Effects of the uterus and mid-pregnancy conceptus on luteal lifespan of the rat

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Summary. Hysterectomy on or after Day 10 resulted in a quick return of pseudopregnant rats to oestrus but the interval to oestrus in pregnant rats was significantly longer ($P < 0.01$), indicating that the gravid uterine horn interfered with uterine-induced luteolysis by Day 10.

When pseudopregnant rats were injected with extracts of conceptuses or pregnant rat serum (PRS), samples collected on Days 10 or 12 of pregnancy delayed the return to oestrus ($P < 0.01$ for conceptus extracts, $P < 0.05$ for PRS) and serum progesterone concentrations on Day 13 of pseudopregnancy were higher ($P < 0.01$) than in controls. Bioassays utilizing mammary development or inhibition of oestrus in virgin cyclic rats as endpoints failed to detect lactogenic activity in Day 10 conceptus extracts or PRS, and radioreceptor assays using bovine prolactin (NIH-B5) as a standard showed that Day 10 extracts had only about 2–3% of the lactogenic activity found in Day 12 extracts. Injections of dilutions of Day 12 extracts with 2 or 4 times the lactogenic activity as Day 10 extracts did not extend pseudopregnancy. These results suggest that by Day 10 the conceptus produces a factor capable of interfering with luteolysis and that this factor is not placental lactogen.

Introduction

Maintenance of pregnancy in the rat is dependent on progesterone production by the corpus luteum (CL). The hypophysis is necessary for luteal maintenance for the duration of pseudopregnancy and for the first half of pregnancy (Pencharz & Long, 1933; Selye, Collip & Thomson, 1933), being a source of prolactin for the first 8 days of pregnancy and of luteinizing hormone (LH) from Days 8 to 12 (Madhwa Raj & Moudgal, 1970; Morishige & Rothchild, 1974; Smith, Freeman & Neill, 1975). Twice daily surges of prolactin occur after induction of pseudopregnancy by cervical stimulation (Day 1) (Smith *et al.*, 1975). These surges end on the morning of the 11th day of pseudopregnancy and with the nocturnal surge on the 10th day of pregnancy (mating = Day 1) (Smith & Neill, 1976). The luteotrophic action of LH from Days 8 to 12 of pregnancy may be due to a stimulatory effect on luteal oestrogen production (Gibori, Keyes & Richards, 1978).

Placental hormones can maintain progesterone production by CL when hypophysectomy is performed on or after the 12th day of pregnancy (Pencharz & Long, 1933). Bioassays have detected lactogenic activity in placental extracts and serum collected at mid-pregnancy (Averill, Ray & Lyons, 1950; Matthies, 1967; Cohen & Gala, 1969; Linkie & Niswender, 1973). Luteotrophic activity peaked on the 12th day and was not detectable before the 11th day (Cohen

& Gala, 1969; Linkie & Niswender, 1973). A radioreceptor assay (RRA) for placental lactogen demonstrated a peak of cross-reactivity on or after the 12th day in serum and placental extracts (Kelly, Shiu, Robertson & Friesen, 1975). Only very low levels were detected before the 11th day. Histological studies of CL (Wayneforth, 1971) and measurements of serum progesterone (Fajer & Barraclough, 1967; Pepe & Rothchild, 1974) have shown luteal stimulation on or after the 12th day of pregnancy. Thus, the ability of the placenta to maintain and stimulate luteal function on and after the 12th day seems well established.

Prostaglandin (PG) F-2 α released from the uterus is responsible for luteolysis at the end of pseudopregnancy (Horton & Poyser, 1976). In a majority of pseudopregnant rats, the CL have been irreversibly affected by the uterus by the 10th day, and hysterectomy after this time does not extend luteal lifespan (Silberger & Rothchild, 1963). The findings that the luteolytic function of the uterus was exerted by the 10th day of pseudopregnancy and the luteotrophic effects of placental lactogen were not fully manifested until the 12th day of pregnancy suggested that a placental factor other than the placental lactogen affected luteal lifespan during mid-pregnancy. The present experiments examined this possibility.

Materials and Methods

All rats were virgin females purchased from Holtzman, Sprague-Dawley, or King Laboratory animal suppliers. Rats were housed under controlled temperature (23°C) and photoperiod (12 h light/12 h dark, lights on 06:30 h). Purina rat chow and water were provided *ad libitum*. At least one normal 4- or 5-day oestrous cycle was observed before rats were assigned to experiments. Pseudopregnancy was induced by cervical stimulation with a glass rod on the evening of pro-oestrus and the morning of oestrus. Operations were done via a mid-ventral incision and using methoxyflurane anaesthetic. Vaginal smears were taken daily, and Day 1 of pregnancy was considered to be the first day spermatozoa were found in the vagina, while Day 1 of pseudopregnancy was the last day on which the smear contained mostly cornified cells. Each pregnant or pseudopregnant rat was then housed continuously with a male. The endpoint used in all experiments was the interoestrous interval defined as the number of days between matings in pregnant rats, or days between the last cornified smear and mating in pseudopregnant rats. Mating was confirmed by the presence of spermatozoa in vaginal smears.

