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# Ovulation response and fertilization failure in immature rats induced to superovulate

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**Summary.** Immature female rats (75 g body wt, aged 29 days) were injected with 4 or 40 i.u. PMSG on Day −2 and were killed at intervals between 18:00 h on Day −2 and 09:00 h on Day 1. Control animals (4 i.u.) ovulated between 00:30 and 05:30 h on Day 1 whereas the number of ova recovered from superovulated rats (40 i.u. PMSG) increased slowly between 06:00 h on Day −1 and 24:00 h on Day 0 and markedly between 24:00 on Day 0 and 06:00 on Day 1.

Similarly treated rats were caged overnight on Day 0 with males of proven fertility and killed between 14:00 and 16:00 h on Day 1. A significantly lower percentage of normal 1-cell ova was recovered from the superovulated rats compared to control animals (71·6 and 98·5%) and of these 1-cell ova a lower percentage was fertilized (69·7 and 99·1%). In the control group all mated animals had a high proportion of ova fertilized whereas 26% of superovulated rats had none or a very low proportion fertilized. In the control animals there was evidence of sperm penetration and pronucleus formation; in superovulated rats significantly fewer ova had pronuclei than were penetrated.

These results suggest that reduced fertility of superovulated immature rats is due to complete or partial failure of fertilization in some animals. The extended period during which ovulation occurs may be a contributory factor.

## Introduction

The use of superovulatory doses of gonadotrophin may result in reduced fertility in large domestic animals (du Mesnil du Buisson, Renard & Levasseur, 1977; Evans & Robinson, 1980) and in small laboratory animals (Beaumont & Smith, 1975; Miller & Armstrong, 1981a). Nuti, Sridharan & Meyer (1975) have shown that ovulation induced in immature rats by a single injection of pregnant mare serum gonadotrophin (PMSG), can be followed by normal pregnancy but superovulatory doses of PMSG result in partial or complete infertility (Miller & Armstrong, 1981a).

Since a large number of ova can be recovered on Day 1 of pregnancy from the oviducts of immature rats induced to superovulate, the ovulatory process is probably not defective (Austin, 1950; Walton & Armstrong, 1981). Between Days 1 and 2 there is a substantial reduction in the number of ova and/or embryos recovered from the reproductive tracts of these animals and this reduction may be partly accounted for by the loss of degenerate ova ovulated 24 h earlier than the normal time of ovulation (Miller & Armstrong, 1981b; Walton & Armstrong, 1981). In these two studies no attempt was made to determine the exact proportions of 1-cell, 2-cell and degenerating ova on Days 1 and 2. It was therefore of interest to determine whether the impairment of fertility in superovulated rats was due to a reduced fertilization rate or to loss of zygotes before Day 2. The

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timing of ovulation in these animals was also investigated, because asynchrony between ovulation and mating could result in reduced fertilization.

## Materials and Methods

### *Animals*

Immature female Sprague–Dawley rats were obtained from Charles River, St Constant, Quebec, at body weight 45–50 g. Animals were allowed free access to food and water; temperature and lighting (14 h light, 10 h dark; lights on 05:00–19:00 h) were controlled. At body weight 75 g ($\sim$29 days of age) rats were randomly allocated to one of two groups; before 09:00 h animals were injected with 4 i.u. PMSG (Equinex: Ayerst), a dose known to induce a physiological number of ovulations (control), or 40 i.u. PMSG (superovulated). The day of injection was designated as Day $-2$.

### *Timing of ovulation*

Animals in the control group were killed at 1-h intervals from 23:00 h on Day 0 until 05:30 h on Day 1, and at 09:00 h on Day 1. The number of ovulation points on each ovary was counted. Rats in the superovulated group were killed at 3- or 6-h intervals from 18:00 h on Day $-2$ until 09:00 h on Day 1. Since the large number of ovulations made it impossible to obtain accurate counts of ovulation points on the ovary the oviducts were flushed and recovered ova were counted. Rats from which no ova were recovered were included in the calculation of means. From 18:00 h on Day 0 to 09:00 h on Day 1 the number of freshly ovulated ova was estimated from the number of distinct masses of cumulus. Since 1-cell ova, with and without cumulus, and fragmented ova tended to become clumped in the dish, the flushings were then exposed to 0·1% hyaluronidase for 5 min and the total numbers of ova and fragmented ova were recorded; the number of 1-cell ova without cumulus was estimated by subtraction. Oocyte recovery over time was analysed by linear regression after arbitrary division into 24-h time periods (see 'Results') and the slopes of the lines were compared to zero (Steel & Torrie, 1960). The effect of time on the total number of ova, and numbers of fragmented ova or freshly ovulated cumulus masses, was compared using one-way analysis of variance and differences between times were assessed using Duncan's New Multiple Range test.

