Relationship between corpora lutea or fetal number and plasma concentrations of progesterone and testosterone in mice

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Summary. Blastocysts (1–14) were transferred unilaterally into 63 pseudopregnant mice which were killed on Day 17. Plasma progesterone concentrations were significantly ($P < 0.05$) lower in animals with one fetus than in those with 2–5 or 9–14 fetuses. Plasma testosterone concentrations were correlated with fetal number in mice with 1–13 fetuses ($P < 0.001$). The total placental content of chorionic gonadotrophin in 13 litters varied directly with the number in the litter (1–6), and was 1.67 ± 0.15 ng/placenta. The number of corpora lutea per mouse was negatively correlated with mean CL volume per mouse ($P < 0.001$), and the number of conceptuses was positively correlated with mean CL volume per mouse ($P < 0.001$). The effect of conceptuses on the ovary was systemic. The relationship between plasma testosterone concentration and conceptus number may be due to gonadotrophins acting on the ovary, or androgens produced by the placenta or fetus.

Introduction

The mouse placenta contains a gonadotrophin similar to human chorionic gonadotrophin (hCG) (Wide & Hobson, 1977, 1978; Wide, Hobson & Wide, 1980; Rao, Pointis & Cedard, 1982). Placental CG reaches peak levels on Days 11 and 16 *post coitum* (Wide & Wide, 1979), and may be partly or wholly responsible for maintaining the secretion of ovarian steroids during the second half of pregnancy, when the continuation of gestation is not dependent on the presence of the pituitary (Newton & Beck, 1939).

A quantitative relationship might exist between the number of implantations and the production of ovarian steroids, but the evidence for this is contradictory in both the mouse and the rat. Plasma progesterone and testosterone concentrations increase in the second half of pregnancy in the mouse, and plasma progesterone values are greater in mice selected for large litters than for small (Michael, Geschwind, Bradford & Stabenfeldt, 1975; Barkley, Michael, Geschwind & Bradford, 1977). In experiments using C3H mice in which the number of fetuses was experimentally adjusted to between 1 and 10, serum concentrations of progesterone were directly proportional to litter size (Soares & Talamantes, 1983). However, neither the number of corpora lutea nor that of fetuses is correlated with plasma progesterone concentrations in Rockland Swiss mouse litters varying in size from 7 to 11 (Simon, Bridges & Gandelman, 1978). In the rat there is a significant correlation between total corpus luteum weight and serum progesterone concentration on Day 16 of pregnancy, but reducing the number of conceptuses from 12 to 5 does not significantly alter the levels of progesterone (Elbaum, Bender, Brown & Keyes, 1975). However, Kato, Morishige &

Rothchild (1979) found a direct relationship between the number of conceptuses and serum progesterone concentration in rats on Day 15 of pregnancy.

Our experiments were designed to test whether the number of fetuses is related to plasma concentrations of testosterone and progesterone in mice, by independently controlling the numbers of fetuses and corpora lutea by using embryo transfer. This design also enabled us to examine whether fetuses exert a local trophic effect on the ipsilateral ovary, as has been found in rats carrying one or two experimentally implanted fetuses on Day 18 of pregnancy (Zambrana & Greenwald, 1971).

Materials and Methods

The mice were from our own closed colony of randomly bred albinos, maintained under a constant schedule of 14 h light and 10 h dark. Food and water were freely available.

Fetal number was controlled by transferring blastocysts obtained from donor mice on Day 4 *post coitum* to hosts on the 3rd day after mating with vasectomized males. Donor mice were prepared for superovulation by i.p. injections of 5 i.u. PMSG and 5 i.u. hCG 48 h apart. Animals were then paired with males of proven fertility, and inspected for a coital plug the following morning (Day 1 of pregnancy). Blastocysts were flushed from the uterine horns into sterile Petri dishes using Medium 199 (Flow Laboratories, Irvine, U.K.) containing 20 mM-Hepes buffer, 10% fetal calf serum and 100 units penicillin-G/ml (Sigma, London, U.K.). Host animals were anaesthetized with 2,2,2-tribromethanol (Avertin: Winthrop, Surbiton upon Thames, U.K.), on the 3rd day after a sterile mating with a vasectomized male, and the uterus was exposed via a flank incision. Between 1 and 18 embryos were collected in a small volume of medium (< 1 µl), in an orally controlled pipette, and injected unilaterally into one uterine horn per animal, choosing alternate sides in successive mice.

