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Effects of Delayed Ovulation on Pregnancy in the PMSG-Treated Immature Rat[1] (41336)

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Abstract. The effects of phenobarbital (PB)-delayed ovulation on embryonic development, implantation, and serum concentrations of progesterone (P), androstenedione (A), and estradiol (E_2) were ascertained in the pregnant mare serum gonadotropin (PMSG)-treated immature rat. Two and three days of ovulatory delay retarded embryonic development as evidenced by the presence of 70–80% of the embryos in the two-cell stage on Day 3 (Day 1 = sperm positive); whereas, nondelayed controls exhibited 40–50% of the embryos in the two-cell stage and 30–40% in the four-cell stage. Ovulatory delay of 3 days induced a higher percentage of abnormal embryos when compared with controls. After 2 days of delay, implantation was inhibited in ~40% of the rats by Days 6 and 8. In controls ~80% of rats exhibited implantation sites on Day 6 and all control rats exhibited implantation sites on Day 8. Serum concentrations of P, A, and E_2 were similar to nondelayed controls on Day 3 of pregnancy suggesting that the retardation in development and inhibition of implantation are consequences of intrafollicular aging and/or to an altered uterine environment. However, the possibility remains that some of these alterations may be related to the effects of PB on the reproductive tract and/or embryo. On Day 14 of pregnancy, an increase in resorption sites was observed after 2 and 3 days of ovulatory delay. This correlated with a significantly lower level of serum P in rats with delayed ovulation indicating that ovulatory delay not only alters embryonic development by altering the oocyte and uterine environment but also may induce abnormalities in the ability of corpora lutea to secrete P and/or alter the clearance rate of P.

Previous studies have shown that pentobarbital-delayed ovulation in the adult rat may alter the oocyte (1, 2), the uterine environment (3), and therefore the subsequent pregnancy if ova from follicles of rats exhibiting delayed ovulation are fertilized (4–6). Similar alterations have been observed in aged rats with spontaneously prolonged (5- and 6-day) cycles (7). Since the PMSG-treated rat is a useful model for barbiturate-induced delayed ovulation (8), it was an aim of the present study to ascertain whether alterations in embryonic development are induced in young (immature) pregnant animals as has been reported for adult and aged rats. In addition, the effects of ovulatory delay on the serum concentrations of steroid hormones during the pre- and postimplantation periods was of special interest.

Materials and Methods. Immature female rats (Holtzman) were obtained from Charles River, Inc. (Wilmington, Mass.) at 21 days of age. Rats were maintained in rooms at ~72°F with a 14 L:10 D daily lighting schedule (lights on at 0600 hr). On Day 28 of age at 0900–1000 hr, rats were injected sc with 5 IU pregnant mare serum gonadotropin (PMSG 1790 IU/mg in 0.1 ml saline) from NICHD. Rats were then randomly assigned to four groups.

Group 0. Group 0, a control group composed of rats experiencing no ovulatory delay that was placed with male rats only on the evening (~1630 hr) of proestrus (Day 30 of age). If sperm were observed in the vaginal lavage the following morning (0900–1030 hr), then this was designated Day 1 of pregnancy. If sperm were not observed, then animals were discarded from the experiment. Rats were then decapitated at 0900–1000 hr of Days 3 and 14 of pregnancy. Serum was saved for RIA of progesterone (P), androstenedione (A) and estradiol (E_2) (see RIA methods). The uteri and oviducts of rats sacrificed on Day 3 were irrigated with Hams F-12 medium (GIBCO, Grand Island, N.Y.). The number of embryos, the number of blastomeres in each embryo, and the number of abnormal

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embryos (either with unequal sized or degenerate blastomeres) were recorded. On Day 14 of pregnancy viable fetuses and resorption sites were counted. Whenever possible, five uterine swellings (fetus, placenta, and uterus) were dissected and weighed. When necessary fetal viability was assessed by observing the cardiac contractions.