### *Fertilization study*

At 21:00 h on Day 0 the vaginae of all rats were opened with a blunt instrument and the rats were caged individually overnight with males of proven fertility. Only females that mated, as judged the following morning (Day 1) by a copulatory plug and/or spermatozoa in a vaginal smear, were included in the experiment. At 14:00 to 16:00 h on Day 1 rats were killed and the oviducts were flushed with Dulbecco's phosphate-buffered saline as described previously (Walton & Armstrong, 1981). Recovered ova were counted and classified as normal or degenerate; fragmented ova or ova with more than 1 cell were considered degenerate. One-cell ova were mounted on a slide with a coverslip suspended at the corners by four spots of vaseline (Chang, 1952) and examined without staining under a phase-contrast microscope for evidence of sperm penetration and pronucleus formation. Statistical comparisons between control and superovulated groups were made using a $\chi^2$ test with Yates' correction.

## Results

### *Time of ovulation*

All control rats ovulated between 00:30 and 05:30 h on Day 1 (Text-fig. 1). By 05:30 h 100% of large follicles visible on the ovary had ovulated and there was no statistical difference in the number

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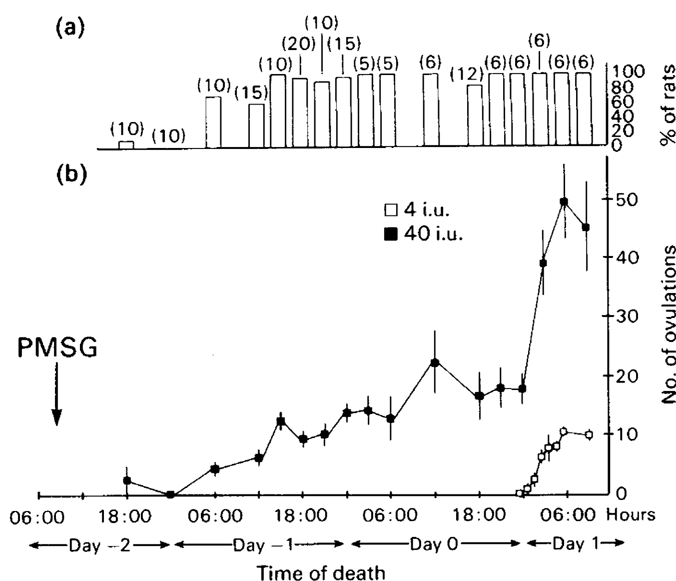
of ovulations from 03:30 to 09:00 h. The superovulated animals showed a more complex pattern of ovulations. With the exception of one rat, which had ovulated by 18:00 h on Day −2, no ovulations were observed before 06:00 h on Day −1. The majority of animals had ovulated by 15:00 h on Day −1 although no oocytes were recovered from 2/12 rats killed as late as 18:00 h on Day 0. The number of oocytes recovered from the oviducts of superovulated rats increased significantly ($P < 0{\cdot}01$) throughout Day −1 with no significant increase throughout Day 0 (Text-fig. 1). The regression of oocyte recovery on time was described by $y = 0{\cdot}52x + 1{\cdot}66$ and $y = 0{\cdot}22x + 14{\cdot}16$ for Day −1 and Day 0, respectively, where $y$ = oocyte recovery and $x$ = time. With the exception of one rat that had 2 fragmented oocytes in the oviduct at 03:00 h on Day 0 no fragmented ova were recovered before 18:00 h on Day 0. However, there were some animals in which there was very little or no cumulus mass surrounding the oocytes. The number of ova recovered varied considerably between animals, but there was a significant ($P < 0{\cdot}001$) increase in both total ova recovered and the number of cumulus masses from 24:00 h on Day 0 to 06:00 h on Day 1 (Text-fig. 2). From then onwards there was no significant increase. The number of fragmented ova did not increase significantly over this 15-h period.




