EFFECT OF DECREASED ESTROGEN ACTIVITY ON INDUCTION OF OVULATION IN BARBITAL-TREATED RATS

Barbara Shirley

Faculty of Natural Sciences, University of Tulsa, Tulsa, Oklahoma 74104

When rats were injected with sodium barbital (25 mg/100 g body weight) at 13.00 h on proestrus in order to prevent their ovulating spontaneously, 70% of the animals that mated in that estrous cycle were induced to ovulate. In contrast mating did not in any case induce ovulation in barbital-treated rats that were injected with 10 mg of the antiestrogenic compound MER-25 at 10.00 h on the day preceding proestrus. The failure of mating to induce ovulation in the second group indicated that mating-induced ovulation, like spontaneous ovulation, requires estrogen activity prior to proestrus. Barbital treatment caused rats with 4-day estrous cycles to switch to 5-day cycles, as evidenced by their exhibiting uterine ballooning, by their mating, and by their ovulating, one day later than expected. Rats with 5-day cycles exhibited no retardation of their cycles due to barbital treatment.

INTRODUCTION

Spontaneous ovulation of rats requires release of a surge of luteinizing hormone (LH) from the pituitary gland during a critical period on proestrus (1) and the LH release is dependent on ovarian hormone (estrogen) secretion earlier in the estrous cycle (2, 3). Administration of a barbiturate on proestrus prior to the critical period can block the LH release and consequently block spontaneous ovulation at estrus (1, 4, 5). However, rats will in some cases mate following barbiturate treatment and mating can induce ovulation (6) by effecting LH release (7). More rats with 5-day than with 4-day estrous cycles ovulate in response to copulatory stimuli following blockage of spontaneous ovulation by barbiturate injection on proestrus (8). Because rats with 5-day estrous cycles have higher levels of estrogen in circulation on proestrus than do 4-day rats (9), it has been postulated that mating-induced ovulation of rats, like spontaneous ovulation, requires estrogen secretion prior to LH release and the higher estrogen levels in 5-day rats may be responsible for their greater capacity for mating-induced ovulation (8).

If a certain estrogen threshold must be achieved in order for mating to induce ovulation of barbiturate-blocked rats, a significant decrease in estrogen activity should reduce the incidence of, or prevent, the induction of ovulation by mating. This hypothesis was tested in the present study. A test of the hypothesis necessitated decreasing estrogen activity in test animals without inhibiting their sexual receptivity to the extent that none would mate, thus preventing a test of their capacity for mating-induced ovulation. The compound 1-(*p*-(2-diethylaminoethoxy) phenyl)-1-phenyl-2-*p*-methoxyphenylethanol (MER-25) has anti-estrogenic properties (10, 11) but rats given low dosages of the compound will mate (3), thereby permitting the influence of decreased estrogen activity on induction of ovulation to be examined.

A study of the influence of a decrease in estrogen activity on the capacity for mating-induced ovulation at the time of expected estrus was the primary objective of this investigation. However, since barbiturate injection has been found in some cases to cause ovulation to be delayed one day (1), those rats which were not found to mate or to ovulate by the time of expected estrus were examined again on the following day to determine whether reduced estrogen activity would affect ovulation delayed by barbiturate treatment.

MATERIALS AND METHODS

Sixty-day-old virgin, female rats of the Sprague-Dawley strain were caged singly and maintained under controlled lighting with lights on from 05.00 to 19.00 h. Other environmental conditions in the animal colony have been previously described (12). Animals were provided with food and water *ad libitum*.

Vaginal smears were taken by saline lavage from all rats before noon each day in order to monitor their estrous cycles and animals were assigned to experimental groups only after completing at least two successive cycles of the same length.

At 10.00 h on the day preceding proestrus, rats were injected intramuscularly with 0.25 m*l* of sesame oil or with 10 mg of MER-25 dissolved in sesame oil at a concentration of 40 mg/m*l*. (MER-25 dissolved in oil when this was heated for 24-48 h at 72 C.) Each rat was injected intraperitoneally with sodium barbital (25 mg/100 g body weight) at 13.00 h on proestrus. (Fresh barbital solution was prepared on the day that it was injected in a concentration of 100 mg/m*l* of saline.)

Female rats were placed in the cages of proven male breeders at 16.00 h on proestrus. On the morning of estrus each female was checked for sperm in the vaginal smear and vaginal plugs in the vagina or on the floor of the cage. Each rat exhibiting either of these evidences of mating at estrus was autopsied before noon that day. Those rats that had not mated by the morning of estrus were laparotomized before noon and returned to the males' cages where they remained until the following morning when they were checked once more for evidence of mating, then autopsied. When a rat was laparotomized, the uterus was examined for ballooning and the right oviduct was examined *in situ* at a magnification of 30x to determine whether the animal had ovulated. Only when a segment of the oviduct appeared both translucent and swollen was ovulation considered to have occurred.

