amounts in response to exogenous LH.

EXPERIMENTAL

Factrel® (synthetic GnRH) was obtained from Ayerst Laboratories, Montreal, Canada. It was dissolved in normal saline before use.

[1,2-^{3}H]Progesterone (sp. act. 57 Ci/mmol), [1,2,6,7-^{3}H] testosterone (sp. act. 93.9 Ci/mmol) and [6,7-^{3}H]estradiol (sp. act. 52 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA).

All rabbits were approx 12-weeks old. Eight animals were used per group. They were housed individually in cages with food and water available *ad libitum*. Experimental conditions were the same as previously described [12]. A Teflon catheter (Angiocath, 20 gauge, $1\frac{1}{4}$ in. Deseret Pharmaceutical Co., Sandy, Utah) was inserted into the central ear artery under local anaesthesia. The catheter was flushed with heparin, closed with a luer-lok cap and held in place with adhesive tape. Rabbits were given an intravenous injection of 1.5 μg GnRH in 0.3 ml normal saline either once (GnRH-1) or every 1.5 h for 5 injections (GnRH-5). Blood was withdrawn through the indwelling catheter and serum stored until later analyses. Control rabbits received normal saline.

The two protocols used are summarized in Fig. 1. For GnRH-1, blood (1 ml) was withdrawn every 15 min for 1 h prior to the injection of GnRH through the catheter. Then 4 more samples of blood were withdrawn at 15 min intervals during 1 h and 6 more samples were taken 30 min apart for 3 h. At 24 h after the GnRH injection animals were killed. In the second group of animals, 5 injections of GnRH were given (GnRH-5). Two samples of blood were withdrawn at 15 min intervals before the first GnRH injection. Then 6 more samples at 15 min intervals over the next 1.5 h. This procedure was repeated for a total of 5 GnRH injections. Normal saline was given to replace blood loss.

Incubation

After a blood sample was taken, rabbits were killed with an overdose of sodium pentobarbital 24 h after the first GnRH injection. Ovaries and uteri were removed, weighed and the presence of fresh corpora lutea noted. A piece of one ovary from each rabbit was fixed in Davidson's fixative for microscopic examination using hematoxylin-eosin staining. Follicles from all rabbits in each group were dissected free of interstitial tissue, pooled and separated with groups of follicles < 1 mm dia and > 1 mm dia. Hemorrhagic follicles and corpora lutea were not used. Large follicles were then incubated singly and small follicles in groups of 2–6 for 0.5 h in Krebs–Ringer bicarbonate buffer containing 0.2% glucose and 0.1% BSA. Media were removed and follicles incubated for a further 2 h in medium alone (control) or in medium containing 1 μg/ml LH (NIH-oLH-S17). The media were then removed and stored for later steroid analysis.

Statistical analyses

P values were determined with Duncan's multiple range test or Student's *t*-test using a HP97 calculator. To maintain homogeneity of variance steroid values were converted to natural logarithms before analyses. Undetectable steroid levels were assumed to be at the limit of sensitivity of the assay before conversion to natural logarithms. A *P* value less than 0.05 was considered significant. All differences noted were significant at greater than 95% level.

Radioimmunoassays

LH was measured by a heterologous double antibody radioimmunoassay using reagents provided by Drs A. F. Parlow, L. E. Reichert, Jr and R. J. Scaramuzzi. The characteristics of the assay have been published [12]. Aliquots of 10–100 μl of sera were usually analysed. Results are expressed in terms of a pure rabbit pituitary LH standard (EX-130 GB) (Dr H. Papkoff).

FSH was measured by a homologous double antibody radioimmunoassay system with reagents obtained though the Hormone Distribution Program NICHHD, Md (courtesy of Dr A. F. Parlow). Assay characteristics were the same as published [13]. Results are expressed in terms of the pure FSH standard, AFP-538C, used in iodination.