**Text-fig. 1.** Timing of ovulation in immature rats induced to ovulate with 4 or 40 i.u. PMSG. (a) Percentage of rats displaying ovulation after 40 i.u. PMSG (numbers indicate the no. of rats killed each time). (b) Number of ova recovered per rat after treatment with 4 ($n$ = 4 rats/point) or 40 i.u. PMSG ($n$ = 5–20 rats/point, as in (a)). Values are mean ± s.e.m.

*Fertilization study*

Fourteen of 27 control rats mated, a significantly lower proportion ($P < 0{\cdot}01$) than 19/21 superovulated animals. The results from all mated rats are presented in Table 1. All ova classified as fertilized from control rats showed evidence of sperm penetration and pronucleus formation. Any cumulus cells attached to these ova generally dispersed during the handling and mounting procedure, whereas ova from most, but not all, superovulated rats had cumulus cells firmly attached after mounting. All mated rats in the control group displayed a high proportion of ova fertilized. Despite evidence of mating, no fertilized ova were recovered from 4 rats in the superovulated group and one further rat had only 1 fertilized ovum of 26 ovulated. Exclusion of these 5 rats from the superovulated group results still gave a significantly lower ($P < 0{\cdot}01$) percentage of one-cell ova fertilized in the superovulated group (89·3%) than in the control group (99·1%).

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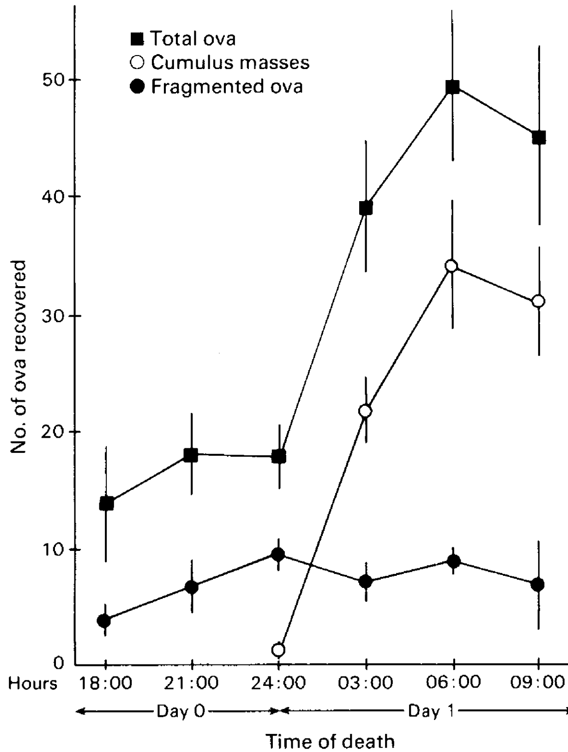


**Text-fig. 2.** Recovery of cumulus masses and fragmented ova from the oviducts of immature rats 58–73 h after induction of ovulation with 40 i.u. PMSG. Values are mean ± s.e.m. (no. of rats as in Text-fig. 1a).

**Table 1.** Ovum recovery, fertilization rates and incidence of polyspermy in immature rats induced to ovulate with 4 or 40 i.u. PMSG

| | No. of ova (%) | | |
|---|---|---|---|
| | 4 i.u. PMSG | 40 i.u. PMSG | Significance |
| Recovered | 132 | 631 | |
| Degenerate | 2 (1·5)* | 179 (28·4)* | $P < 0·001$ |
| One-cell | 130 (98·5)* | 452 (71·6)* | $P < 0·001$ |
| One-cell examined | 112 | 373 | |
| One-cell with pronuclei | 111 (99·1)† | 234 (62·7)† | $P < 0·001$ |
| One-cell with sperm tail in vitellus | 111 (99·1)† | 260 (69·7)† | $P < 0·001$ |
| One-cell with polyspermy | 4 (3·6)† | 12 (4·6)† | N.S. |

* % of recovered eggs.
† % of 1-cell eggs examined.

