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Effects of Administration of a LH-RH Inhibitory Analogue on Stages of the Rat Estrous Cycle[1,2] (40305)

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It has been demonstrated that synthetic inhibitory analogues of LH-RH can block pre-ovulatory surges of gonadotropins and ovulation in hamsters (1) and rats (2, 3). The subcutaneous administration of 750 μg of [D-Phe^2, D-Leu^6]-LH-RH four times on proestrous afternoon in hamsters, produced an 80% suppression of the LH surge and a 30% blockade of ovulation (1). In rats, 6 mg of [D-Phe^2, D-Ala^6]-LH-RH, injected in several doses on the afternoon of proestrus, brought about a 90% inhibition of ovulation (2) whereas a single dose of 1.5 mg of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH at noon on proestrus strongly inhibited LH and FSH surges and suppressed ovulation by 85% (3). Recently, Beattie *et al.* (4) reported that [D-Phe^2, D-Ala^6]-LH-RH significantly inhibited ovulation when it was injected on days other than estrus in rats. Using *in vivo* assays, such as inhibition of LH-RH-induced LH release in immature male rats, and blockade of ovulation in normal rats, [D-Phe^2, Phe^3, D-Phe^6]-LH-RH is more potent and longer acting than [D-Phe^2, D-Leu^6]-LH-RH (3, 5), which in turn is more potent than [D-Phe^2, D-Ala^6]-LH-RH (5). We have therefore investigated the effects of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH on ovulation in rats when injected at different stages of the estrous cycle or daily during estrus (E), diestrus 1 (D1) and diestrus 2 (D2).

Materials and methods. Adult female rats (Charles River CD strain), weighing 200–250 g were maintained under conditions of controlled lighting (14 hr light and 10 hr darkness) and temperature (22°). Following a one week period of adjustment to the animal house, their estrous cycles were determined by inspection of daily vaginal smears. Only those animals presenting at least two successive, regular 4-day cycles were used.

In the first experiment, the animals were injected s.c. with a single 1.5 mg dose of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH in 0.5 ml of vehicle or with vehicle alone (20% propylene glycol/saline solution) at noon of either E, D1, D2 or proestrus. Another group was injected at 9 AM on proestrus. On the following estrus, the animals were sacrificed and ovulation was checked by counting the number of ova under a dissecting microscope. The number of rats which ovulated compared to the total number of rats was considered an index of the antiovulatory activity of the analogue.

In a second experiment, rats were injected s.c. with a 1.5 mg dose of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH twice a day (9 AM and 4 PM; total dose: 3 mg/day) during E, D1 and D2. No injection was given on proestrus. Control rats were injected with 0.5 ml of vehicle alone. On the following E, ovulation of both control and experimental animals was checked as described above. At 4 PM on each day of treatment, a blood sample from the jugular vein of control and experimental animals was collected within 20–30 sec under light ether anesthesia. The blood was centrifuged and sera separated and stored at −20° until assayed for LH and steroids. Some ovaries from control and [D-Phe^2, Phe^3, D-Phe^6]-LH-RH treated rats were removed at the time ovulation was being determined. The ovaries were fixed in Bouin's solution and then stained with hematoxylin-eosin (Bay Histology Service, San Rafael, CA). Vaginal smears were also examined daily during the period of treatment. Serum LH was determined by the double antibody radioimmunoassay method

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of Niswender *et al.* (6) as described elsewhere (7, 8). NIH-LH-S_{17} was used as the standard preparation.

Estradiol and progesterone were measured in duplicate by the method of Abraham *et al.* (9) with slight modifications. About 1000 dpm of 2,4,6,7-^{3}H-17β estradiol (SA:91, 3 Ci/m*M*) and of 1,2,6,7-^{3}H progesterone (SA 103 Ci/m*M*) were added to one ml of plasma to estimate the recovery of the steroids. Each sample was extracted twice with anesthetic ether (Mallinckrodt). The ether extract was evaporated and the steroids were then resuspended in 1 ml of isooctane and were chromatographed on celite micro-columns. Progesterone was eluted with isooctane and estradiol with isooctane: ethyl acetate (3:2). The estradiol fraction was diluted in 0.5 ml of 0.1 *M* phosphate buffer (pH 7.8) containing 0.14 *M* NaCl, 0.01 *M* EDTA, 0.015 *M* sodium azide, and 0.1% gelatin. After an aliquot was taken to estimate steroid recovery, 0.2 ml of the solution was incubated with 2,4,6,7-^{3}H-17β estradiol (0.1 ml/40,000 dpm) and with estradiol antiserum (0.1 ml at 1/100,000). The antiserum (S-310) was obtained from Abraham's laboratory. The estradiol recovery was 80%. The sensitivity of the assay was 2.5 pg/tube with an interassay coefficient of 8.5%.

