Critical progesterone requirement for maintenance of pregnancy in ovariectomized rats

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Summary. The minimum progesterone concentration required to maintain the pregnancy was studied by varying doses of progesterone given subcutaneously to rats ovariectomized on Day 8 of pregnancy. Injecting 3 mg progesterone plus 200 ng oestradiol benzoate daily provided serum progesterone values between 25.4 ± 7.0 and 35.2 ± 6.2 ng/ml throughout Days 10–19 which were significantly lower than normal levels ($P < 0.05$), but resulted in 93.6% of fetal survival on Day 19 which was not significantly different from 93.3% in the control group. Injecting 2 mg progesterone plus 200 ng oestradiol benzoate daily gave progesterone values between 13.2 ± 4.6 and 19.0 ± 6.2 ng/ml and could not maintain fetal viability to Day 19 (14.2%, $P < 0.05$ compared with control group).

Critical times to supplement progesterone in rats ovariectomized on Day 8 or Day 15 were studied by varying the time of progesterone implantation after ovariectomy. Progesterone implants were administered 8, 12 and 24 h after ovariectomy on Day 8 and 24, 36 and 48 h after ovariectomy on Day 15. On Day 8, progesterone replacement could be delayed to 8 h but not 12 h, while on Day 15, progesterone replacement could be delayed up to 36 h but not 48 h after ovariectomy without affecting fetal survival.

Keywords: progesterone; rat pregnancy; abortion; ovariectomy

Introduction

Progesterone is essential to initiate embryo implantation and to maintain pregnancy in eutherian mammals (Rothchild, 1983). The depletion of sex hormones in pregnant rats induced by ovariectomy results in pregnancy termination (Deanesly, 1973). However, pregnancy termination in ovariectomized rats can be prevented by giving hormone supplements (Pepe & Rothchild, 1973; MacDonald, 1982). Progesterone used in the ovariectomized rats to maintain the pregnancy in various studies ranged from 3 to 20 mg/day. Lerner *et al.* (1962) suggested that the optimum subcutaneous dose to maintain the pregnancy in rats ovariectomized on Day 8 of pregnancy was 20 mg/day. Tamada & Ichikawa (1980) injected only 4 mg progesterone/day plus 200 ng oestradiol/day to rescue the fetuses of Day-14 pregnant ovariectomized rats. MacDonald (1982) injected 3 mg progesterone/day plus oestradiol to rats ovariectomized on Day 3 in order to maintain the free blastocyst and support the implantation and pregnancy through to Day 20. Unfortunately, in those studies, serum progesterone concentrations were not determined. Some investigators have suggested that the progesterone concentrations that are required in pregnant rats need not be as high as are actual physiological values (Pepe & Rothchild, 1973; Legrand *et al.*, 1979). Pepe & Rothchild (1973) showed that serum progesterone concentrations between 30 and 80 ng/ml could maintain the pregnancy to Day 22 in rats ovariectomized on Day 6, but they did not show whether pregnancy could be maintained by lower serum progesterone levels. The critical progesterone requirement for pregnancy maintenance, therefore, has not been fully determined. The purposes of this study were to determine the minimum serum progesterone concentration needed to maintain

the pregnancy under conditions of adequate oestrogen replacement, and also to investigate the critical time of progesterone supplement after ovariectomy for the rescue of the fetuses in rats.

Previous studies have documented the changes in physiological progesterone concentrations in pregnant rats. These changes can be divided into a pre-implantation rise of the values from Days 1 to 4, a plateau phase from Days 5 to 10 then a rise and fall with the maximum levels during Days 15–19 (Morishige *et al.*, 1973; Bridges, 1984). In the present study, Day 8 was selected for investigation as typical of the post-implantation plateau phase and Day 15 as the time of the physiological peak levels. These times were of particular interest in view of the current clinical use of anti-progestagens as abortifacients and represent suitable times for detailed further study of the mechanisms involved. This information will be of value in determining the optimal pharmacokinetic properties of antiprogestagens.

Materials and Methods

Female Sprague–Dawley rats aged 9–13 weeks were housed as described previously (Kendle, 1975). Vaginal smears were taken every morning after pairing with male rats overnight. The day of a positive sperm smear was defined as Day 1 of pregnancy.

