Advancement of meiotic resumption in Graafian follicles by LH in relation to preovulatory ageing of rat oocytes

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After ovulation, the fertile life of oocytes is short. In the present study, the fertile life of oocytes was studied in relation to the resumption of meiosis. Early on the day of pro-oestrus, meiotic resumption was advanced in rats by a brief infusion of LH; ovulation was induced 8 h later by Ovalyse, a GnRH analogue; rats were mated 13 h after receiving Ovalyse, that is at about the time of ovulation. Rats in which meiosis was not advanced were injected with Ovalyse and mated at various intervals after ovulation. Rats were killed 13 h after mating or on day 20 of pregnancy. In rats in which meiosis was not advanced that were mated around ovulation, fetal survival was about 90%. In rats with meiosis advanced by 8 h and mated around ovulation, only 44% of the ovulated oocytes with advanced meiosis developed into healthy fetuses; mortality before and after implantation was 37 and 19%, respectively. Rats in which meiosis was not advanced that were mated 8–9 h after ovulation had similar fetal survival and similar mortality before and after implantation. Thus ageing of the oocyte may occur either before, or after, ovulation. Preovulatory ageing is related to the resumption of meiosis.

Introduction

The fertile life of oocytes is the period during which they can give rise to a normal fetus. After ovulation, this period is relatively short (see review by Austin, 1970). In rats, ovulation occurs 3–5 h after the oocyte has reached metaphase II stage (Tsafiriri and Kraicer, 1972). Blandau and Jordan (1941) found that in rats if fertilization occurred 10 h after ovulation, then normal young were delivered in only 21% of resulting pregnancies and that litter size was significantly reduced. Meiotic resumption and ovulation are both induced by the gonadotrophin surge on the day of pro-oestrus (see review by Thibault and Levasseur, 1988). We showed that FSH given in combination with Nembutal advances meiotic resumption with respect to ovulation and that advancing meiotic resumption has a deleterious effect on fertility (Mattheij *et al.*, 1993). Under physiological conditions, LH may trigger meiotic resumption (see review by Lipner, 1988). In the present study, dissociation of meiotic resumption from ovulation was induced by a brief infusion of LH. Embryonic mortality was measured after the advancement of meiosis, and compared with mortality after postovulatory ageing of oocytes.

Materials and Methods

Animals

Virgin F₁ hybrids of two Wistar substrains (U-inbred males and R-inbred females) aged 3–4 months (to avoid age-related

effects on fertility: Mattheij and Swarts, 1991) were housed individually under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$) and photoperiod (14 h light:10 h dark; lights on at 03:00 h) with free access to drinking water and standard food pellets. Oestrous cyclicity was monitored daily by vaginal lavage and observation of oestrous behaviour, for which a male was briefly allowed into each cage at 16:00 h. Only rats with regular 5-day cycles were used. Rats in which meiotic resumption was advanced were fitted with a jugular cannula (Mattheij and Van Pijkeren, 1977). Rats in which LH was to be measured during infusion of LH had cannulae fitted into a branch of both jugular veins. Oestrous cyclicity in cannulated rats was assessed from the day after surgery. Advancement of ovulation by 1 day does not induce embryonic or fetal mortality (Mattheij *et al.*, 1986). Under the light cycle used, the pro-oestrus gonadotrophin surge begins around 12:00 h (Mattheij *et al.*, 1992). Metaphase stage II is reached 8–10 h after LH injection and most ovulations occur 13–14 h after LH (Mattheij *et al.*, 1992).

Hormone treatment

Rat LH used was rLH-I-9 (AFP-10250C). Contamination with rFSH as determined by radioimmunoassay was less than 0.1%. The infusion solution ($840 \mu\text{g rLH-I-9 l}^{-1}$) was prepared in saline containing 10 g BSA l^{-1} , radioimmunoassay grade: Sigma, St Louis, MO. Infusions were made with a Harvard pump (type 975A) at a rate of 1.0 ml h^{-1} . Ovalyse (des-Gly¹⁰-GnRH-ethylamide: Upjohn, Ede) was also dissolved in saline containing BSA.

