Effect of a Single Injection of Estradiol Benzoate (EB) on Ovulation and Reproductive Function in 4-day Cyclic Rats[1] (36228)

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In intact rats undergoing rapid follicular growth, direct and indirect evidence indicate that estrogen is being produced. Vaginal cornification, uterine distention with luminal fluid, and sexual receptivity have served as qualitative indicators of estrogen secretion during the estrous cycle. Quantitative measurements of ovarian secretion by Miyake (1) and Yoshinaga *et al.* (2) indicate that an estrogen "surge" occurs ahead of the ovulatory surge of gonadotropins. It appears that estrogen secretion normally accompanies follicular ripening under the combined influence of FSH and LH. The estrogen surge precedes the cyclic peak of LH, FSH and prolactin release (3–5). That estrogen itself may constitute an essential signal in the chain of events leading to the acute release of gonadotropins seems well established.

Chemical or immunological coverage of estrogen receptor sites blocks ovulation at the expected estrus. Estrogen antagonists such as MER-25, ICI 46,474, or Clomiphene have been shown to interfere with ovulation in the 4-day cyclic rat (6–9). Ovulation was restored by the administration of estrogen. Using antibodies to 17β-estradiol (Anti-E_2), Ferin *et al.* (10) have inhibited the LH surge and the incidence of ovulation in 4-day cyclic rats. However, diethylstilbestrol, a synthetic estrogen that is not inhibited by anti-E_2, restored ovulation in anti-E_2-treated rats. These findings further indicate that in the adult rat the initiation of the gonadotropin surge is under the control of estrogen.

Everett (11) found that administration of estrogen to 5-day cyclic rats on the second day of diestrus induced ovulation one day early. Brown-Grant (12) demonstrated advancement of ovulation in 4-day cyclic rats by injections of estrogen on the first and progesterone on the second day of diestrus, Recently, Ying and Greep (13, 14) further extended Hohlweg's observation of inducing ovulation with a single injection of estradiol benzoate in immature rats and found that estrogen plays an important role in both maturation of follicles and the ovulatory surge of gonadotropin.

One aim of the present study was to determine whether, in 4-day cyclic rats, estrogen has the same role in the initiation of the ovulatory surge of gonadotropin with consequent advancement of ovulation as described in 5-day cyclic rats. We also wished to examine what effect, if any, estrogen treatment would have on events of the estrous cycle and reproductive function.

Materials and Methods. Mature female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. The animals were fed Purina laboratory chow and water *ad lib*. The lights were automatically turned on at 5:00 AM and off at 7:00 PM, producing a 14:10-hr light:darkness period. The midpoint of the dark period is considered as "midnight, colony time" and all times are reported in relation to that. Vaginal smears were taken daily before 11:00 AM for at least three consecutive cycles before any treatment was administered. Only animals showing regular 4-day cycles were used in the experiments. The stages of the 4-day cycle are defined as

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estrus, 2 days of diestrus, and proestrus. Estradiol benzoate (EB) was given at 10:00 AM on the first day of diestrus as a single subcutaneous injection in 0.1 ml sesame oil in the following amount: 6.25, 12.5, 25, 50, 100 and 200 μg. Forty-eight hr after EB injection, the animals were killed with ether. The ovaries were removed and examined. The dissected oviducts were compressed between two glass slides and ova were counted under a low-power light microscope. Groups of animals receiving the same doses of EB were used for the study of pseudropregnancy. Vaginal smears were then made daily until the termination of the experiment. The cervices of ten sexually mature 4-day cyclic rats showing fully cornified vaginal smears were stimulated with a glass rod on the day of estrus and served as controls. In the experiment wherein phenobarbital was used to test the neurological release of ovulating hormone, 26 rats received 50 μg of EB at 10:00 AM on the first day of diestrus. On the second day of diestrus at 1:30 or 5:30 PM, nine rats each received phenobarbital sodium (75 mg/kg body weight in 1 ml physiologic saline). This amount of phenobarbital has been demonstrated to be capable of blocking the ovulatory surge of gonadotropin in the afternoon of proestrus in cyclic rats (15). Eight rats receiving saline at 1:30 PM served as controls.