Progesterone, oestradiol and sesame oil used in this study were purchased from Sigma, St Louis, MO, USA.

Minimum progesterone required to maintain pregnancy

Day-8 pregnant rats were randomly allocated to ovariectomy or sham ovariectomy under light ether anaesthesia. Sham-ovariectomized rats received 0.2 ml sesame oil by subcutaneous injection while ovariectomized rats received 3 mg or 2 mg progesterone plus 200 ng oestradiol benzoate injection daily from Day 8 to Day 18. Blood samples were taken for progesterone assay by cardiac puncture under light ether anaesthesia on Days 10, 13, 16 and 19 just before the time of injections. All rats were killed on Day 19 when fetuses were quickly removed from the uterus to examine for survival as indicated by the presence of heart beat. The number of surviving fetuses and number of implantation sites were counted, and proportion of fetal survival was calculated in each treatment group. Fetal growth was determined by measuring length and by weighing the live fetuses. Placental weight was also separately recorded.

Critical time for progesterone replacement

The critical time to supplement progesterone after ovariectomy was studied by varying the time of progesterone replacement to rats ovariectomized on Day 8 or Day 15 of pregnancy. Progesterone was administered by dorsal subcutaneous implants which were prepared by mixing progesterone with curing agent and then mixing with medical grade Silastic elastomer (Dow Corning, Midland, MI, USA) in a proportion of curing agent to elastomer of 1:10 to make 10% (w/w) progesterone. The mixture was left to cure and was cut to 5 × 40 mm size pieces which weighed 308.5 ± 6.5 mg. The implants were tested to ensure that they provided serum progesterone concentrations above the minimum required level throughout the period of study: 8 rats were ovariectomized on Day 8, given implants and blood sampled on Days 9, 11, 13 and 15. Similarly, 6 Day-15 pregnant rats were ovariectomized, implanted with progesterone, then blood sampled on Days 16, 17, 18, 19 and 20.

Day 8 ovariectomy. The serum progesterone profile after ovariectomy was studied by taking blood samples from 8 Day-8 pregnant rats immediately before ovariectomy and then again at 4, 8, 12, 24, 48, 72, 96, 120 and 144 h after operation.

Nine rats were sham-operated and received daily injections of sesame oil. Further groups of 10, 10, 9 and 5 rats were ovariectomized on Day 8 and received daily injections of 200 ng oestradiol benzoate and progesterone implants immediately or at 8, 12 or 24 h after ovariectomy. All rats were killed on Day 15 and proportions of fetal survival were calculated.

Day 15 ovariectomy. Similarly, 7 Day-15 pregnant rats were ovariectomized and blood samples were taken immediately before, then at 2, 4, 8, 12, 24, 48, 72, 96, 120 and 144 h after operation for the study of progesterone profile after ovariectomy.

Five rats were sham operated and received placebo implants, then were killed on Day 18. Groups of 5, 9, 5, 11 and 11 rats were ovariectomized on Day 15 and received placebo implants or progesterone implants immediately after ovariectomy, or 24, 36, or 48 h later. The animals were killed on Day 18 and examined for survival of fetuses.

The effect of oestrogen on the critical time of progesterone replacement was studied by injecting 200 ng oestradiol benzoate/day to 7 rats ovariectomized on Day 15 and receiving progesterone implants after a 48-h delay.

Serum progesterone assay

All blood samples were centrifuged after clotting to separate serum which was kept at -20°C until assay which was done within 1 month. The assay was done by RIA using a commercial Gamma B Progesterone Kit (IDS, Washington, UK) with a sensitivity of 0.5 nmol/l . The intra-assay and interassay coefficients of variation were 9.6% and 12% respectively.

Statistics

Significance of difference in the proportions of fetal survival in each treatment group was determined by the χ^2 test. Fetal length, fetal weight and placental weight were recorded from every live fetus, then expressed as mean $\pm$ s.d. for each treatment group. One-way analysis of variance, followed when appropriate by the Kramer modification of Duncan's multiple range test, was used to establish significance of difference of the means (Milton & Tsokos, 1983).