The mice were anaesthetized on Day 17 of pregnancy with an injection of amylobarbitone sodium. The thorax was opened and the mouse exsanguinated via the heart. The blood was placed in heparinized tubes, centrifuged and the plasma kept for the radioimmunoassay (RIA) of progesterone and testosterone.

The number of implantation sites and fetuses was counted, and placentae and fetuses were weighed. The maternal ovaries were weighed after fixation for 24 h in Bouin's fluid. The ovaries from 14 mice with 1 fetus, 15 with 2–5 fetuses, 5 with 6–8 fetuses and 11 with 9–14 fetuses were cut at 10 µm and stained with haematoxylin and eosin. The number of corpora lutea in each ovary was counted, and their volumes measured by the method of Rowlands (1961).

Plasma from 62 mice was assayed for progesterone by the method of Scaramuzzi, Corker, Young & Baird (1975). Two 50 µl aliquants from the plasma of each animal were extracted with 2 ml petroleum ether (Analar reagent grade). The ether was evaporated to dryness under nitrogen and the residue dissolved in 1 ml phosphate buffer. Duplicate 0.1 ml portions of the extract were assayed with a specific RIA using sheep anti-progesterone antiserum 91929/9 as described by Scaramuzzi *et al.* (1975). There was little or no cross-reaction between this antiserum and androstenedione, cortisol, pregnenolone, testosterone, or 17-hydroxyprogesterone. There was a significant cross-reaction with progesterone, 11 α - and 11 β -hydroxyprogesterone and 11-ketoprogesterone.

Testosterone was assayed in plasma from 26 mice, with litters of 1–13, by the method of Collins, Mansfield, Alladina & Sommerville (1972). The testosterone antiserum (NEA-042B; New England Nuclear, Boston, MA, U.S.A.) was from a rabbit immunized with a testosterone-3-CMO-bovine serum albumin conjugate. Cross-reactivity at 50% displacement was about 50% for dihydrotestosterone and was negligible for other androgens, progesterones or oestrogens. The efficiency of the extraction method was 85% (range 75–94%). The intra-assay variation was 4.8% and the inter-assay variation about 9%. The detection limit was 0.05 nmol/l.

Table 1. Effects of variation in numbers of fetuses on fetal and placental weights, and plasma progesterone and testosterone concentrations

No. of host mice	No. of fetuses per host	No. of blastocysts transferred to host	No. of implantation sites/host	Mean placental wt in litter (mg)	Mean fetal wt in litter (mg)	Progesterone conc. (nmol/l)	Testosterone conc. (nmol/l)
17	1	2.41 ±0.64	1.24 ±0.11	179.5 ±6.8	831.2 ±40.37	127.9 ±7.02 ^a (16)	0.89 ±0.07 ^c (8)
23	2-5	5.00 ±0.58	4.26 ±0.18	144.3 ±3.7	756.04 ±24.04	164.4 ±9.61 ^b	1.38 ±0.14 ^d (8)
12	6-8	11.00 ±0.89	9.00 ±0.52	124.0 ±2.7	735.75 ±33.08	155.03 ±11.39	2.06 ±0.23 ^e (5)
11	9-14	14.00 ±0.83	12.64 ±0.68	114.0 ±3.6	689.82 ±32.78	173.79 ±16.5 ^b	2.39 ±0.27 ^f (5)

Values are mean ± s.e.m.

Figure in parentheses is the number of mice if different from that in column 1.

Analysis of variance established significant differences between the groups (progesterone, $P < 0.05$; testosterone, $P < 0.001$), and pairs of groups were subsequently compared using error estimates from the analysis of variance: a-b, $P < 0.05$; c-d, $P < 0.01$; d-f, $P < 0.01$; c-e, $P < 0.001$; c-f, $P < 0.001$.

Placentae from 13 mice with litters of 1–6 were homogenized in ice-cold acetone (5 ml/g) plus ice-cold ether (1 ml/g) and the tissue extracts were kept at $+4^{\circ}\text{C}$ overnight and centrifuged. The tissue residue was washed with acetone plus ether, air dried at room temperature and kept at -20°C until analysed. The acetone–ether precipitates were then dissolved in saline (9 g NaCl/l), left overnight at $+4^{\circ}\text{C}$ and centrifuged. The saline extracts were then assayed by a solid-phase RIA (Wide, 1969). The reagents were ^{125}I -labelled hCG and a rabbit hCG-antiserum coupled to CNBr-activated Sephadex. The samples were incubated with the solid phase-coupled antibodies for 6 days at room temperature and subsequently for 1 day with addition of labelled antigen. The unknown samples were tested in 5 or 6 concentrations with a log dose interval of 0.301 and in duplicates at each dose. A highly purified preparation of hCG was used as a standard: 1 ng hCG corresponded to 5 arbitrary units (U_{arb}) of mouse CG (Wide & Wide, 1979).