Groups 1, 2, and 3. Groups 1, 2, and 3 are composed of PMSG-treated rats in which ovulations have been blocked with phenobarbital for 1–3 days, respectively. A single sc injection of 6.5 mg PB given at 1400 hr of Day 30 blocked ovulation for 1 day. Two days of ovulatory delay were induced by injections of 6.5 mg PB at 1400 hr Day 30 and 13 mg at 1300 hr Day 31; for 3 days of delay another injection of 19.5 mg at 1300 hr Day 32 was required. Only on the evening of expected proestrus, PB-treated rats were mated (~1630 hr). On the following morning if sperm were observed in the vaginal lavage, then this was considered Day 1 of pregnancy. Rats were then decapitated at 0900–1000 hr of Days 3 and 14 of pregnancy. Sera and embryos were evaluated the same as controls. In order to ascertain the effects of ovulatory delay on the number of ova shed, oviducts of separate groups of rats were irrigated. Ova were found in a plug of granulosa cells in the oviducts on Days 31–34 following 0–3 days of blockade, respectively. Other groups of rats were decapitated at 0900–1000 hr of Day 30 (0-day delay, proestrus), Day 31 (1-day delay group), Day 32 (2-day delay), and Day 33 (3-day delay); their ovaries were placed in Bouin's solution, dehydrated in alcohol, embedded in paraffin, serial sectioned at 10 μm, and stained with hematoxylin and eosin. The number of follicles $\geq$520 μm were counted and the diameter measured in that section containing the nucleolus of the oocyte by averaging two perpendicular measurements, one being at the largest cross section. The large follicles were adjudged atretic if degenerating granulosa cells or pyknotic nuclei in the oocytes were observed.

In order to ascertain the effects of ovulatory delay on the time of onset of implantation, additional groups were studied. After 0 or 2 days of delay, rats were anesthetized with ether on the morning of Day 6 or 8 of pregnancy. Then, approximately 0.5 ml 2% Chicago blue B (Fisher Scientific Co., St. Louis, Mo.) in saline was injected slowly (10 sec) into the tail vein. The anesthesia was removed and rats decapitated ~20 min following the administration of the blue dye. At necropsy, the uterus was carefully dissected from the body cavity and each uterine horn was irrigated with 0.5 ml Hams F-12 medium. Thereafter, the number of blue (implantation) sites and/or uterine swellings and embryos in the uterine flushing were recorded. The uteri were then blotted and weighed to the nearest milligram.

Steroid radioimmunoassay. Methods for RIA of P, A, and E_2 in sera are similar to those previously described for hamsters (9), the exception being that the ether phase was dried under reduced pressure with gentle mixing at 45°C. The lower limit of sensitivity in the P assay was 5 pg/tube, and serum from chronic ovariectomized (OVX-ADX) rats contained less than 1 ng P/ml (n = 7). Anti-P was provided by Dr. Gordon Niswender, Colorado State University, Fort Collins, Colorado. Results from characterization of anti-P are similar to that previously described (10). Androstenedione antiserum was provided by Dr. John Resko (Oregon Regional Primate Center, Beaverton, Ore.) and has been previously characterized (11). This assay was sensitive at a minimum of 4 pg/tube and serum values for OVX-ADX rats were 68 ± 13 (n = 5). In the E_2 assay, antiserum was provided by Dr. Delwood Collins (Emory University, Atlanta, Ga.). Characterization of this antiserum was similar to that previously reported (12). The lower limit of assay sensitivity was 0.9 pg/tube, and serum from OVX-ADX rats contained 7.3 ± 1.4 pg E_2/ml serum (n = 6).

Statistics. Data from steroid RIAs, number of blastomeres, and fetal weights were analyzed by Duncan's multiple range test. Differences were considered significant if $P < 0.05$.

Results. Neither the number of follicles

≥520 μm diameter nor the number of ova shed changed significantly throughout 3 days of ovulatory delay (Table I). However, the diameter of the follicles gradually increased ($P < 0.05$) after 1 day of delay. Atretic follicles were not observed during the experimental period.

Preimplantation and early implanting embryos. On Day 3 of pregnancy, rats delayed from ovulating for 0 and 1 day exhibited 40–50% of two-cell embryos and 40–50% of three- and four-cell embryos (Fig. 1). Two and three days of ovulatory delay increased the percentage of two-cell embryos found on Day 3 of pregnancy ($P < 0.05$) compared to 0 and 1 day of delay. Concomitant with the increase in two-cell embryos was a decline ($P < 0.05$) in the three- and four-cell embryos. After 3 days of ovulatory delay, the percentage of abnormal embryos was significantly greater ($P < 0.05$) than that of any other day.