Extracts of conceptuses and pregnant rat serum (PRS)

To obtain conceptus extracts, rats were anaesthetized, the uterus was removed, a slit was made on the anti-mesometrial side and the entire contents of each embryonic swelling were removed with forceps. The contents were homogenized immediately in cold (2°C) sterile saline (9 g NaCl/l) in a ground-glass homogenizer. The homogenate was centrifuged at 2500 g for 20 min (3°C) and the supernatant adjusted with sterile saline to a final volume of 10 conceptuses per ml. The homogenates contained all products of the uterine swellings (i.e. fetal placenta, fetus and any associated decidual tissue). For serum samples, blood was collected from anaesthetized rats via cardiac puncture, allowed to clot overnight (3°C) and centrifuged at 2500 g for 20 min. Sera from a given day for any one experiment were pooled, as were extracts of conceptuses, and the samples were placed in disposable syringes which were kept frozen (−20°C) until use.

Experiment I

The effect of acute hysterectomy during pregnancy and pseudopregnancy upon luteal function (i.e. return to oestrus) was investigated. Rats were randomly assigned to one of 10 groups in an experiment of 2 $\times$ 5 factorial design with pregnancy status (pregnant or pseudopregnant) and day of hysterectomy (6, 8, 9, 10, 12) as the factors.

Experiment II

The effect of treatment of pseudopregnant rats with conceptus extract was investigated. Rats were randomly assigned to one of 5 treatments: non-injected control, injection of muscle extract, or injections of conceptus extract obtained at Day 8, 10 or 12 of pregnancy. Rats were injected with the extract of two conceptuses twice daily from Days 9 to 14 or from Day 9 until return to oestrus. Increasing the duration of treatment produced no apparent effect, so the results were pooled. The experiment was done in two replicates with $N=8$ and $N=6$, respectively. Serum samples were collected from the second replicate. Serum progesterone concentrations on Day 13 and the interval to oestrus were measured.

Experiment III

Except that pseudopregnant rats were treated with PRS (0.2 ml/injection) the experimental treatments were as in Exp. II.

Experiments IV and V

Extracts of conceptuses (Exp. IV) and PRS (Exp. V) were tested for activity corresponding to rat placental lactogen by a modification of the method of Cohen & Gala (1969). Vaginal smears of young rats were followed, from the time of vaginal opening, to ensure that none experienced pseudopregnancy. Only rats with 4- or 5-day cycles were utilized. In Exps. IV and V the experimental groups were the same as in Exp. II except that the non-injected control group was eliminated. Commencing at oestrus rats were injected directly over the fourth mammary gland with the extract of two conceptuses or 0.2 ml PRS twice daily. After 8 days of treatment, rats were killed and the fourth mammary glands were removed and fixed in Bouin's solution, stained with haematoxylin, dehydrated, and cleared with methyl salicylate. Mammary gland development was rated as unstimulated (ducts with little or no alveoli) or stimulated (moderate to extensive lobulo-alveolar development). The luteotrophic activity was determined by whether or not rats showed vaginal oestrus during the treatment period.

Experiment VI

The effect of treatment of pseudopregnant rats with dilutions of conceptus extract was investigated. Rats were randomly assigned to one of 5 treatments: control injection of muscle extract, injection of extract of Day-10 conceptuses, injection of extract of Day-12 conceptuses, or injection of a 1:10 or 1:20 dilution of Day-12 extracts. Rats were injected with the extract of two conceptuses (or 0.2 and 0.1 conceptuses in the last two groups) on Day 7 in the afternoon (16:30 h) and every morning (08:00 h) and afternoon (16:30 h) from Day 8 to Day 14. Serum progesterone concentrations on Day 13 and the time to return to oestrus were used as endpoints of luteal function. Although 8 rats were initially assigned to each group, some rats failed to become pseudopregnant and were discarded.

Progesterone assay

Approximately 1 ml aliquots of blood were collected from the tail vein of unanaesthetized rats. Blood was allowed to clot at 3°C overnight, and serum was collected and stored at -20°C until use. Progesterone concentrations were measured by double-antibody radioimmunoassay using a highly specific antiserum (Staigmiller, Short, Bellows & Carr, 1979). Serum samples were extracted with hexane and not subjected to further purification. There were no reagent blanks and the efficiency of extraction averaged 78.4%. All samples were analysed at two dose levels with two tubes per dose. Validation of the assay was based on failure to find heterogeneity

of regression among unknowns, parallel dose responses between standards and unknowns and on the quantitative recovery of various amounts of progesterone (11.25–90 ng/ml) added to hypophysectomized male rat serum ($r = 0.99$, $n = 4$). The sensitivity of the assay was 60 pg/tube; utilizing 0.1 ml samples, the lower limit of the assay was 3 ng/ml. In 8 assays, a standard serum pool gave a mean progesterone value of 38.0 ng/ml with a coefficient of variability of 8.8%.