## Discussion

The experiments reported here demonstrate that fertilization failure, both complete and partial, is higher in 1-cell ova from immature rats induced to superovulate than in control animals. An earlier study utilizing a different superovulatory treatment regimen (Austin, 1950) had shown that 51% of

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the ova were unfertilized but in that particular study no distinction was made between unfertilized degenerate and unfertilized 1-cell ova. It was suggested that sperm transport might be a problem and that even when spermatozoa were present in the oviduct the numbers were lower than in adult animals. However, in the absence of control immature rats with normal ovulation numbers it was possible that a lower number of spermatozoa was normally present in the oviducts of the immature animals. In the present experiment, a total absence of spermatozoa in the ampulla may have been the cause of fertilization failure in rats that gave no evidence of fertilization, but even in rats in which several ova were fertilized the proportion fertilized was still significantly lower than in control animals. This reduction might be attributed to a decreased number of spermatozoa in the ampulla, genetic abnormalities in the ovum, asynchronies between maturation, ovulation and mating or a less favourable endocrine environment, either within the follicle or in the oviduct immediately after ovulation.

No published data are available on the exact timing of ovulation and subsequent fertilization in the superovulated rat. After injection of immature rats with 30 i.u. PMSG and 20 i.u. human chorionic gonadotrophin (hCG) 56 h later (Zarrow & Gallo, 1969) or a single injection of 40 i.u. PMSG (Miller & Armstrong, 1981b), two distinct populations of ova have been observed on the 3rd day after PMSG. No data appear to be available on whether these superovulatory treatments result in two distinct sets of ovulations on two consecutive nights or whether there is a period of 24 h or longer over which ovulations occur, either singly or in small groups. Indirect evidence has suggested that the first ovulation 38–46 h after PMSG is a fairly discrete event since ova recovered on Day 0 are homogeneous, apparently normal ova always surrounded by a cumulus mass (Miller & Armstrong, 1981b). However, the experiments reported here suggest that this early ovulation may occur over a considerable time period; this is not evident at death on Day 0 since degenerative changes in the oocyte are not visible at that time. Since the rise in degenerate oocytes approximately parallels, with a lag of approximately 36 h, the rise in oocytes recovered, degeneration may not occur for at least 36 h after ovulation. Therefore, the decreased fertilization seen in superovulated rats may be partly due to oocytes ovulated before the night of Day 0 to Day 1; these would still be 1-celled and apparently normal but too aged to undergo fertilization.

Due to the large variations between animals it is difficult to establish definitely what pattern of ovulation is occurring. A large number of ovulations occurs in superovulated rats at a time corresponding to that seen in control animals (between 24:00 h on Day 0 and 06:00 h on Day 1), which suggests that the lower percentage of ova displaying formation of pronuclei compared to sperm penetration is unlikely to be due to a later fertilization resulting from later ovulation. However, it is possible that, within the experimental variability, a small proportion of this large number of ovulations in superovulated rats occurred relatively late and these ova had not yet progressed to the pronuclear stage by the time of death; formation of pronuclei is complete within 10–12 h of insemination *in vitro* (Toyoda & Chang, 1974).

The actual timing of ovulation relative to the LH surge appears to be an important factor for normal fertilization. Niwa & Chang (1975), working on immature rat ova cultured *in vitro*, showed that insemination of 'early' oocytes (collected 0–4 h after hCG and before normal ovulation) or of 'late' oocytes (collected 12–14 h after ovulation) was associated with sperm penetration but the sperm head was not transformed into a male pronucleus. In the present experiment, therefore, the lower percentage of superovulated ova with pronuclei compared to sperm penetration could be explained by a failure of transformation of the sperm head to a pronucleus.

Changes in the endocrine environment, particularly elevated levels of sex steroids, occur in immature rats induced to superovulate (Walton & Armstrong, 1981). The percentage of rats mated in the control group was inexplicably low compared with 75% reported by Miller & Armstrong (1981a) in this laboratory, but nevertheless there appears to be a real difference between control (52%) and superovulated animals (90%) in the numbers mating. The role of oestrogen in the mating response in rats has long been established (Boling & Blandau, 1939). The relationship between the level of oestrogen and the intensity of the oestrous response has been studied in more detail in sheep

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(Lindsay, 1966) in which the response has been shown to be dose dependent. Concentrations of ovarian and plasma oestradiol are higher in immature superovulated rats (Walton & Armstrong, 1981) and this may result in more intense mating behaviour in these animals.

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