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Radioimmunoassay

Blood samples (150 μ l) were collected into heparinized tubes on ice. Plasma (50 μ l) was diluted with two volumes of phosphate-buffered saline (pH 7.5) and stored at -25°C . LH was measured with the kit provided by NIDDK: rLH-I-6 was labelled with ^{125}I ; anti-rLH-S10 was used as first antibody; rLH RP2 diluted in serum of hypophysectomized rats was used as reference material. The second antibody was donkey-anti-rabbit (Sac-cel, Wellcome Reagents, Beckenham). Variation within and between assays in relative standard deviation was 5.3 and 11.2%, respectively. The detection level of the assay was 0.1 ng per tube at binding ratio (B:B₀) 90%.

Experiments

After a single injection of LH, meiotic resumption is triggered by a lower dose than is necessary for ovulation; however, subsequent ovulation is induced in only a few oocytes (Mattheij *et al.*, 1993). In the present study, LH was administered by infusion which was started at 03:30 h on the day of pro-oestrus.

Preliminary experiments. The dose of LH infused was varied between 6 and 14 ng min⁻¹, and the duration of infusion was varied between 30 min and 2 h to find an LH dose that induced meiotic resumption but not ovulation. Rats were killed by cervical dislocation under ether anaesthesia 10 h or 18 h after the beginning of the infusion and the ovaries and oviducts examined histologically.

Experiment 1a. Plasma LH was measured during and after the infusion of 14 ng LH min⁻¹ for 50 min. Blood, 150 μ l, was collected before infusion was started, at 10 min intervals during, and at 10 and 30 min after infusion had stopped. In rats not receiving LH infusion, blood was taken at 30 min intervals between 10:00 h and 13:00 h, to assess whether LH infusion affected the time of onset of the LH surge on the day of pro-oestrus.

Experiment 1b. Meiotic resumption was measured after 50 min of LH infusion (14 ng min⁻¹). Rats were killed 10 h or 18 h after the beginning of infusion. Ovaries and oviducts were collected for histological examination.

Experiment 1c. The ability of follicles with advanced meiosis to undergo ovulation was determined. Rats were infused with LH (14 ng min⁻¹) beginning at 03:30 h on the day of pro-oestrus, and 100 ng of the GnRH analogue Ovalyse (Upjohn Company, Ede) was injected i.p. 8 h later. Ovalyse injected during pro-oestrus induces a considerable increase in plasma LH lasting 3 h and results in the ovulation of virtually all Graafian follicles (Mattheij *et al.*, 1992). Rats were killed 10, 16 or 25 h after the injection of Ovalyse and the ovaries and oviducts collected for histological examination.

Experiment 2. Embryonic mortality was assessed in rats in which meiotic resumption had been advanced by 8 h. Starting at 03:30 h on the day of pro-oestrus, LH (14 ng min⁻¹) was infused for 50 min. Ovalyse (100 ng) was given at 11:30 h and

the rats mated with males of proven fertility 13 h after Ovalyse injection. Ovulation was assumed to have occurred 13–14 h after Ovalyse injection (Mattheij *et al.*, 1992) and fertilization of oocytes was assumed to begin 2 h after copulation (Austin and Braden, 1954). Copulation was usually observed within 10 min of mating; so oocytes were fertilized 1–2 h after ovulation. Controls received no LH infusion, but were injected with Ovalyse at 11:30 h during pro-oestrus, and were mated 13 h after Ovalyse injection. Groups of controls and of rats with advanced meiotic resumption were killed either 13 h after mating and examined histologically for fertilization, or on day 20 of pregnancy for assessment of embryonic mortality and fetal survival.

Experiment 3. Embryonic mortality was also measured after postovulatory ageing of oocytes. Rats were injected with Ovalyse (100 ng) at 11:30 h on the day of pro-oestrus. Males were allowed to copulate with females 16, 18, 20 or 22 h after Ovalyse injection. Some females caged with males 20 h after Ovalyse injection did not copulate and about 50% of the females caged with males 22 h after Ovalyse injection did not copulate. Rats were only included if at least five copulations were seen within 30 min of mating; so fertilization occurred 4–5 h, 6–7 h, 8–9 h or 10–11 h after ovulation, respectively. The rats were killed on day 20 of pregnancy and the ovaries and uterine horns were examined.