Steroid assays were carried out on dilutions of the incubation medium using antisera produced in our laboratories. The progesterone antiserum was raised against progesterone-11α-hemisuccinyl-BSA and had less than 0.1% cross reactivity with 20α-dihydroprogesterone, testosterone, dihydrotestosterone, androstenedione, estrone and estradiol. Only corticosterone cross-reacted 1.2%. The limit of sensitivity of the assay was 10 pg. Testosterone was measured using an antiserum raised against testosterone-3-oxime BSA [13]. It cross-reacted significantly with dihydrotestosterone (35%). Although further metabolism of testosterone to other products under similar experimental conditions is less than 8% [14] the assay was considered to be measuring total androgens. The limit of sensitivity of the assay was 25 pg. Estradiol was measured using an antiserum raised against estradiol-6-carboxymethyloxime BSA [15]. Its cross reaction with estrone, estriol, progesterone, testosterone and androstenedione was less than 0.1%. The limit of sensitivity of the assay was 10 pg. All steroid assays had an interassay coefficient of variation of less than 15%. Steroid levels in medium without follicles were below the limit of sensitivity of the assay. Several dilutions of medium from LH-stimulated follicles showed parallelism with standard curves.

RESULTS

There was no significant difference in uterine and body weights between groups. Uterine weights were approx 2 g. The ovarian weights of GnRH-1 rabbits were 84.7 ± 4.5 (SEM) mg compared to controls (70.8 ± 3.3 mg) and GnRH-5 were 98.4 ± 7.2 versus 90.4 ± 9.9 for controls. Histological examination of ovarian pieces showed atretic follicles as well as large antral follicles with 7–10 layers of granulosa cells. Because a small piece of ovary was taken for histology it was not possible to quantitate normal and atretic follicles. In 5 of 8 GnRH-5 rabbits 1–2 hemorrhagic follicles and corpora lutea were seen. No ovulations occurred in any of the 8 GnRH-1 animals.

Serum gonadotropins after GnRH

The LH and FSH responses to one injection of GnRH are shown in Fig. 2. Within 15 min of the injection there was a peak in the secretion of both LH and FSH. The LH increase was more than 40-fold whereas the FSH increase was only 3-fold. Baseline levels of LH were reached within 2 h of injection whereas FSH remained elevated for the duration of the sampling period. By 24 h FSH had also returned to preinjection levels.

The effects of multiple doses of GnRH (GnRH-5) on gonadotropin secretion are shown in Fig. 3. After the second injection there appeared to be a progressive loss of ability of the pituitary to secrete LH. As GnRH-1 the FSH increases due to GnRH was similar to the amount of FSH normally released prior to ovulation. The high standard errors were due to variable responses of two animals where FSH increases were small. Unlike the GnRH-1 group FSH levels 24 h after the first injection appeared to be higher than preinjection levels but were not significantly different when compared with immediate preinjection levels by the paired *t*-test.

Follicular response in GnRH-1

The steroid response of follicles from rabbits given a single injection of GnRH is shown in Table 1. In

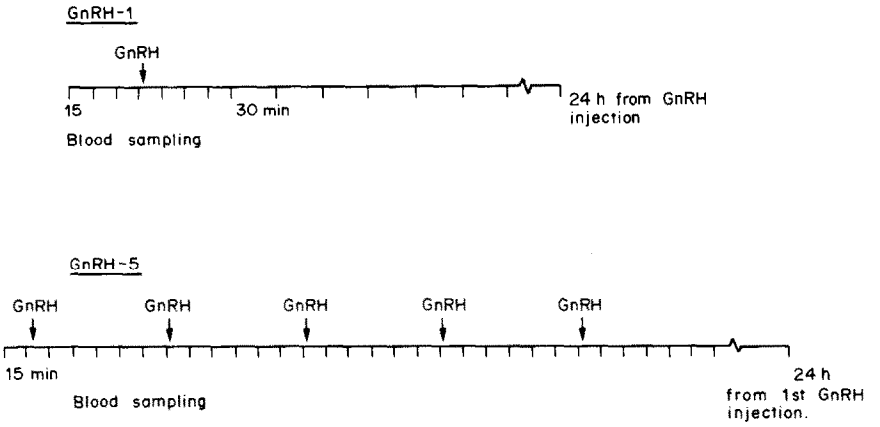

Fig. 1. Pubertal female rabbits were given two different schedules of GnRH. In GnRH-1, a single injection of GnRH (1.5 μg) was given after 4 × 15 min blood samples were taken. In GnRH-5, multiple injections of GnRH were given at the times indicated. Blood sampling was done every 15 min or 30 min as indicated.