Results

Minimum progesterone required to maintain pregnancy

Table 1 shows serum progesterone concentrations of sham-ovariectomized rats and of ovariectomized rats treated with 3 or 2 mg progesterone/day plus 200 ng oestradiol benzoate/day. Serum progesterone values in rats treated with 3 mg progesterone/day were significantly lower than in sham-operated rats, and the concentrations in rats given 2 mg/day were significantly lower than in those given 3 mg progesterone/day ($P < 0.05$).

Table 1. Mean ($\pm$ s.d.) serum progesterone concentrations (ng/ml) in sham-operated rats and rats ovariectomized on Day 8 of gestation and treated with 3 or 2 mg progesterone + 200 ng oestradiol benzoate/day

Day	Sham ovariectomy + vehicle (N = 5)	Ovariectomy + 3 mg progesterone (N = 9)	Ovariectomy + 2 mg progesterone (N = 8)	ANOVA
Day 10	43.1 ± 4.3	25.4 ± 7.0	13.2 ± 4.6	$P < 0.05^*$
Day 13	55.4 ± 11.0	35.2 ± 6.2	19.0 ± 6.2	$P < 0.05^*$
Day 16	70.4 ± 16.3	27.0 ± 8.9	14.1 ± 2.6	$P < 0.05^*$
Day 19	60.8 ± 12.8	26.8 ± 5.6	17.7 ± 3.5	$P < 0.05^*$

*Each value is significantly different from each of the others in the same day (one-way analysis of variance).

The proportion of fetal survival on Day 19 in rats ovariectomized on Day 8 and given 3 mg progesterone plus 200 ng oestradiol benzoate daily was not significantly different from the value of sham-operated rats. Those given 2 mg progesterone/day plus 200 ng oestradiol benzoate/day had significantly reduced fetal survival on Day 19 (Table 2). Fetal length in progesterone-treated animals was not significantly different from the value in sham-operated rats. Fetal weight in rats receiving 3 mg progesterone/day was not significantly different from that in sham-operated rats, but rats receiving 2 mg progesterone/day had significantly lower fetal weight. Placental weights in both treatment groups were higher than in the sham-operated group (Table 2).

Critical time for progesterone replacement

Day 8 ovariectomy. As shown in Fig. 1(a) serum progesterone concentrations in ovariectomized rats without progesterone replacement dropped from $57.4 \pm 14.4\text{ ng/ml}$ before ovariectomy to $10.6 \pm 2.6\text{ ng/ml}$ at 4 h after operation, and then slightly increased to $14.9 \pm 3.1\text{ ng/ml}$ at 8 h after ovariectomy. Values declined again with an elimination rate constant of 0.02 h^{-1} to a level of around 6 ng/ml at 48 h and remained around this concentration throughout the period of study.

Table 2. Fetal survival, fetal growth and placental weight in rats sham ovariectomized on Day 8 of pregnancy and given vehicle and rats ovariectomized on Day 8 treated with 3 or 2 mg progesterone + 200 ng oestradiol benzoate/day

Groups	Fetal survival on Day 19	Fetal growth		Placental weight (g)
		Length (mm)	Weight (g)	
Sham- ovariectomy	102/109 (93.6%) (N = 7)	26.4 ± 1.2 (N = 78)	1.4 ± 0.2 (N = 78)	0.47 ± 0.06 (N = 78)
Ovariectomy + 3 mg progesterone	78/83 (93.9%) (N = 9)	26.2 ± 1.2 (N = 102)	1.4 ± 0.2 (N = 102)	0.56 ± 0.08* (N = 102)
Ovariectomy + 2 mg progesterone	32/226 (14.2%)** (N = 19)	25.8 ± 1.8 (N = 32)	1.3 ± 0.2* (N = 32)	0.53 ± 0.09* (N = 32)

Values are means ± s.d.

*Significantly different from sham-ovariectomized rats, $P < 0.05$ (ANOVA).

**Significantly different from values of the other 2 groups, $P < 0.01$ (χ^2).

Serum progesterone concentrations in ovariectomized rats implanted with progesterone declined from 52.5 ± 5.5 ng/ml on Day 9 to 25.9 ± 5.9 ng/ml on Day 15, but the values were above the minimum required level for maintenance of pregnancy throughout (Fig. 1a).