Histology and dissection

Ovaries with oviducts were fixed in Bouin's solution and embedded in paraffin wax. Serial sections (10 μ m thick) were stained with haematoxylin and eosin. Healthy follicles (exceeding 600 μ m in diameter) and ovulated follicles from both ovaries of each rat were studied; atretic follicles were not considered.

Ovaries and uterine horns from rats killed on day 20 of pregnancy were studied with a dissection microscope. On day 20 of pregnancy, corpora lutea could not be distinguished from luteinized, unruptured follicles. For each rat in Expt 2, the number of corpora lutea representing ovulated oocytes was calculated after subtracting the average percentage of luteinized, unruptured follicles found in the group in Expt 1c killed 16–25 h after Ovalyse injection. No correction for luteinized, unruptured follicles was made in Expt 3, as there had been no infusion of LH (Mattheij and Swarts, 1991). Fetuses of normal size were classified as living. Large, dead embryos or dead fetuses were rarely seen. Postimplantation mortality was assessed by counting deciduae without an embryo or with a dead embryo. Preimplantation loss was calculated by subtracting living fetuses and postimplantation mortality from the number of corpora lutea per rat. Mortality during early implantation was considered as preimplantation loss because no decidua would be found if an embryo died during early implantation.

Statistical analysis

In Expts 2 and 3, fetal survival and embryonic mortality were calculated relative to the numbers of corpora lutea. In rats with meiotic resumption advanced by 8 h, the number of

Table 1. Ovulation rate in rats infused with LH for 50 min from 03:30 h on the day of pro-oestrus at various intervals after Ovalyse was given at 11:30 h

Interval (h) from Ovalyse to section	Number of rats	Number of follicles	Number of ruptured follicles	Percentage of follicles ovulating
10	8	13.2 ± 0.8	0.5 ± 0.3	3.5 ± 2.0
16	7	12.2 ± 0.5	7.6 ± 1.0	62.5 ± 8.0
25	12	12.8 ± 0.5	7.3 ± 0.5	57.3 ± 3.8
16 plus 25	19	12.6 ± 0.4	7.5 ± 0.5	59.2 ± 4.3

Values are means ± SEM.

corpora lutea corresponding to oocytes with advanced meiosis was calculated after correction for luteinized, unruptured follicles and for oocytes with unadvanced meiosis. Statistical significance of fetal survival between the groups was calculated with the Mann-Whitney test (Snedecor and Cochran, 1989). Data are reported as means ± SEM.

Results

Preliminary experiments

Infusion of 6 ng LH min⁻¹ for 2 h induced some expansion of the cumulus but not meiotic resumption. Infusion of 10, 12 or 14 ng LH min⁻¹ for 2 h caused meiotic resumption in nearly all Graafian follicles. Infusion of 10 or 12 ng LH min⁻¹ for 60 min caused meiotic resumption in 29 and 75% of oocytes, respectively. Infusion of 14 ng LH min⁻¹ for 30 min did not induce meiotic resumption, but infusion for 40, 50 or 60 min caused meiotic resumption in 53, 85 and 95% of Graafian follicles, respectively; ovulation did not occur 18 h after infusion of 14 ng LH min⁻¹ for 50 min, whereas ovulation sometimes occurred 18 h after LH had been infused for 60 min. On the basis of these data, 14 ng LH min⁻¹ for 50 min was chosen as the dose to dissociate meiosis from ovulation in the following experiments.

Experiment 1a

Plasma LH was 0.6 ± 0.1 µg l⁻¹ before the beginning of LH infusion at 03:30 h during pro-oestrus (number of rats, *n* = 16). After 20 min infusion, plasma LH had increased to 10.3 ± 0.7 µg l⁻¹ and was maintained at 10.9 ± 0.6 µg l⁻¹ during the remaining 30 min of infusion. Ten minutes after the LH infusion was stopped, plasma LH was 3.9 ± 0.6 µg l⁻¹, falling to 1.9 ± 0.6 µg l⁻¹ 25 min later. After infusion of LH for 50 min (*n* = 9), plasma LH was 0.8 ± 0.1 µg l⁻¹ at 10:00, 10:30 and 11:00 h. A slight increase of LH was observed in four rats at 11:30 h and 12:00 h, resulting in an average plasma LH concentration of 1.0 ± 0.1 and 1.2 ± 0.2 µg l⁻¹ at 11:30 h and 12:00 h, respectively. A significant rise in plasma LH was measured at 12:30 and 13:00 h: 1.8 ± 0.3 and 5.7 ± 1.5 µg l⁻¹, respectively.