At autopsy, the ovaries with oviducts attached were removed and weighed. The oviducts were then dissected free of the ovaries, pressed between microscope slides and examined at a magnification of 100x for the presence of ova. The uterus was also removed, trimmed, and, after any intraluminal fluid or semen that it contained was expressed, the tissue was weighed, dried to a constant weight in a warming oven, and reweighed.

Tables for use with binomial samples (13) or chi square analyses were used to compare groups of animals with regard to incidence of mating, ovulation, uterine ballooning, and vaginal cornification. Organ weights of different treatment groups, as well as body weights, were compared by using Student's t test.

RESULTS

Since all rats received an injection of barbital on proestrus to block LH release, the only treatment that will be mentioned when reporting experimental results will in most cases be that which differed between groups, i.e., whether rats were injected with oil or with MER-25 in oil prior to barbital injection.

Observations at proestrus.

Although results of some autopsies at the time of predicted estrus suggested that estrus had been blocked or delayed (Table 1), vaginal smears taken the previous day at proestrus were typical-proestrous smears and were predictive that estrus would ensue. In the oil-injected group vaginal smears consisting of only cornified cells, or a mixture of nucleated and cornified cells, were taken from 5 of 8 rats with 4-day cycles and all 10 rats with 5-day cycles. Similarly, in the MER-25-injected group proestrous smears comprised of these cell types were taken from 8 of 9 rats with 4-day cycles and 7 of 10 rats with 5-day cycles.

Observations at estrus.

In the control group of rats, fewer animals with 4-day than with 5-day estrous cycles mated at the time of expected estrus (Table 1). Because barbital inhibited mating of 4-day cyclic controls, MER-25 given in addition to barbital caused no greater inhibition of mating in the 4-day cyclic experimental rates. However, significantly fewer MER-25-treated 5-day rats mated than in the control group although, based on the appearance of proestrous vaginal smears taken from both groups, the MER-25-treated rats were considered to be in the same phase of the estrous cycle as oil-injected controls.

Composite data from all MER-25-treated rats when compared with composite data from all control rats revealed no differences between the groups in the numbers of rats that mated or in the numbers of rats that ovulated, but data from only those rats that *did* mate in each of the two treatment groups indicated that MER-25 was effec-

TABLE 1. *Effects of barbital and an estrogen antagonist (MER-25) on rat reproductive functions at estrus.*

Treatment and cycle length	*N* (observed)	Mated	Ovulated	Ballooned uterus	Vaginal cornification	*N* (autopsied)	Body wt. (g) ± S.E.[a]	Ovarian wt. (mg) ± S.E.[a]	Uterine wt. (mg) ± S.E.[g] Wet	Dry
Oil + barbital										
4-Day	8	1/8	1/8	7/8	7/8	1	276	59.0	508.4	100.8
5-Day	10	9/10[b]	6/10	2/10[c]	4/10	9	254	50.8	488.1	95.5
Combined	18	10/18	7/18	9/18	11/18	10	256±5.8	51.6±2.3	490.1±14.8	96.0±2.6
MER-25 + barbital										
4-Day	9	1/9	0/9	0/9[d]	0/9[d]	1	248	45.5	319.8	66.8
5-Day	10	3/10[e]	0/10	0/8[g]	0/10	3	252	45.7	347.5	69.2
Combined	19	4/19	0/19	0/17[d]	0/19[d]	4	251±4.9	45.6±1.8	340.6±10.2[f]	68.6±1.6[f]

[a] Only combined data from 4- and 5-day rats were used when comparing effects of different treatments; S.E. not calculated for each group.
[b,c] Significance of difference between 5-day rats and 4-day rats receiving the same treatment: b ($p<0.01$); c ($p<0.05$).
[d,e,f] Significance of difference between MER-25-treated rats and corresponding control group: d ($p<0.01$); e ($p<(0.05$); f ($p<0.001$).
[g] Uteri of only eight of ten rats in this treatment group were examined at estrus.