The proportion of fetal survival on Day 15 in rats ovariectomized on Day 8 and receiving progesterone implants immediately or after 8 h was not significantly different from the figure in the sham-operated rats, but when progesterone replacement was delayed up to 12 h after ovariectomy, fetal survival on Day 15 was just 17.1%, significantly different from that in the sham-operated group. Delay of progesterone replacement to 24 h after ovariectomy reduced fetal survival to zero (Table 3).

Day 15 ovariectomy. Serum progesterone concentrations dropped from 63.8 ± 13.1 ng/ml before ovariectomy on Day 15 to 19.2 ± 3.0 ng/ml at 8 h after operation and then slightly increased to 23.8 ± 5.5 ng/ml at 12 h (Fig. 1b). Again, the level declined with an elimination rate constant of 0.02 h^{-1} to values around 6 ng/ml at 96 h, then remained around this level throughout the study period.

Progesterone implants in rats ovariectomized on Day 15 provided serum progesterone concentrations above the minimum required level throughout the period. The levels declined from 58.9 ± 9.7 ng/ml on Day 16 to 46.4 ± 9.6 ng/ml on Day 18 (Fig. 1b).

In rats ovariectomized on Day 15 of pregnancy, progesterone alone could rescue fetuses up to Day 18, while placebo could not. Delay in progesterone replacement of 24 or 36 h after ovariectomy did not make any difference to the survival of fetuses compared to sham-ovariectomized rats. However, delay in progesterone replacement up to 48 h after ovariectomy resulted in a significantly lower proportion of fetal survival than that in sham-operated rats (Table 4).

When 200 ng oestradiol benzoate/day were injected together with progesterone implants, a delay of 48 h resulted in a proportion of fetal survival on Day 18 not significantly different from that in ovariectomized rats receiving progesterone alone after a 48-h delay (Table 4).

Discussion

To maintain the pregnancy in rats ovariectomized on Day 8, Lerner *et al.* (1962) had to inject progesterone in doses as high as 20 mg/day because oestrogen was not also given, but if oestrogen was co-injected, the progesterone requirement was lower. In our study, an adequate dose of oestrogen was injected and different doses of progesterone were administered to determine the minimum progesterone concentrations required. It was found that 3 mg progesterone/day, given sub-

Fig. 1. Serum progesterone concentrations in (a) rats ovarietomized on Day 8 and with (■) or without (●) progesterone implants and (b) rats ovarietomized on Day 15 and with (■) or without (●) progesterone implants.

cutaneously, gave progesterone concentrations around 30 ng/ml, which was enough, whereas 2 mg progesterone/day, which provided serum progesterone values of less than 20 ng/ml, was not enough to maintain full fetal viability. The need for oestrogen in addition to progesterone to maintain pregnancy has also been shown by Pepe & Rothchild (1973), Tamada & Ichikawa (1980) and MacDonald (1982). Especially in late pregnancy, oestrogen is necessary to ensure sufficient plasticity of the uterine tissue (Tamada & Ichikawa, 1980).

Progesterone concentrations in rats injected with 3 mg progesterone/day were significantly lower than in the control group, but the proportions of fetal survival on Day 19 in both groups were not significantly different. Serum progesterone values in pregnant rats can therefore be lower

Table 3. Fetal survival on Day 15 in rats sham ovariectomized (OVX) on Day 8 of pregnancy and in rats ovariectomized on Day 8 and given 200 ng oestradiol benzoate/day + progesterone implants at various times after ovariectomy

Treatment groups	No. of rats	Fetal survival
Sham OVX + placebo	9	66/73 (90.4%)
OVX + progesterone immediately	10	128/140 (91.4%)
OVX + delay progesterone 8 h	10	129/145 (88.9%)
OVX + delay progesterone 12 h	9	28/164 (17.1%)**
OVX + delay progesterone 24 h	5	0

**Significantly different ($P < 0.01$, χ^2) from each of the first three groups which are not significantly different from each other.