Experiment 1b

Ovaries from rats killed 10 h after infusion of LH (*n* = 13) contained 13.2 ± 0.8 Graafian follicles; 86.5 ± 4.5% of these

follicles showed expanded cumulus cells surrounding an oocyte in metaphase stage II. Ovaries from rats killed 18 h after LH infusion (*n* = 7) contained 12.0 ± 0.3 Graafian follicles, but no ruptured follicles; of the large follicles, 98.8 ± 1.1% showed an oocyte in metaphase stage II surrounded by an expanded cumulus mass. This result indicated that the endogenous LH surge had triggered meiotic resumption in virtually all remaining Graafian follicles. Follicles that did not resume meiosis (1.2%) were excluded from subsequent experiments. In several of the large follicles, the basal lamina between the granulosa and theca cells had partly disappeared.

Experiment 1c

When Ovalyse was given 8 h after the beginning of the LH infusion, few ovulations had occurred 10 h after the injection of Ovalyse (Table 1). Ovulation rates were similar 16 and 25 h after Ovalyse injection, so rats killed at these times were considered as one group (*n* = 19), in which Ovalyse caused 59.2 ± 4.3% ovulations per rat. Unovulated follicles in this group had large antra and contained metaphase stage II oocytes surrounded by strongly expanded cumulus cells. In the walls of these follicles, the basal lamina between the granulosa and theca layers had disappeared; capillaries were seen penetrating the granulosa layer. These follicles (40.8 ± 4.3% of the follicles per rat) were considered as luteinized, unruptured follicles.

Experiment 2

In controls killed 13 h after mating (*n* = 10), the ovulation rate was 98.3 ± 1.3%; almost all oocytes in the oviducts were fertilized. In rats in which meiotic resumption had been advanced by 8 h and that were killed 13 h after mating (*n* = 9), ovulation rate was 64.6 ± 5.8% and 97.7 ± 1.5% of these oocytes contained a male and a female pronucleus. Data obtained from rats killed on day 20 of pregnancy are summarized (Table 2, Fig. 1a). Rats that received Ovalyse but no LH infusion had less than 10% embryonic mortality. In rats infused with LH and injected with Ovalyse 8 h later, 40.8% of the corpora lutea were luteinized, unruptured follicles (see Expt 1c) and 13.5% were from Graafian follicles with unadvanced meiosis (see Expt 1b). The number of corpora lutea corresponding to ovulated oocytes with advanced meiosis was: [12.5 - 6.6 = 5.7 (100% - (40.8 + 13.5)%)]. Oocytes with

Table 2. Effect of advancing meiotic resumption by 8 h on embryonic mortality and fetal survival. Rats with unadvanced meiotic resumption received Ovalyse at 11:30 h on the day of pro-oestrus, and were mated 13 h later. In the rats with advanced meiotic resumption, Ovalyse was preceded by an infusion of LH lasting 50 min, beginning at 03:30 h

Meiotic resumption	Number of rats	Interval from meiotic resumption to fertilization (h)	Interval from ovulation to fertilization (h)	Corpora lutea	Dead embryos		Living fetuses
					Preimplantation	Postimplantation	
Not advanced	14	15	1–2	12.9 ± 0.4 (100)	0.8 ± 0.4 (6.2 ± 3.1)	0.4 ± 0.1 (3.1 ± 0.8)	11.7 ± 0.4 (90.7 ± 3.1)
Advanced	22	23	1–2	12.5 ± 0.4	nc	1.2 ± 0.3	4.0 ± 0.6
Advanced; data corrected for luteinized, unruptured follicle and non-advanced meiosis	22	23	1–2	5.7 ± 0.2 (100)	2.1 ± 0.7 (36.8 ± 12.1)	1.1 ± 0.3 (20.0 ± 4.5)	2.5 ± 0.4 (43.2 ± 10.9)

Values are means ± SEM of numbers and percentage of corpora lutea in parentheses.
nc: Not possible to calculate from uncorrected data.