Delayed ovulation tended to retard the initiation of implantation. On Day 6, three of eight PB-treated rats exhibited implantation (blue) sites; whereas, 10 of 12 control rats exhibited implantations (Table II). Also indicative of the delayed implantation in PB-treated rats was the presence of blastocysts encased by the zona pellucida on Day 6. On Day 8, all control rats exhibited uterine swellings; whereas in rats with delayed ovulation only 2 of 7 exhibited early stages (nonswollen sites) of implantation and two rats exhibited uterine swellings comparable to controls (Table II). However, three rats did not exhibit a blue reaction and a blastocyst without a zona pellucida was observed in each of these rats. As expected in delayed implantation, on Day 8, uterine weight was significantly less ($P < 0.05$) than nondelayed control.

Conceptuses on Day 14 of pregnancy. On Day 14, one day of ovulatory delay had no effect on percentage of viable fetuses or resorptions (Table III). However, the average number of viable fetuses and conceptuses/animal after 2 and 3 days of ovulatory delay were significantly lower ($P < 0.05$) than controls and those after 1 day of delay. In addition, the percentage resorption was greater after 2 and 3 days of delay than controls.

Serum steroid concentrations (Fig. 2). On Day 3 of pregnancy serum concentrations of P, A, and E_2 were not altered by ovulatory delay. On Day 14 analysis of variance revealed that P levels in rats with delayed ovulation were lower than nondelayed controls. Using Duncan's test, serum levels of A were lower than controls only after 3 days of delay in the Day 14 group. Serum concentrations of E_2 in rats with delayed ovulation were not different from 0-day delay on Day 14 of pregnancy.

Discussion. A 2-day delay in ovulation clearly retards development of preimplantation embryos and inhibits the onset of implantation when compared with nondelayed controls. Also associated with delay were higher incidences of abnormal embryos as observed on Day 3 (for 3-day delay only; Fig. 1) and an increase in resorption for 2 and 3 days of delay on Day 14 of pregnancy (Table III). The reasons for these alterations may be related to several factors.

TABLE I. EFFECTS OF DELAYED OVULATION ON FOLLICULAR DEVELOPMENT AND THE NUMBER OF OVA SHED IN THE PMSG-TREATED IMMATURE RAT[a]

	Days of delay			
	0	1	2	3
No. of follicles/ovary ≥520 μm	6.3 ± 1.2	5.9 ± 2.4	5.7 ± 1.1	6.4 ± 0.6
Mean follicular diameter (μm)	583 ± 22	637 ± 17	694 ± 19*	760 ± 32*
No. of ova shed	9.9 ± 1.1	8.5 ± 1.4	8.3 ± 1.2	7.1 ± 1.5

[a] Autopsy was on estrus.

* $P < 0.05$, respectively, when compared with 0 day of delay by Duncan's multiple range test.

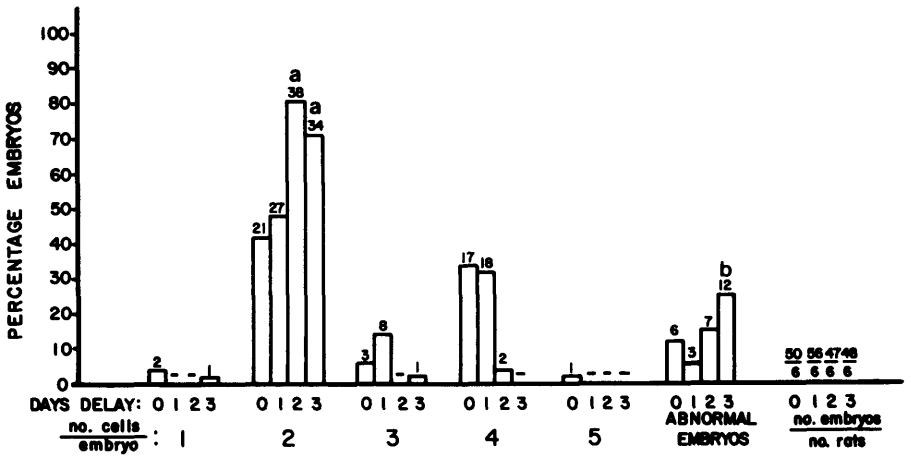

FIG. 1. Percentage of embryos found in the uterus on Day 3 of pregnancy in the PMSG-treated immature rat whose ovulation had been blocked for 1, 2, or 3 days (and controls were not delayed = 0 days delay). Details of injection schedules are given under Materials and Methods. The numbers above the open bar represent the actual number of embryos observed. Numerator/denominator on the right hand side of the figure represents the total number of embryos observed/total number of rats for each day of delay. (a) $P < 0.05$ when compared with Days 0 and 1 of delay; (b) $P < 0.05$ when compared with 0–2 days of delay.