The progesterone fraction was diluted with 1 ml of phosphate buffer; an aliquot was taken to estimate recovery and another (50 μl) was incubated with 1,2,6,7,^{3}H progesterone (0.1 ml/40,000 dpm) and with progesterone antiserum (0.1 ml at 1/7,000). The antiserum 3-oxime-BSA cross reacted with the following steroids: Testosterone and 20α-OH-progesterone less than 1%, and 17β-OH-progesterone and deoxycorticosterone 2%. The recovery was 86%, the interassay coefficient of variation was 10%, and the sensitivity was 25 pg/tube. The free and bound hormones were separated using 0.2 ml of dextran-coated charcoal.

Duncan's new multiple range test (10) was used to analyze the significance of the differences in LH serum levels among the groups. The results from the ovulatory test were expressed as binomial data using one for ovulation and 0 for no ovulation; they were subjected first to analysis of variance (11, 12) and then compared by Duncan's new multiple range test (10) as described previously (5–8). The LH-RH analogue was prepared in our laboratory by the solid phase method (5). Its purity was confirmed by TLC and amino acid analysis.

Results. Table I shows the effect on ovulation of a single dose of [D-Phe^2, Phe^3, D-Phe^6] -LH-RH injected at different days of the estrous cycle. It can be seen that when the analogue was injected at noon of proestrus, a 100% blockade of ovulation was observed. The degree of ovulation blockade decreased to 33% and 17% when the analogue was

TABLE I. BLOCKADE OF OVULATION IN THE RAT BY [D-Phe^2, Phe^3, D-Phe^6]-LH-RH (ANALOGUE) ADMINISTERED AT DIFFERENT STAGES OF THE ESTROUS CYCLE.[a]

Group	# of rats ovulated/total # of rats	% of blockade of ovulation	Mean ± SE of ova in ovulating rats
A. Proestrus (noon)			
1. Vehicle	4/4	0	12.2 ± 0.5
2. Analogue	0/6*	100	—
B. Proestrus (9 AM)			
3. Vehicle	4/4	0	13.0 ± 0.6
4. Analogue	4/6†	33.3	10.7 ± 0.9
C. Diestrus 2 (noon)			
5. Vehicle	4/4	0	12.7 ± 0.2
6. Analogue	5/6†	16.7	9.8 ± 1.1
D. Diestrus 1 (noon)			
7. Vehicle	4/4	0	12.0 ± 0.4
8. Analogue	6/6	0	11.6 ± 0.2
E. Estrus (noon)			
9. Vehicle	4/4	0	11.0 ± 0.4
10. Analogue	6/6	0	10.2 ± 0.6

[a] Dose of analogue: 1.5 mg/rat at the time shown in parenthesis. Duncan's new multiple range test: * Significantly different from the respective control value. † Significantly different from the value of Group 2.

injected at 9 AM on proestrus and on D2, respectively. Ovulation was not blocked after injecting the analogue on D1 and the previous E (Table I).

The effect of daily injections for three days (E, D1, and D2) of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH is presented in Table II. Two out of fourteen rats treated with the analogue ovulated (86% blockade of ovulation), one of them fully (12 ova) and the other partially (4 ova). On the other hand, only one of the control rats failed to ovulate (Table II).

Figure 1 shows the effect of daily injections of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH on the LH serum levels. In the analogue-treated rats, serum LH was significantly lower ($P < 0.01$) than the control group, when they were compared on the evening of proestrus. This demonstrates that the injection of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH during E, D1 and D2 inhibited the LH surge that was seen in the control animals on the afternoon and evening of proestrus.

Figure 2 shows the effects of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH on serum levels of steroids. While estradiol in the treated rats was not affected, progesterone was higher on D1 and lower on the afternoon of proestrus in those rats. Moreover, the animals treated with the analogue showed a normal vaginal smear pattern during the period of observation.

The ovaries of the anovulatory animals treated with [D-Phe^2, Phe^3, D-Phe^6]-LH-RH showed a histological pattern similar to ovulating untreated animals; normal follicular developent including antral follicles were present, although corpora lutea hemorrhagica were absent in the latter animals.

Discussion. Beattie *et al.* (4) reported that [D-Phe^2, D-Ala^6]-LH-RH significantly inhibited ovulation when it was injected on

TABLE II. BLOCKADE OF OVULATION IN THE RAT BY [D-Phe^2, Phe^3, D-Phe^6]-LH-RH (ANALOGUE) ADMINISTERED ON E, D1 AND D2.[a]

Group	# of rats ovulated/ total # of rats	% of blockade of ovulation	Mean ± SE of ova in ovulating rats
1. Vehicle	13/14	7.1	11.8 ± 0.3
2. Analogue	2/14	85.7*	8.0 ± 4.0$^\nabla$

[a] Dose of analogue: 1.5 mg/rat twice a day (total dose: 3 mg daily). ∇One rat ovulated four ova. Duncan's multiple range test: * Significantly different from the value of the control group.