Table 3. Effect of postovulatory ageing of oocytes on embryonic mortality and fetal survival. Ovulation was induced by Ovalyse at 11:30 h on the day of pro-oestrus

Interval from Ovalyse to mating (h)	Number of rats	Interval from meiotic resumption to fertilization (h)	Interval from ovulation to fertilization (h)	Number of corpora lutea	Dead embryos ^a		Living fetuses ^a
					Preimplantation	Postimplantation	
16	10	18	4–5	12.8 ± 0.5	0.4 ± 0.3 (3.1 ± 2.3)	1.3 ± 0.5 (10.2 ± 4.1)	11.1 ± 0.7 (86.7 ± 4.7)
18	12	20	6–7	13.0 ± 0.4	1.3 ± 0.5 (10.0 ± 4.0)	2.8 ± 0.6 (21.5 ± 5.1)	8.9 ± 1.0 (68.5 ± 6.6)
20	15	22	8–9	12.9 ± 0.4	4.7 ± 0.8 (36.4 ± 5.9)	2.8 ± 0.4 (21.7 ± 3.2)	5.4 ± 0.7 (41.9 ± 5.6)
22	12	24	10–11	12.6 ± 0.3	8.2 ± 1.0 (65.1 ± 8.0)	1.6 ± 0.5 (12.7 ± 4.0)	2.8 ± 1.0 (22.2 ± 8.2)

^aDead embryos and living fetuses are expressed as numbers and as a percentage of corpora lutea in parentheses.

unadvanced meiosis (13.5%) showed similar fetal survival and embryonic mortality to oocytes in the control group, that is 90.7% of these 13.5% oocytes presumably developed into surviving fetuses, whereas 6.2 and 3.1% of these 13.5% oocytes resulted in preimplantation and postimplantation mortality, respectively. Fetal survival and embryonic mortality therefore corresponding to 5.7 corpora lutea from follicles with advanced meiosis (Table 2) were calculated as follows: fetal survival: $4.0 - (13.5\% \times 90.7\%) \times 12.5 = 2.5$; postimplantation mortality: $1.2 - (3.1\% \times 13.5\%) \times 12.5 = 1.1$; preimplantation mortality: $2.1 - (6.2\% \times 13.5\%) \times 12.5 = 2.1$. Fetal survival was significantly lower in rats with meiosis resumption advanced by 8 h ($P < 0.01$).

Experiment 3

The results of the effect of postovulatory oocyte ageing on embryo mortality are summarized (Table 3, Fig. 1b). Compared with the controls of Expt 2, a significant decrease in fetal

survival was measured if fertilization occurred 6–7 h after ovulation ($P < 0.05$). With greater intervals between ovulation and fertilization, a further reduction in fetal survival was measured ($P < 0.01$). This reduction in fertility was mainly attributed to preimplantation mortality.

Discussion

In laboratory rats, the pro-oestrus LH surge usually begins 2 h after the midpoint of the daily light period (Everett *et al.*, 1949), that is around 12:00 h in this study. Infusion of 14 ng LH min^{-1} for 50 min mimicked a brief LH surge of similar magnitude to that reached during the endogenous surge (Mattheij *et al.*, 1992). This endogenous surge on the day of pro-oestrus induces a transient increase in plasma progesterone (Hashimoto *et al.*, 1987); infusion of LH during early pro-oestrus may therefore also have caused a transient rise in plasma progesterone. Prior administration of progesterone may advance the LH surge on the day of pro-oestrus (Brown-Grant

Fig. 1. Embryonic mortality and fetal survival in rats (a) after meiotic resumption has been advanced by 8 h and (b) after increasing the interval between ovulation and fertilization. Data are expressed as means $\pm$ SEM relative to the number of corpora lutea. Data from the rats with meiosis advanced by 8 h were corrected for luteinized, unruptured follicles and for follicles with meiosis not advanced (Table 3, last line). MR: meiotic resumption; MR-Fert: interval from meiotic resumption to fertilization; Ov-Fert: interval from ovulation to fertilization. (▨) Preimplantation mortality; (■) postimplantation mortality; (□) fetal survival.

and Naftolin, 1972; Blake, 1977). However, in the present study, the assumed rise of plasma progesterone caused by LH infusion may have been too weak and endogenous LH release was not advanced; so Ovalyse was given at 11:30 h before the beginning of the surge.