TABLE 2. *Effects of barbital and an estrogen antagonist (MER-25) on rat reproductive functions at metestrus.*

Treatment and cycle length	*N*	Mated	Ovulated	Ballooned uterus	Vaginal cornification	Body wt. (g) ± S.E.	Ovarian wt. (mg) ± S.E.	Uterine wt. (mg) ± S.E. Wet	Dry
Oil + barbital									
4-Day	7	7/7	6/7	0/7	0/7	254±5.6	48.7±1.9	503.3±27.9	96.2±4.9
5-Day	1	0/1	0/1	0/1	0/1	253[a]	54.5[a]	388.8[a]	73.2[a]
Combined	8	7/8	6/8	0/8	0/8	254±4.8	49.5±1.7	489.0±28.4	93.4±5.2
MER-25 + barbital									
4-Day	8	5/8	6/8	0/8	0/8	242±6.5	48.9±2.2	324.1±14.8[b]	63.8±2.6[b]
5-Day	7	2/7	2/7	0/7	0/7	244[a]	43.9[a]	325.0[a]	63.6[a]
Combined	15	7/15	8/15	0/15	0/15	243±4.2	46.5±1.4	324.5±10.1[b]	63.7±1.7[b]

[a] Data from groups of 5-day cyclers were not compared; S.E. not calculated for these groups.
[b] Significance of difference between MER-25-treated rats and corresponding control group: ($p<0.001$).

tive in preventing mating-induced ovulation; of the 10 rats that mated in the control group, 7 were induced to ovulate while none of the 4 MER-25-treated rats that mated also ovulated ($p<0.05$). There were no rats in any group that ovulated at estrus without having mated.

Uterine ballooning at estrus, a day later than its usual occurrence, was seen in significantly more control rats than MER-25-treated rats. More specifically, the control group of 4-day rats had a higher incidence of uterine ballooning at estrus than either the control 5-day rats or the 4-day rats given MER-25.

Vaginal cornification at estrus was inhibited in rats with 4-day cycles following injection with MER-25. So few rats with 5-day cycles in the control group exhibited cornification of vaginal cells at estrus that MER-25 was not found to cause any further reduction in the incidence of vaginal cornification in experimental 5-day rats.

Because few 4-day rats mated at estrus, and rats were autopsied then only if they mated, autopsy data from 4-day rats were insufficient to permit a comparison of the body or organ weights of these animals with those of 5-day rats at estrus. However, composite data from groups of experimental and of control rats (Table 1) indicate that MER-25 did not significantly alter body weights or ovarian weights of experimental rats but did cause both wet and dry uterine weights to be significantly less than those of oil-injected controls.

Observations at metestrus.

There was no difference at metestrus between control and MER-25-treated rats with regard to the incidence of mating, ovulation, uterine ballooning, or vaginal cornification (Table 2). Only one 5-day cyclic rat given oil failed to mate at estrus so only one 5-day rat remained in the control group for study and autopsy at metestrus. Consequently no comparison was made at metestrus between 5-day cyclic controls and 4-day rats in the control group or 5-day rats in the MER-25-treated group. There were sufficient numbers of 4- and 5-day rats in the MER-25-treated group to permit their comparison but they did not differ from one another in the incidence of mating, ovulation, uterine ballooning, or vaginal cornification.

Neither body weights nor ovarian weights at metestrus were affected by treatment of rats with MER-25, but both wet and dry uterine weights of MER-25-treated rats were significantly less than those of controls. This was evident when 4-day cyclic rats in the control group were compared to 4-day rats given MER-25 and when uterine weights of all controls were compared to uterine weights of all MER-25-treated rats.

DISCUSSION

The dosage of sodium barbital used (25 mg/100 g body weight) was effective in blocking spontaneous ovulation (as indicated by the failure of any rats to ovulate at estrus unless mating occurred) and in fact this dosage exceeded that of 15 mg/100 g body weight that was reported in one study to block spontaneous ovulation in all rats to which it was administered (1). Since uterine weights can be correlated with estrogen titers (14), the uterine weights of MER-25-treated rats (Tables 1 and 2) indicated that MER-25 administered on the day preceding proestrus was effective in achieving the desired reduction of estrogen activity in experimental rats. Despite a decrease in their estrogen activity, some rats that were treated with MER-25 and with barbital mated at estrus; however, mating did not overcome the barbiturate blockage of ovulation in any of these rats, although 70% of the barbital-injected control rats that mated also ovulated. Estrogen activity prior to proestrus has been found to be necessary if the ovulatory surge of pituitary LH is to be released (2, 3) and these data suggest that estrogen activity prior to proestrus is a requirement for mating-induced ovulation as well.

Zarrow and Clark (15) found that the number of ova released at ovulation following vaginal stimulation of PMS-primed immature rats with a glass rod was proportional to the amount of stimulation given, but Rodgers (16) found no correlation between numbers of ova released and numbers of intromissions received when high-intromission and low-intromission groups of rats were compared. Since Rodgers further reported that 9 to 12 intromissions are usually required before an experienced male rat will ejaculate and rats in the

present study were considered to have mated only if evidence of ejaculation was found, the failure of mating to induce ovulation in MER-25-treated rats was not considered to be due to fewer intromissions having occurred than in the oil-injected group.