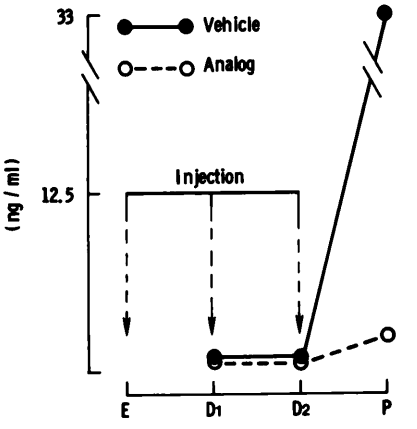

FIG. 1. Effect of the administration of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH on serum LH levels. Animals were injected s.c. with either the analogue (1.5 mg) or vehicle at 9 AM and 4 PM on E, D1 and D2. Blood was taken at 4 PM. Each point represents the mean ± SE of six rats.

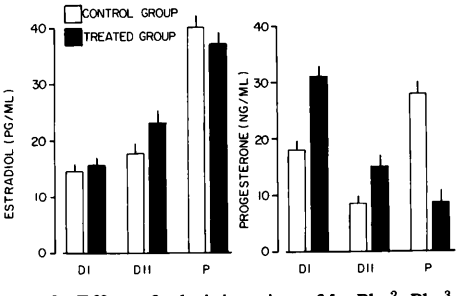

FIG. 2. Effect of administration of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH on serum steroid levels. Animals were injected s.c. with either the analogue (1.5 mg) or vehicle at 9 AM and 4 PM on E, D1 and D2. Blood was taken at 4 PM. Each point represents the mean ± SE of six rats.

different days of the estrous cycle in 4-day cycling rats. Ovulation was inhibited by 97%, 87% and 79% after proestrus, D2 and D1 injection of the analogue, respectively. Using a more potent analogue, [D-Phe^3, Phe^3, D-Phe^6]-LH-RH (5), we were able to inhibit ovulation considerably when this analogue was injected either on the morning or at noon of proestrus. Only a 17% inhibition of ovulation was observed when [D-Phe^2, Phe^3, D-Phe^6]-LH-RH was injected at noon on D2 and no inhibition of ovulation was seen when it was injected on the previous E. Apparently, the length of the action of [D-Phe^2, Phe^3, D-Phe^6]-LH-RH is not sufficient to block ovulation when it is injected before D1. Furthermore, the injection of 1.5 mg twice a day at 9 AM and 4 PM (total dose of 3 mg/rat/day)

on E, D1 and D2, brought about almost complete suppression of the LH surge on the next day (Proestrus) and an 86% blockade of ovulation on the following estrus morning, without altering the normal vaginal smear pattern. This might be due to unaltered serum levels of estradiol after the treatment (Fig. 2). Discrepancies between our results and those obtained by Beattie *et al.* (4) might be due to the different schedule of treatment and doses used.

It is interesting to point out the changes in serum progesterone levels observed in the animals treated with the analogue on E, D1 and D2 throughout the experiment. They were higher on D1 and lower on proestrus when they were compared with those of the control rats. Because the peak of serum LH levels in the rats of our colony occurs between 3 and 4 PM, the low proestrous afternoon levels of progesterone could be due to a blockade of LH release produced by direct effects of the analogue on the pituitary and hypothalamus. The lack of LH release could have impaired the subsequent ovulation and luteinization. On the other hand, high progesterone levels during D1 might have contributed to the blockade of the LH surge and ovulation. It has been demonstrated that administration of progesterone or synthetic analogues early in the cycle depresses proestrus serum LH and FSH and delays ovulation (13–17).

In conclusion, using antagonist analogues of LH-RH it is possible to block ovulation without affecting the rat estrous cycle. Thus, the possibility exists to develop an even more potent analogue which can be used in humans without altering plasma estrogen levels.

Summary. [D-Phe^2, Phe^3, D-Phe^6]-LH-RH, a potent antagonist of LH-RH, was injected during the different stages of the estrous cycle in rats at a dose of 1.5 mg/rat. When it was administered at noon or proestrus, a 100% blockade was observed. This decreased to 33% and 17% when the analogue was injected at 9 AM on proestrus and diestrus 2, respectively. No blockade of ovulation was observed after the injection of the analogue on diestrus 1 or on the previous estrus. The sequential administration of the analogue twice daily on E, D1 and D2, brought about almost complete suppression of LH surge on proestrus and an 86% blockade of ovulation without altering the cyclic vaginal smear pattern. In this case, serum levels of estradiol were not modified but progesterone levels were significantly lower on proestrus and higher on diestrus 1 in the analogue treated group as compared to control rats. This higher level of progesterone on diestrus 1 might account in part for the inhibition of the LH-surge and blockade of ovulation by the inhibitory analogues of LH-RH.

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