Results

The weight of ovaries on the implantation side of the 63 mice was 9.48 ± 0.27 mg (mean $\pm$ s.e.m.), and on the contralateral side 8.77 ± 0.24 mg (paired $t = -1.8$; $P > 0.05$). There was no significant difference between the mean volumes of corpora lutea (CL) in ovaries on the implantation side (0.350 ± 0.79 mm³), and those of CL in the other ovary (0.354 ± 0.008 mm³; paired $t = 0.692$; $P > 0.4$). There was no correlation between the combined weights of both ovaries and fetal number (intercept = 17.65; slope = 0.13; $r = 0.19$; $P > 0.1$). The fetal and placental weights (Table 1) were within the normal range for our colony of mice.

There was no correlation between the number of CL/mouse and plasma progesterone concentrations (Text-fig. 1a). Analysis of variance showed that the concentration of plasma progesterone in mice with 1 fetus was significantly less than that in mice with 2–5 and 9–14 fetuses

Text-fig. 1. The relationship between plasma concentrations of (a) progesterone and (b) testosterone and the number of corpora lutea in the pregnant mouse. In (a) intercept = 142.9, slope = 0.84, $r = 0.07$, $P > 0.6$, and in (b) intercept = 1.47, slope = 0, $r = -0.01$, $P > 0.9$.

Text-fig. 2. The relationship between plasma concentrations of (a) progesterone and (b) testosterone and the number of fetuses in the litter in the mouse. In (b) intercept = 0.84, slope = 0.15, $r = 0.78$, $P < 0.001$.

Text-fig. 3. The relationship between placental CG content/litter and number of fetuses per mouse. Intercept = 0.58, slope = 1.96, $r = 0.90$, $P < 0.001$.

(Table 1; Text-fig. 2a). There was no correlation between the number of CL/mouse and plasma testosterone concentrations (Text-fig. 1b) but the correlation between plasma testosterone values and fetal number was significant (Table 1; Text-fig. 2b). The mean chorionic gonadotrophin content was 1.67 ± 0.15 ng/placenta, equivalent to $8.35 U_{arb}$ mouse CG (Wide & Wide, 1979). The

total amount of chorionic gonadotrophin in all placentae in each of the 13 litters varied directly with the number present (range 1–6) (Text-fig. 3), but the placental CG value was not related to the plasma progesterone concentration in these 13 mice (intercept = 134.46; slope = 2.33; $r = 0.24$; $P > 0.4$).

To see whether there was any effect of the total number of CL/mouse, or of conceptuses on the mean CL volume/mouse, a multiple regression was done using the data shown in Text-fig. 4. The

Text-fig. 4. The relationship between the average volume of corpora lutea per mouse and the number of corpora lutea per mouse.

Table 2. Multiple regression analysis showing the effect of the number of corpora lutea/mouse, and of the number of conceptuses on the mean volume of the corpora lutea/mouse (mm³) (y)

Term	Estimate	Standard error	Significance
Intercept of line on y axis	0.47765		
Effect of number of fetuses (partial regression coefficient b_1)	0.00726	0.00152	$P < 0.001$
Effect of no. of CL/mouse (partial regression coefficient b_2)	-0.01175	0.00187	$P < 0.001$

Text-fig. 5. The relationship between the number of corpora lutea and the mean volume of corpora lutea in each ovary of mice with unilateral implantations. Intercept = -0.0053 , slope = 0.021 , $r = 0.1747$, $P > 0.2$.

results (Table 2) show that both factors have a small but significant correlation with mean CL volume/mouse. For example, in a mouse with 7 fetuses and 13 CL we can predict that the mean volume of the CL would be 0.376 mm^3 . If the number of fetuses was increased by 1 the mean volume would be 0.383 mm^3 , an increase of 1.9%. If, instead, the number of CL was increased to 14, the mean volume would be reduced to 0.364 mm^3 , a decrease of 3.1%. The multiple regression analysis was repeated after the data from two mice with 23 and 27 CL (Text-fig. 4) had been excluded, since such large numbers are unusual. The significance levels were only marginally increased, and our conclusions are unaffected.