Infusion of LH for 50 min caused meiotic resumption but not ovulation. This finding supports the view that less gonadotrophin is required to trigger oocyte maturation than for induction of ovulation (Vermeiden and Zeilmaker, 1974; Bomsel-Helmreich *et al.*, 1989; Mattheij *et al.*, 1993). This dose of LH also resulted in the disappearance of the basal lamina between theca and granulosa cells in many Graafian follicles; this is a morphological characteristic of incipient luteinization (Bjersing, 1978). When luteinization of a follicle had proceeded beyond a certain stage, the follicle no longer responded to an 'ovulatory' dose of Ovalyse and differentiated into a luteinized, unruptured follicle. Luteinization affects steroid synthesis in Graafian follicles (Gore-Langton and Armstrong, 1988); steroid hormones may have a role in ovulation (Tsafiri *et al.*, 1987; LeMaire, 1989). A change in steroid synthesis may therefore inhibit the ability of Graafian follicles to ovulate.

After Ovalyse injection preceded by LH infusion, few ovulations had occurred 10 h later, whereas all follicles with ovulation capability had ovulated 16 h after Ovalyse injection. When injection of Ovalyse was not preceded by LH infusion, most follicles had ovulated 14 h later, and ovulation was completed 16 h after the Ovalyse injection (Mattheij *et al.*, 1992). Infusion of LH followed by Ovalyse injection does not therefore markedly affect the timing of ovulation.

When Ovalyse injection was not preceded by LH infusion, a mean of 11.7 living fetuses were found. If Ovalyse injection was preceded by LH infusion, there was a dramatic reduction in fertility. This was partly due to about 40% of corpora lutea becoming luteinized. Even after correction for this luteinization and for fetuses originating from unadvanced oocytes, mortality was still high. Infusion with LH might cause a slight asynchrony between embryo 'age' and secretory 'age' of the

oviduct and uterus. The embryo may compensate for a minor asynchrony by a quicker development (Shalgi *et al.*, 1985). The high mortality after LH infusion is therefore caused by advancing meiotic resumption by 8 h, which induces ageing of oocytes before ovulation.

In Expts 2 and 3, the interval between meiotic resumption and the start of fertilization can be estimated. In rats with meiosis advanced by 8 h, the interval between the beginning of infusion with LH and fertilization was 23 h; in the preliminary experiments, infusion of 14 ng LH min⁻¹ for 30 min did not trigger meiotic resumption; the interval between meiotic resumption and fertilization was therefore about 22.5 h. In rats mated 8 h after Ovalyse injection, the interval between meiotic resumption and ovulation was 22 h. Rats with meiosis advanced by 8 h therefore had a similar interval between meiotic resumption and fertilization to rats mated 8 h after Ovalyse injection; these two groups of rats had a similar embryonic mortality. Ageing of the oocytes could have been triggered at the resumption of meiosis and continued after ovulation. In rats with meiosis advanced by 8 h, almost all of the ova had a male and female pronucleus 13 h after mating. Thus, the observed preimplantation loss was not caused by reduced fertilization but by post-fertilization defects.

It is not known whether preovulatory oocyte ageing occurs under natural physiological conditions. A weak LH surge on the day of pro-oestrus might dissociate meiotic resumption from ovulation. This finding supports the view that a premature preovulatory surge of LH causes reduced fertility in women (Stanger and Yovich, 1985; Howles *et al.*, 1986; Jacobs and Homburg, 1990). Recent studies indicate the existence of an ovarian factor that is a functional antagonist of GnRH (Koppelaar *et al.*, 1992; Messinis and Templeton, 1991). The physiological significance of this factor may be that it contributes to a steep increase in LH during pro-oestrus and prevents a weak LH surge that should start meiotic resumption too early with respect to ovulation. Various studies have indicated that stress affects LH secretion (see review by Grossman and Besser,

1988; Rivier and Rivest, 1991). Currently, we are investigating whether stress induces preovulatory ageing of oocytes through partial inhibition of the LH surge at pro-oestrus.

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