Placental lactogen assay

Samples from the conceptus extract and PRS pools used in Exps II, III and VI were stored at -20°C until used. Rat placental lactogen concentration was measured by radioreceptor assay using mammary membranes from lactating rabbits, bovine prolactin (NIH-PRL-B5) standards, and ^{125}I -labelled bovine prolactin (Shiu, Kelly & Friesen, 1973). For the assay of serum samples, 0.1 ml hypophysectomized male rat serum was added to each standard tube. The same volume of serum was then assayed. The sensitivity of the assay was 10 ng per tube.

Statistical analysis

Treatment effects in Exps I, II, III and VI were determined by analysis of variance using the appropriate pre-planned orthogonal comparisons. For Exps IV and V, the proportion of animals showing mammary development and inhibition of oestrus were tested by χ^2 analysis using a contingency table (Snedecor & Cochran, 1967).

Results

Experiment I

Pregnant rats hysterectomized on or after Day 10 had longer ($P < 0.01$) intervals to next oestrus than did identically treated pseudopregnant rats (Text-fig. 1). The large variation for

Text-fig. 1. The effect of acute hysterectomy in pregnant (●) and pseudopregnant (○) rats on the interoestrous interval. Values are mean $\pm$ s.e.m. for 8 rats/group. *Values significantly different from those for pregnant rats, $P < 0.01$ (analysis of variance).

pseudopregnant rats on Day 10 or 12 was the result of a bimodal distribution of the data: for the Day-10 group 5/8 rats had intervals of <16 days, while 3 had intervals ≥ 20 days and for the Day-12 group, the intervals were <16 days for 6 rats and ≥ 20 days for 2 rats.

Experiments II and III

The results for the injected and non-injected controls were not different and were pooled. Treatment with extracts of conceptuses or PRS resulted in longer interoestrous intervals and higher progesterone concentrations (Table 1).

Table 1. The effect of treatment of pseudopregnant rats with extracts of conceptuses (Exp. II) and serum collected from pregnant rats (Exp. III)

Treatment	Lactogenic activity ($\mu\text{g}/\text{injection}$)	Interoestrous interval (days) N = 14	Serum progesterone (ng/ml) N = 6
Conceptus extracts			
Control		14.3 ± 0.2	16.0 ± 3.4
Day 8		14.6 ± 0.6	23.3 ± 9.2
Day 10	0.15	$17.1 \pm 0.4^*$	$59.7 \pm 13.6^*$
Day 12	10.60	$17.2 \pm 0.4^*$	$58.8 \pm 13.6^*$
Pregnant rat serum			
Control		14.3 ± 0.2	17.4 ± 3.8
Day 8		14.9 ± 0.3	9.7 ± 1.6
Day 10	0.04	$16.4 \pm 0.4^\dagger$	$32.5 \pm 5.1^*$
Day 12	1.15	$16.0 \pm 0.5^\dagger$	$32.5 \pm 8.1^*$

Values are mean $\pm$ s.e.m.

Values significantly different from the others in that Experiment, $*P < 0.01$; $^\dagger P < 0.05$.

Experiments IV and V

Only the treatments with Day-12 conceptus extracts or PRS caused mammary development ($\chi^2 = 16.0$, 3 d.f. $P < 0.01$) and inhibited oestrus ($\chi^2 = 11.07$, 3 d.f., $P < 0.025$) (4/4 mammary stimulation in both experiments, 3/4 cycle inhibition in both experiments).

Experiment VI

Only the rats treated with extracts of 2 conceptuses had longer intervals to oestrus and higher progesterone concentrations (Table 2).

Table 2. The effect of treatment of pseudopregnant rats with the extract of conceptuses and dilutions of extracts (Exp. VI)

Treatment	Lactogenic activity ($\mu\text{g}/\text{injection}$)	Interoestrous interval (days)	Serum progesterone (ng/ml)
Control	—	13.9 ± 0.3 (7)	25.9 ± 4.1 (7)
Day 10	0.40	$*17.0 \pm 0.4$ (6)	$*71.0 \pm 7.8$ (6)
Day 12	17.00	$*17.0 \pm 0.4$ (8)	$*76.4 \pm 8.1$ (8)
1:10 dilution, 2 conceptuses	1.70	14.8 ± 0.6 (8)	42.2 ± 6.3 (7)
1:20 dilution, 2 conceptuses	0.85	14.3 ± 0.4 (6)	31.0 ± 7.4 (6)

Values are mean $\pm$ s.e.m. for the no. of rats indicated in parentheses.