First, a study has shown that prolonged exposure of the intrafollicular oocyte to estradiol during the period of ovulatory delay is causally related to abnormal development (13). This was deduced by neutralization of circulating estrogen (and probably intrafollicular estrogen) during ovulatory delay with an antiserum to estradiol (ASE) (13). Subsequent fertilization of ova from ASE-treated rats produced an incidence of abnormal embryos similar to nondelayed controls (13). In the present study, estradiol levels are elevated (72–100 pg/ml serum) during 3 days of ovulatory delay (P. F. Ter-

TABLE II. EFFECTS OF DELAYED OVULATION ON IMPLANTATION IN THE PMSG-TREATED IMMATURE RAT

Day of pregnancy:	6		8	
Days of delay:	0	2	0	2
n	12	8	6	7
Implantation Sites[a]	70(10) 0(2)	13(3) 0(5)	49(6)	31(4) 0(3)
$\bar{x}$ ± SEM[b]	7.0 ± 0.6(10)	4.3 ± 1.2(3)	8.2 ± 1.1(6)	7.8 ± 1.7(4)
Blastocysts	33(9)	17(5)	—	4(3)
No zona pellucida	0(3)	0(2)	—	0(4)
$\bar{x}$ ± SEM	3.7 ± 0.6(9)	3.4 ± 1.0(5)	—	1(3)
Encased by zona pellucida	0(12)	5(3)	—	0(7)
$\bar{x}$ ± SEM	—	1.7 ± 0.7(3)	—	—
Wet uterine wt (mg)	153 ± 12(7)	158 ± 17(5)	323 ± 20(6)	195 ± 37(7)*

[a] As determined by blue reactions; number of blue sites (number of rats).
[b] $\bar{x}$ ± SEM = average ± standard error of mean.
* $P < 0.05$ when compared with 0-day delay on Day 8 of pregnancy.

TABLE III. EFFECTS OF DELAYED OVULATION ON FETAL VIABILITY IN THE PMSG-TREATED IMMATURE RAT[a]

	Days of delay			
n	0 (6)	1 (6)	2 (6)	3 (6)
No. viable fetuses	55	49	30	20
Average No. viable fetuses/animal	9.2 ± 0.7	8.2 ± 0.8	5.0 ± 1.0*	3.3 ± 0.8*
No. resorption sites[b]	0(3) 1(3)	0(4) 1(1) 2(1)	0(3) 2(1) 2(2)	0(2) 1(2) 4(1) 7(1)
$\bar{x}$ = avg resorptions/animal	$\bar{x} = 0.5$	$\bar{x} = 0.5$	$\bar{x} = 0.8$	$\bar{x} = 2.2$
Average No. conceptuses/animal	10.0 ± 0.7	8.7 ± 0.8	5.8 ± 0.7*	5.5 ± 0.8*
Average viable "swelling" wt (mg)	527 ± 33	396 ± 51	402 ± 31	503 ± 25
Percentage resorption	5.2	5.8	14.3*	39.4*

[a] Autopsy was on Day 14 of pregnancy.
[b] Total No. resorption sites (number of rats).
* $P < 0.05$ when compared with Day 0 or 1 value.

ranova, unpublished). Therefore, prolonged exposure of the reproductive tract to E_2 in the immature rat is consistent with that reported for adult delayed rats. The mechanism by which estrogen induces abnormal embryos is unknown but related to alterations in the oocyte prior to fertilization (1, 2) and alterations in the intrauterine environment (3, 6). Second, the alterations may be the result of a detrimental action of PB on the ovary (ova), embryo, and uterus. Previous studies have shown that PB reduces the *in vitro* production of P, A, and E_2 by ovary and adrenal of the immature rat (14, 15); however, pregnenolone synthesis was increased. *In vitro* incubation of hamster preovulatory follicles with PB reduces the steroidogenic capacity of the follicles to respond to LH (P. F. Terranova, unpublished). Therefore, it seems likely that PB may directly alter ovarian steroidogenesis and interact with the detrimental effect of ovulatory delay on ova. Previous studies have shown that PB reduces the uterotrophic action of estrogens (16) and increases the activity of liver enzymes that metabolize P and 17β-E_2 (17). Although the serum levels of E_2 were not altered in the present study it is possible that the level of E_2 in sera was insufficient (due to the refractoriness of the uterus to E_2 induced by PB treatment; see (16)) for maintenance of normal embryos and implantation. It is well established that E_2 and P are required for implantation in the rat (18); in addition previous studies have shown that embryos from rats hypophysectomized on Day 1 of pregnancy and treated with P develop slower than those treated with P and E_2 (19) indicating that E_2 enhances the development of the embryo. The mechanism by which PB could reduce the effectiveness of E_2 at the embryonic and/or uterine level is unknown. It is known that PB decreases energy production and ATP (20) which are necessary for embryonic development (21) and for expression of E_2 action on rat uteri (22–24).