Vaginal stimulation can induce ovulation by effecting a release of pituitary LH (7, 15, 17). Since MER-25 treatment has been demonstrated to increase pituitary LH stores (3), the failure of mating to overcome barbiturate blockage of ovulation in MER-25-treated rats appears due to a failure of the release mechanism for LH rather than to a lack of available pituitary LH.

Experimental results indicated in several ways that barbital injection caused rats with 4-day cycles to switch to 5-day cycles. Only 1 of 8 4-day control rats injected with barbital mated on the day that the rats were expected to be in estrus (Table 1); the other 7 rats in the group mated the following day and 6 of the 7 ovulated (Table 2). Ovulation of barbiturate-treated rats one day after expected estrus has been reported to occur even when rats are not placed with males (1) and, therefore, cannot be considered to be mating-induced ovulation. Each of the rats with 4-day cycles that mated a day later than expected had a ballooned uterus on the day of expected estrus (Table 1) and since ballooning of the uterus is characteristic of proestrus (18), its occurrence a day late also indicated retardation of the cycle. The uterine weights recorded for 4-day control rats at expected metestrus were as high as those of 5-day rats at estrus and provided further evidence that the 4-day rats switched cycle lengths and were in estrus rather than exhibiting characteristics of metestrus. No delay in mating of barbital-injected 5-day rats was evident since 9 of 10 controls with this cycle length mated at estrus and the rat that failed to mate was not found to mate the day after expected estrus. In addition 5-day rats did not exhibit uterine ballooning on the day of expected estrus as 4-day rats did.

At metestrus the uterine weights of 4-day rats treated with MER-25 were significantly less than those of controls, indicating less estrogen activity, but when these rats were compared to 4-day control rats with regard to the numbers of rats that ovulated and the numbers that mated in the two groups, no differences were found. Although MER-25-treated rats with 4-day cycles mated and ovulated at metestrus, some of the data failed to support that these rats had switched to 5-day cycles as control rats did, e.g., they exhibited no uterine ballooning on the previous day and their uterine weights on the day that mating and ovulation occurred were not suggestive of estrus. A comparison of data collected at metestrus with those collected at the time of expected estrus shows that uterine weights of 4-day rats given MER-25 were no greater at metestrus than at estrus (Tables 1 and 2), indicating that estrogen levels had not increased. Yet, the number of 4-day rats that ovulated at expected metestrus (6/8) was significantly greater ($p<0.01$) than the number of rats that ovulated at the time of expected estrus (0/9). This suggests that the estrogen threshold for ovulation decreases with time and is less on the second day after barbiturate blockage of ovulation than on the first day. A similar indication of a change in the LH-release apparatus the second day after proestrus was reported by Everett and Sawyer (1), who found that a single injection of a barbiturate prior to the critical period for LH release on proestrus blocks ovulation that night, but ovulation occurs the second night, even when a second injection of barbiturate is given 24 h after the first injection.

Cornification of vaginal cells, a characteristic of estrus (19), is estrogen dependent (2, 3) and the failure of any MER-25-treated rats to exhibit vaginal smears containing exclusively cornified cells on either estrus or metestrus was attributed to inhibition of estrogen activity in these animals. Vaginal smears taken from rats shortly after they have mated often contain many leukocytes, unless comparatively few intromissions have occurred (20), and the mating of control animals in this study on estrus or metestrus (delayed estrus in the case of 4-day rats) would account for failure of some of these rats to have only cornified cells in vaginal smears. Retardation of vaginal cornification can be caused by barbiturate treatment (1), but 7 of 8 control rats with 4-day cycles had cornified vaginal smears at the time of expected estrus (Table 1) even though they failed to ovulate or to mate at that time.

Repeated injections of MER-25 will decrease rate of growth (10) but the single dosage administered in this study had no significant effect on body weight at estrus or metestrus nor did it cause ovarian weights of treated rats to be different from those of control rats on either of these days. Ovarian weights of rats that have ovulated are usually greater than ovarian weights of rats that fail to ovulate. The total number of control rats that ovulated at estrus or metestrus was not significantly greater than the number of MER-25-treated rats that ovulated on these days (Tables 1 and 2) and, similarly, there was no significant difference in ovarian weights of rats in the two groups. Yet, a significant decrease in uterine weight of MER-25-treated rats was observed at estrus and at metestrus and indicated that the single injection of this substance administered on the day preceding proestrus continued to decrease estrogen activity at least three days after its administration.

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