order to simplify later discussions follicles from saline and GnRH treated rabbits are referred to as Sal-follicles and GnRH-1 or GnRH-5 follicles. Endogenous progesterone accumulation by large GnRH-1 follicles was greater than that from small and large Sal-follicles and small GnRH-1 follicles which was negligible. LH stimulated progesterone release by all Sal- and GnRH-1 follicles. The magnitude of the LH response was greater (>100-fold) in small GnRH-1 and large Sal-follicles compared to large GnRH-1 and small Sal-follicles.

The endogenous accumulation of androgens by all Sal- and GnRH-1 follicles was similar. LH caused a 10-fold increase in the release of androgens by all follicles except the large Sal-follicles where a 50-fold increase was found.

Estradiol accumulation by small Sal-follicles was negligible and did not change with LH. Large Sal-follicles released 57 ± 18 (SEM) pg per follicle and was increased to 212 ± 75 pg per follicle in the presence of LH. Small GnRH-1 follicles released 44 ± 8 pg estradiol per follicle and large GnRH-1 follicles 166 ± 58 pg per follicle. The presence of LH in the medium caused a 30% decrease ($P < 0.001$) in the release of estradiol by small and large GnRH-1 follicles.

Follicular response in GnRH-5

The accumulation of steroids in the medium of follicles isolated from rabbits 24 h after the first of 5 multiple injections of GnRH is shown in Table 2. There was significantly more progesterone accumulation in medium with small and large GnRH-5 follicles compared to Sal-follicles where progesterone was undetectable. LH stimulated progesterone accumulation by all follicles. In the GnRH-5 group LH caused a 9.5-fold increase in progesterone accumulation by small follicles and a 2.5-fold increase by large follicles.

Multiple injections of GnRH inhibited androgen release to undetectable levels by small and large follicles incubated *in vitro*. Both small and large Sal-follicles of the control group released significant amounts of androgen which was similar to levels accumulated by small and large GnRH-5 follicles stimulated with LH. LH caused a 6.3-fold increase in

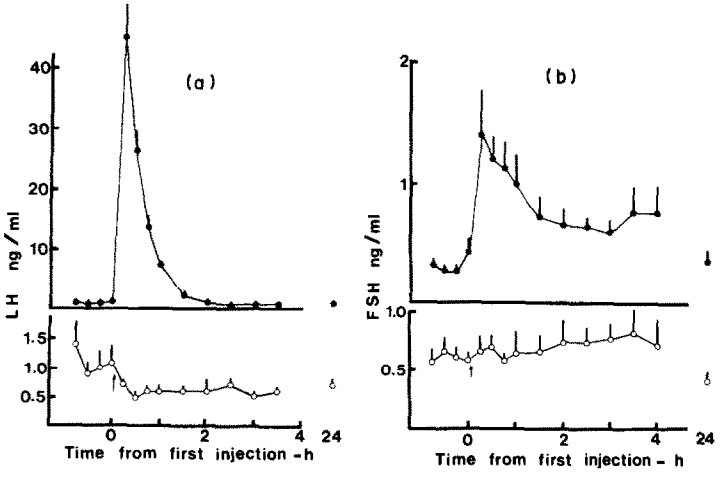

Fig. 2 Effect of a single dose of GnRH (1.5 μg) on the release of (a) LH and (b) FSH in pubertal female rabbits. Solid circles denote GnRH treated and open circles saline treated. Results are mean ± SEM. $n = 8$