Table 4. Fetal survival on Day 18 in rats sham ovariectomized (OVX) or ovariectomized on Day 15 of pregnancy and given progesterone implants at various times after ovariectomy and in rats given 200 ng oestradiol benzoate/day in addition to a 48 h progesterone delay

Treatment groups	No. of rats	Fetal survival
Sham OVX + placebo	5	67/67 (100%)
OVX + progesterone immediately	9	175/184 (95.1%)
OVX + delay progesterone 24 h	5	73/76 (96.1%)
OVX + delay progesterone 36 h	11	135/150 (90.0%)
OVX + delay progesterone 48 h	11	77/148 (52.0%)**
OVX + oest. + delay progesterone 48 h	7	39/74 (52.7%)**
OVX + placebo	5	7/76 (9.2%)**

**Significantly different ($P < 0.01$, χ^2) from each of the first four groups which are not significantly different from each other.

than the actual physiological level without affecting survival or the growth of fetuses. Labhsetwar & Watson (1974) have shown the rise of oestradiol and the fall of progesterone serum concentrations, resulting in an increasing oestrogen:progesterone ratio at term, which is believed to initiate the parturition. Inoue (1981) has also shown that parturition could not occur, even at term, if the oestrogen:progesterone ratio was not correct. It is therefore possible that pregnant rats may sustain higher progesterone concentrations than is required for maintenance of pregnancy in order to prepare for the change of oestrogen:progesterone ratio which is essential for parturition when the pregnancy comes to term.

In our experiment, uterine progesterone was not assayed because, in rats, the placenta is not a site of progesterone production. Legrand *et al.* (1979) had already shown that uterine progesterone concentrations were proportional to plasma progesterone concentrations, and it originated from peripheral blood and not from the placenta.

Reduction of the progesterone concentration to about 50% did not affect the normal growth of fetuses, at least from Days 8 to 19, as shown by fetal length and fetal weight in ovariectomized rats treated with 3 mg progesterone/day. However, if the level fell too far, as in rats treated with 2 mg progesterone/day, fetal weight was significantly lower although no difference in fetal length was seen. This implies that fetal weight is a more sensitive index of growth than fetal length. Uterine blood supply may be altered by progesterone deprivation, and this may explain altered growth. This possibility is being currently investigated. Placental weights in ovariectomized rats treated with 2 or

3 mg progesterone/day were significantly higher than placental weight in sham-ovariectomized rats. Csapo & Wiest (1969) also demonstrated hypertrophy of the placenta in ovariectomized rats. Wilson & Lindsey (1987) have shown that progesterone withdrawal is a potent stimulus for uterine prostaglandin synthesis. Prostaglandins are the known cause of tissue inflammation and a cause of increasing capillary permeability (Moncada *et al.*, 1980). Therefore, this placental hypertrophy may be a result of fluid infiltration caused by an increasing level of prostaglandins when progesterone concentration drops.

Serum progesterone profiles in rats ovariectomized on Day 8 or Day 15 of pregnancy showed a dramatic fall after the operation, but the concentrations increased slightly at 8 h and 12 h after ovariectomy on Day 8 and Day 15 respectively. These slight rises of about 6 ng/ml could be explained by a compensatory mechanism of progesterone production by the adrenal gland. While the effects of combined ovariectomy and adrenalectomy was not investigated in the present study, Lu & Judo (1982) found a similar rise of progesterone level after ovariectomy in cyclic rats which was abolished by simultaneous adrenalectomy. Accordingly, after chronic ovariectomy, progesterone concentrations were around 6 ng/ml (Fig. 1a, b). In addition, ovariectomy in early and late pregnancy resulted in the same progesterone elimination rate constant of 0.02 h^{-1} .

The study of critical time for progesterone replacement after ovariectomy was determined by administering progesterone implants at various times to ovariectomized rats in early or mid-pregnancy. Rats ovariectomized on Day 8 were given progesterone plus oestradiol while rats ovariectomized on Day 15 were treated with progesterone alone because the results in Table 4 show that progesterone alone could maintain the pregnancy up to Day 18. Progesterone implants were used in this study to prevent fluctuation of serum concentrations. Our unpublished data confirm that the implants released progesterone above the required levels within 2 h. Progesterone replacement could be delayed until serum progesterone concentrations dropped to about 15 ng/ml, but the minimum required level had to be above 20 ng/ml (Tables 1 & 2) otherwise fetal survival was significantly reduced. This means that serum progesterone can fall below the minimum required level for a period provided that it is restored to over 20 ng/ml within a critical period which depends upon the stage of the pregnancy. Injecting 200 ng oestradiol benzoate/day did not help to prolong this critical time of progesterone replacement, since fetal survival was not significantly different from that in ovariectomized rats receiving progesterone alone.

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