Twenty-two other animals treated with a single injection of 50 μg of EB on the first day of diestrus were tested for reproductive ability. They were caged with proven fertile males on the night of the second day of diestrus. The females were first examined for the presence of a copulation plug and/or spermatozoa the following morning. Part of the mated rats were laparotomized and examined later to ascertain fertilization, blastocyst development, or implantation. Sixteen rats in which EB advancement of ovulation was verified by laparotomy were caged with fertile male rats until they carried implantation sites. The length of time from EB injection to the first exhibition of normal reproductive function was recorded.

Results. Ovulation was induced during advanced proestrus in 30% of the rats treated with a single injection of 6.25 μg of EB on the first day of diestrus. The percentage increased to 60% when 12.5 or 25 μg of EB was given, respectively. With 50–200 μg of EB, over 70% of the animals ovulated (Table I). The average number of ova shed with different doses of EB did not differ significantly. A given dose of EB induced ovulation ranging from 1 or 2 ova to 14 ova, probably due to the individual responsiveness to EB stimulation.

TABLE I. Advancement of Ovulation by a Single Injection of Estradiol Benzoate (EB) at 10:00 AM of the First Day of Diestrus in 4-Day Cyclic Rats.

Dose of EB (μg)	No. rats ovulated	% Ovulating	Average number of ova per ovulating rat ± SE
6.25	3/10	30	5.3 ± 1.5
12.5	6/10	60	6.2 ± 1.5
25	6/10	60	6.3 ± 1.1
50	8/10	80	9.0 ± 1.1
100	9/10	90	6.0 ± 0.9
200	7/10	70	6.4 ± 1.5

Phenobarbital injected at 1:30 PM on the second day of diestrus prevented ovulation from occurring on the expected proestrus in animals treated with 50 μg EB on the first day of diestrus (Table II). Ovulation was not prevented if phenobarbital was given at 5:30 PM. Saline injected at 1:30 PM did not affect the EB-induced ovulation.

The periods of diestrus induced by treatment with varying doses of EB in 4-day cyclic rats are summarized in Table III. All the rats were treated with EB on the first day of diestrus. With doses of 6.25 or 12.5 μg of EB, all animals showed prolonged vaginal diestrus lasting about 9 days even in those that did not show advancement of ovulation. Animals treated with higher doses of EB (25–100 μg) displayed a period of diestrus not significantly different from that recorded for the control group. Rats treated with 200 μg EB had a significantly longer period than those of controls ($p < 0.05$).

Mating occurred in 20 of 22 verified ovulators treated with 50 μg EB, however, further examination indicated that the mating was not fertile. Six mated rats were killed and the

TABLE II. Effect of Phenobarbital on EB-Induced Ovulation in 4-Day Cyclic Rats.

Treatment	No. rats ovulated	% Ovulating	Average number of ova per ovulating rat ± SE
Saline, 1:30–2:00 PM	7/8	88	6.4 ± 0.9
PB,[a] 1:30–2:00 PM	0/9	0	—
PB,[a] 5:00–5:30 PM	6/9	67	5.5 ± 1.1

[a] PB: Phenobarbital (75 mg/100 g body weight) given subcutaneously on the second day of diestrus.

oviducts were examined on the first day of pregnancy. The average number of fertilized ova was 7.1 ± 1.6. In another 7 mated rats killed on the afternoon of day 4 or 5 of pregnancy, no blastocysts were found in any of the animals. In duplicate experiments, mating and ovulation were confirmed in 21 out of 26 rats. Seven animals were killed on day 9 of pregnancy and no implantation sites were found in any of them. The remaining 16 animals were maintained with fertile males. Three laparotomies were carried out and the length of time from EB injection to the appearance of implantation sites comparable to those observed on day 9 of normal pregnancy was 23.6 ± 3.7 days, *i.e.*, approximately 9 days after the pseudopregnancy induced by the injection of EB.