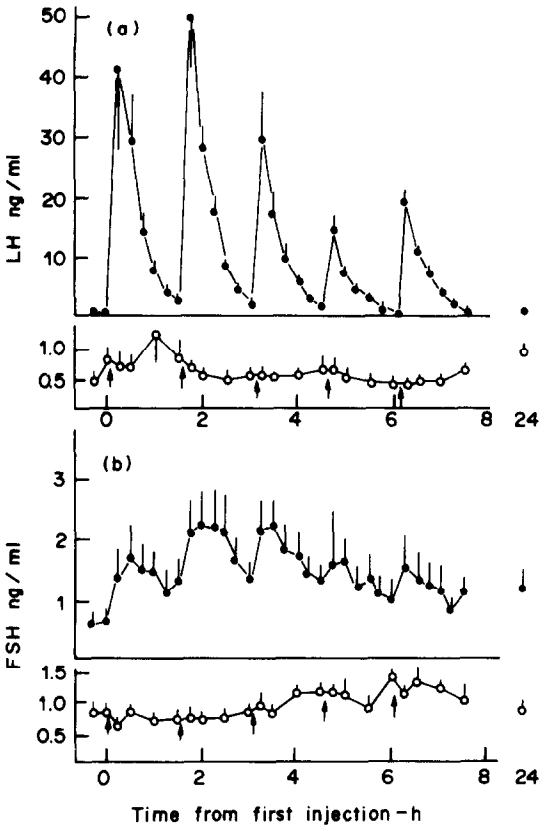

Fig. 3. Effect of 5 injections of GnRH (1.5 μg) given 1.5 h apart, on the release of (a) LH and (b) FSH in pubertal female rabbits. Symbols are the same as in Fig. 1. $n = 8$

androgen accumulation by small Sal-follicles and a 17.6-fold increase by large Sal-follicles.

Estradiol release by isolated follicles after 5 injections of GnRH was not detectable. Stimulation with LH caused large Sal-follicles to release 1.8-fold more estradiol from 146 ± 64 pg per follicle to 268 ± 93 pg per follicle. No response to LH was elicited in small Sal or GnRH-5 follicles. Large GnRH-5 follicles released 89 ± 49 pg estradiol per follicle in the presence of LH.

DISCUSSION

Histological examination of ovarian pieces from saline treated rabbits showed follicles at different stages of development. Cell debris was also observed in about 50% of the antral follicles seen in some sections, indicating that atresia was well advanced. Because of the method of sampling it was not possible to obtain a quantitative analysis of all the different types of follicles in each ovary. A more detailed morphological study would be necessary to obtain such data. Serum levels of LH and FSH were identical to those reported by Turckheim *et al.* [16] for day 90 rabbits.

The administration of single or multiple doses of GnRH caused ovulatory increases in LH secretion. Levels of LH attained were similar to those previously found for normal adult females [7–10, 17]. With a comparable single dose of GnRH, Amoss *et al.* [11] found 100% ovulation frequency in adult female rabbits. The failure of a single dose of GnRH to induce ovulation indicates the absence of mature follicles within the ovary. Although well developed antral follicles were observed in ovarian pieces it is possible that they were not sufficiently primed or that the number of LH receptors was not optimal. Such an explanation would be consistent with the few ovulations observed in the GnRH-5 group. Further support for this concept is provided by the demonstration in the hypophysectomized immature female rat that pulsatile release of LH can increase follicular LH receptors [18].

To date, the pulsatile release of LH in the female rabbit has not been demonstrated. The data in the

Table 1. Effects of one injection of GnRH on steroid accumulation during 2 h incubation without and with LH (1 μg/ml)

			Steroid accumulation (pg/follicle/2h)					
			Progesterone		Androgen		Estradiol	
(a)	Saline treated		−LH	+LH	−LH	+LH	−LH	+LH
	Small follicles	($n = 5$)	<25	39 ± 11	67 ± 12	886 ± 245	<25	<25
	Large follicles	($n = 7$)	<25	269 ± 66	92 ± 34	4658 ± 1434	57 ± 18	212 ± 75
(b)	GnRH-1							
	Small follicles	($n = 5$)	25	131 ± 25	81 ± 34	729 ± 334	44 ± 8	31 ± 6
	Large follicles	($n = 10$)	209 ± 90	1575 ± 657	55 ± 21	782 ± 257	166 ± 58	114 ± 31

Small follicles were less than 1 mm dia and large greater than 1 mm. All data were converted to natural logarithms before analysis by the Student's t-test. LH altered steroid accumulation in all instances except for estradiol by small follicles from saline treated rabbits. Results are mean ± SEM.