It is possible that the preovulatory follicles from rats with delayed ovulation were producing more E_2 than controls (therefore exposing the oocyte to excessive amounts of E_2). This is based on the observations that serum E_2 levels were similar in delayed and control rats (Fig. 1) and on the assumption that PB increased the activity of liver enzymes that metabolize E_2 (17). Therefore, it seems plausible that if the E_2 was

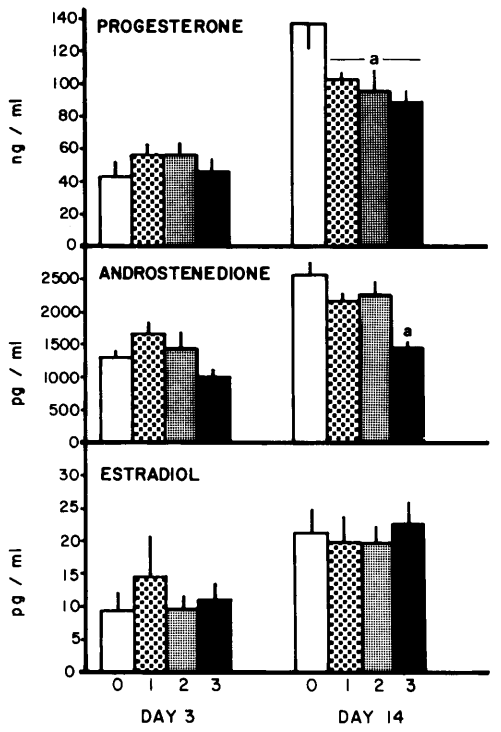

FIG. 2. Serum concentrations of progesterone (ng/ml), androstenedione (pg/ml), and estradiol (pg/ml) on Days 3 and 14 of pregnancy (Day 1 = sperm positive) following 0 (□), 1 (▩), 2 (▩), and 3 (■) days of delay in PMSG-treated immature rats given phenobarbital (for injection schedules see Materials and Methods). (a) $P < 0.05$ when compared with 0-day delay.

being cleared at a faster rate in PB-treated animals and serum levels were similar to controls, the ovary would have to be producing more E_2 than controls in order to maintain a control level of E_2 in sera.

Two days of ovulatory delay did not increase the number of abnormal preimplantation embryos in the immature rat (Fig. 1). In fact, after 2 days of delay only seven abnormal embryos were found in six animals on Day 3 of pregnancy (Fig. 1); whereas on Days 6 and 8 of pregnancy no abnormal/degenerating embryos were observed. This finding contrasts with previous reports for 2 days of delay in adult 4-day cyclic rats (1, 4). The reason for the differences are probably related to several technical differences in the two models used to delay ovulation, i.e., namely PB vs pentobarbital, immature vs adult rats, the PMSG priming vs adult 4-day cyclic rats (in the present study 95% of the immature rats will become 5-day cyclers). By Day 14, the number of viable fetuses declined significantly below that of nondelayed controls (Table III) indicating postimplantation alteration in development induced by 2 days of delay (and therefore similar to the adult model, Refs. (6, 25)). In addition, the lower P values observed on Day 14 after 2 days of delay may be related to the low fetal viability indicating inadequate luteotropic stimuli from the placenta to maintain elevated circulating P similar to controls. The low serum P indicated the possibility of an inability of the corpora lutea, derived from follicles of PB-treated rats, to produce sufficient P and/or the enhanced clearance of P by the PB-induced liver enzymes (17).

From the data shown in Table I, only one set of preovulatory follicles (>520 μm) were observed in the ovary during the experimental period. Therefore the ova that were shed were indeed from the delayed follicles. Interestingly the number of ova shed correlated well with the number of preimplantation embryos observed on Days 3, 6, and 8 (Tables I, II, and Fig. 1) but not on Day 14 (Table II) indicating degeneration of embryos between Days 8 and 14.

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