Discussion. The present study demonstrates that a single dose of estradiol benzoate exerts a facilitative effect on the process of ovulation in 4-day cyclic rats, presumably on the neurological control of ovulating hormone release. At the time of preparation of this manuscript, Krey and Everett (16) reported that estradiol benzoate injection on the first day of diestrus advances LH release and ovulation 24 hr. This advancement of ovulation depends on the time of injection and the dose of EB. Ovulation occurs without histological evidence of luteolysis. Pseudopregnancy consistently follows, characterized by two sets of histologically sound and physiologically active corpora lutea. Our finding of EB-induced ovulation and the subsequent pseudopregnancy is in agreement with that reported by Krey and Everett.

TABLE III. Effect of Different Doses of Estradiol Benzoate (EB) Given as a Single Injection on the First Day of Diestrus on the Length of Vaginal Diestrus.

Dose of EB (μg)	No. of rats	Length of diestrus (days ± SE)
Control	10	11.8 ± 1.7
6.25	7	8.8 ± 1.7
12.5	7	9.4 ± 2.4
25	8	10.9 ± 2.5
50	7	12.5 ± 1.6
100	8	15.4 ± 1.9
200	8	18.3 ± 1.7[a]

[a] Significant from controls which were stimulated with glass rod on day of estrus.

The advancement of ovulation with a single injection of EB has been demonstrated in 5-day cyclic rats (11) and immature rats nearing their sexual maturity (13, 14). However, the dose of EB needed to facilitate the process of ovulation in the adult rat is much higher than that in the immature rats. Only a single dose of EB, a known long-acting estrogen, is effective. With short-acting estrogens, two or more injections are needed to induce ovulation (17). Nevertheless, estrogen of short action dissolved in a delay vehicle mimics the EB to cause advancement of ovulation (J. W. Everett, personal communication). The first indicator, either physiologic or radioimmunologic, of the action of EB occurs approximately 12 hr after EB injection as reported elsewhere (18). It seems that a minimum of 12 hr in which the estrogen acts on the neurological and/or subcellular sites is requisite to the facilitation of the process of ovulation. On the other hand, a combination of EB and progesterone has been reported to effectively advance ovulation 24 hr in adult rats or to induce ovulation in immature rats. It appears that EB not only stimulates maturation of follicles but

also triggers the ovulatory surge of pituitary ovulating hormone. The action of progesterone seems to provide an optimal environment enhancing the mechanism of triggering the release of pituitary ovulating hormone.

That the pituitary gonadotropins are involved in EB-induced ovulation has been demonstrated (14, 18). Evidence that the pituitary is involved in the advancement of ovulation in 4-day cyclic rats was obtained from the barbiturate experiment. The data indicate that the ovulatory surge of gonadotropin occurred between 2:00 and 5:00 PM on the afternoon of the presumptive second day of diestrus. Further studies using radioimmunoassay confirmed a marked increase in serum LH with a peak at 3:00 PM on the second day of diestrus (19).

It has been reported that estrogen, either given daily or implanted, maintains large functional corpora lutea for considerable periods (20–23). A single injection of estrogen, given during proestrus or estrus, induces pseudopregnancy (24). Estrogens are able to induce prolactin release either by a direct effect on the pituitary or indirectly via the hypothalamus (25). However, normal pregnancy was not observed until after the termination of pseudopregnancy. High levels of estrogen are known to have adverse effects on the survival of blastocysts and to interfere with implantation (26). The data presented here demonstrate that EB has a deleterious effect on the normal reproductive performance.

Summary. Ovulation was advanced 24 hr by a single injection sc of estradiol benzoate (EB) at 10:00 AM on the first day of diestrus in 4-day cyclic rats. Phenobarbital prevented this EB-induced ovulation if given at 1:30 PM on the second day of diestrus, but not when given at 5:30 PM. However, pseudopregnancy occurred in all animals receiving EB. When animals treated with EB were exposed to fertile males on the second day of diestrus, over 80% mated but normal pregnancy did not ensue. Normal pregnancy of EB-treated rats occurred only after the termination of pseudopregnancy subsequent to EB-induced ovulation. These results suggest that EB injection initiates the ovulating hormone release and results in the advancement of ovulation.

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