Table 2. Effects of 5 injections of GnRH on steroid accumulation during 2 h incubations without and with LH (1 μg/ml)

			Steroid accumulation (pg/follicle/2 h)					
			Progesterone		Androgen		Estradiol	
(a)	Saline treated		−LH	+LH	−LH	+LH	−LH	+LH
	Small follicles	($n = 7$)	<25	50 ± 17	112 ± 49	707 ± 160	30 ± 5	32 ± 4
	Large follicles	($n = 7$)	<25	261 ± 100	122 ± 63	2150 ± 622	146 ± 64	268 ± 93
(b)	GnRH-5							
	Small follicles	($n = 9$)	33 ± 4	320 ± 59	<25	81 ± 33	<25	<25
	Large follicles	($n = 10$)	2374 ± 887	5975 ± 149	<25	364 ± 173	<25	89 ± 49

Data were analysed in the same manner as in Table 1. All values obtained with LH treatment were significantly different from those without LH except estradiol accumulation by small follicles from both groups.
Results are mean ± SEM.

present study suggest that pulsatile gonadotropin stimulation of the pubertal ovary can advance follicular maturation and lead to ovulation. As shown in Fig. 3, serum levels of FSH in GnRH-5 animals remained higher than pre-injection levels throughout the sampling period. Thus FSH probably has a major role in controlling follicular maturation.

In view of the major action of LH to down regulate its own receptors as seen in corpus luteum cells [19] it was hypothesized that steroid synthesis would be inhibited 24 h after an ovulatory surge of LH as occurs in the mature rabbit [20]. However both progesterone and androgen accumulation from small and large GnRH-1 and GnRH-5 follicles were stimulated by LH *in vitro* (Tables 1 and 2). This suggests that there may be a dissociation between adenylate cyclase and steroidogenesis at some stages of development. Further support for this suggestion is provided by the data of Bahr *et al.* [21] who used a perifusion system and showed that isolated rabbit follicles could be stimulated to produce progesterone when pulsed with LH at regular intervals.

Large follicles from GnRH treated rabbits consistently released more progesterone when compared to follicles from saline treated rabbits (Tables 1 and 2). Whether this was due to atretic follicles which are known to produce progesterone [15] could not be determined. However, this observation is similar to data reported by others [22,23] when preovulatory follicles were isolated after the endogenous LH surge. With such follicles progesterone accumulation was stimulated while both testosterone and estradiol accumulation were inhibited.

While it is possible that GnRH effects are probably mediated through an influence of LH on the ovary a direct effect of GnRH cannot be completely ruled out. It has recently been shown that LHRH can have a direct *in vitro* effect on increasing progesterone production by rat granulosa cells [24,25] and preovulatory follicles [26]; the production of progesterone precursor, pregnenolone, can also be stimulated by GnRH [27]. Ekholm *et al.* [28] have also shown that GnRH can stimulate progesterone secretion on the morning of proestrus in PMS primed hypophysectomized immature rats. On the other hand, a single injection of GnRH agonist to intact prepubertal rats leads to loss of ovarian LH receptors and diminished progesterone secretion by ovarian cells [29], further substantiating the view that responses to GnRH depend on the test system used.

In summary, these data demonstrate that multiple injections of GnRH may induce ovulation in the prepubertal rabbit and that the dose schedule of GnRH can influence the subsequent pattern of steroid accumulation when follicles are incubated *in vitro*.

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