To see whether there was any correlation between the number and mean volume of CL in each individual ovary of the mouse we plotted the difference in the number of CL on the implanted and unimplanted sides against the corresponding differences in their mean volumes. There was no correlation (Text-fig. 5). There is no evidence that within each mouse the mean CL volume is less in the ovary with the greater number of CL than in the ovary with the smaller number.

Discussion

We have shown that concentrations of plasma progesterone on Day 17 of pregnancy are independent of fetal numbers in our strain of mice when more than 1 fetus is present. In this strain the

gestation period is related to litter size: in 1246 dated mouse pregnancies 60% of litters with one fetus, but < 18% of those with 8 or more fetuses were born after the 20th day of pregnancy (Dewar, 1968). Our results cannot exclude the possibility that a correlation between litter size and progesterone levels existed earlier in pregnancy. By Day 17 progesterone levels in mice with larger litters might have already declined. The period of functional activity of the mouse corpus luteum lasts from the 8th to the 16th day of gestation, i.e. up to 3 days before parturition, and from the 18th day the corpus luteum accumulates fat and gradually shrinks (Deanesly, 1930). Soares & Talamantes (1983) showed that the level of serum progesterone correlated with litter size on Day 15 of pregnancy in C3H mice in which fetuses were destroyed on Day 7 to adjust litter sizes to groups of 1–2, 3–4 or 8–10. However Simon *et al.* (1978), using Rockland Swiss mice, found that neither the numbers of corpora lutea nor fetuses correlated with levels of plasma progesterone on Day 15 of pregnancy. These differences may be due to the strains of mice used.

It has been suggested that the mouse placenta synthesizes progesterone during the second half of gestation, and that at least part of this progesterone is secreted into the maternal circulation, although the contribution of the placenta to overall progesterone concentration is very low compared with that of the ovary (Pointis, Rao, Latreille, Mignot & Cedard, 1981). In the mouse, removal of the ovaries or corpora lutea leads to the termination of pregnancy (Parkes, 1928; Newton & Lits, 1938). Progesterone and oestrogen are necessary for the maintenance of gestation (Robson, 1938a, b; Jaitly, Robson, Sullivan & Wilson, 1966). Hypophysectomy after the 10th day does not interrupt pregnancy, and the trophic support of the ovary thereafter is dependent upon the presence of the placenta (Mirskaia, 1929; Selye, Collip & Thomson, 1933; Newton & Beck, 1939).

There are two placental hormones which may maintain the production of steroid by the ovary. Mouse placental lactogen is positively correlated with litter size (Markoff & Talamantes, 1981) and may play a part in the maintenance of luteal cell LH receptors as has been suggested in the rat (Gibori & Richards, 1978). The role of endogenous CG in the mouse is not known but in the rat the injection of hCG stimulates the production of both oestradiol and testosterone, although it has no effect on ovarian progesterone secretion. *In vitro*, hCG activates the enzymes involved in the conversion of progesterone to testosterone (Kalison & Gibori, 1983).

We have shown that the amount of CG is related to the number of fetuses in the litter, and this is undoubtedly a function of total placental mass. The amount of CG per placenta in our strain of mice at Day 17 of gestation is similar to that found by Wide & Wide (1979) in the NMRI strain. The amount of mouse CG is not related to the mean plasma progesterone concentration.

Both placental CG and plasma testosterone concentration increase with increasing litter size, and the CG may stimulate the production of testosterone by the mouse ovary. In the rat the ovaries secrete only a limited amount of testosterone in the second half of pregnancy, when the conceptuses become an important source of testosterone (Sridaran, Basuray & Gibori, 1981), and a similar situation may exist in the mouse.

Bartholomeusz & Bruce (1976) showed that mean corpus luteum weight per rat was negatively related to the number of corpora lutea per rat. We have shown the same for luteal volume in the mouse, and that there is a positive correlation between the number of conceptuses and mean CL volume, suggesting a trophic effect of the conceptuses on the corpora lutea.

In our mice there was no local effect of 1 or 2 fetuses on the weight of the ipsilateral ovary, as was found in the rat on Day 18 of pregnancy (Zambrana & Greenwald, 1971), nor was there any unilateral effect of the number of fetuses on the volume of the corpora lutea. The effect of the conceptuses on the ovaries was therefore systemic.

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