* Values significantly different from all other values, $P < 0.01$.

Discussion

These results appear to provide the first direct evidence that the Day-10 rat conceptus produces a factor other than placental lactogen capable of extending luteal lifespan. The non-gravid uterus exerted an irreversible luteolytic effect by Day 10, but this could be overcome by the presence of the conceptus. The results agree with and extend those of Silberger & Rothchild (1963), who also found that hysterectomy on or after Day 10 of pseudopregnancy did not result in extended luteal lifespan.

The effect of the conceptus might be through a luteotrophic or an antiluteolytic stimulus. Alloiteau (1957, 1958) first suggested a role of the gravid uterus in controlling luteal lifespan before Day 12 because pregnancy and luteal function were maintained in rats hypophysectomized on Day 6 of pregnancy and treated with progesterone for 6 days. Yoshinaga & Adams (1967) transferred blastocysts into 'cyclic' rats treated with progesterone: pregnancy was maintained when progesterone treatment was terminated after 6 days. Zeilmaker & Verhamme (1978) found that ectopically developing rat blastocysts could affect CL. All these results provided indirect evidence that the young conceptus (prior to 12 days of age) was capable of influencing luteal lifespan. The results of Kisch & Shelesnyak (1968) suggested that a factor from the Day-10 conceptus could substitute for prolactin under certain experimental conditions but in our experiments the factor in Day-10 conceptus extracts which prolonged luteal lifespan did not have characteristics of prolactin (see below).

It is clear from the results of Exps II and III that injection of Day-10 conceptus extracts and PRS extended the length of pseudopregnancy by prolonging luteal lifespan. The extension of the interval to oestrus was not as great as that seen during pregnancy and serum progesterone concentrations at Day 13 were lower than preinjection values (89.8 ± 4.76 ng/ml at Day 9, $n = 60$). This suggested that regression of CL was slowed or delayed rather than blocked and that factors other than those present at Day 10 were involved in maintaining luteal function for the duration of pregnancy. This may suggest an antiluteolytic rather than a luteotrophic role of the Day-10 extracts.

Injections of conceptus extracts and PRS from Day 12 of pregnancy in virgin cyclic rats produced the expected effects on mammary development and inhibition of oestrus. However, although the Day-10 injections were of the same doses as in Exps II and III, there was no effect in Exps IV and V which indicated the presence of placental lactogen. The results are consistent with what is known about the production and biological effects of placental lactogen (Averill *et al.*, 1950; Matthies, 1967; Cohen & Gala, 1969; Linkie & Niswender, 1973). When lactogenic activity was measured by RRA, Day-10 conceptus extracts and PRS had only about 2–3% of the activity found at Day 12 (Table 2). Therefore, both receptor assay and bioassay suggested that the factor in Day-10 conceptus extracts responsible for extending luteal lifespan was not placental lactogen. The results of Exp. VI add additional support to this conclusion, because dilutions of Day-12 samples which contained 2 or 4 times the amount of lactogenic activity as Day-10 samples were not able to extend luteal lifespan in pseudopregnant rats. Since treatment with Day-10 conceptus extract significantly prolonged luteal lifespan, the effect cannot be due solely to the luteotrophic action of placental lactogen.

The nature of the substance acting at Day 10 or its site of action cannot be determined from our results. Basuray & Gibori (1980) suggested that decidual tissue may produce a substance affecting luteal lifespan before Day 12. In both this study and ours, progesterone concentrations were not elevated above initial values but rather regression of CL appeared to be delayed. This could be indicative of an antiluteolytic effect of the early conceptus upon the uterus. Although oestrogens and androgens have been reported to be luteotrophic during mid-pregnancy (Gibori & Keyes, 1978) it seems unlikely that serum concentrations of these steroids during pregnancy (Presl, Horsky, Herzman, Mikutas & Menzl, 1967; Labhsetwar, 1972; Gibori, Chatterton & Chen, 1979) are sufficient to produce the effect observed in Exp. III. A substance which has

receptor activity corresponding to a chorionic gonadotrophin has been identified in rat placenta collected on Days 10–15 of gestation (Haour, Tell & Sanchez, 1976). Although it has not yet been identified in serum, it is possible that this substance is involved in the effects observed in Exps II, III and VI.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Grant No. 5 T32 HD 0700703, National Institute of Child Health and Human Development, DHEW, and by Grant No. 630-0505 B, C from the Ford Foundation. This is paper number 746 from the Department of Meat and Animal Science.

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Received 19 January 1981