Induction of superovulation in DD mice at different stages of the oestrous cycle

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This study examined the developmental capacity of oocytes in DD mice after they had been injected with pregnant mares' serum gonadotrophin at different stages of the oestrous cycle. The superovulation of mature DD mice at pro-oestrus, oestrus and metoestrus resulted in a large yield of viable embryos. The proportion of abnormal embryos was highest after injection of pregnant mares' serum gonadotrophin at dioestrus. The pool of viable oocytes was most synchronized with normal development after the hormone was injected at oestrus. The results demonstrate that oocytes of different morphology could be induced to ovulate. The developmental capacity of oocytes of different morphology is discussed.

Introduction

Administration of exogenous gonadotrophins to female mammals causes superovulation, which is used as a routine procedure for producing greater yields of embryos in developmental and genetic studies. However, compared with spontaneous ovulation, superovulation is associated with a greater loss of embryos during pregnancy (Allen and McLaren, 1971; Beaumont and Smith, 1975; Miller and Armstrong, 1982; Walton *et al.*, 1983).

The mechanisms by which gonadotrophins increase the incidence of abnormal ova are unclear, but some suggestions have been made. Degenerative changes occur in some ova as a result of fertilization failure (Walton *et al.*, 1983; Evans and Armstrong, 1984), possibly owing to an imbalance in the concentrations of oestradiol and progesterone (Miller and Armstrong, 1981, 1982; Jutaro *et al.*, 1982). Injections of pregnant mares' serum gonadotrophin (PMSG) result in the appearance of a number of cumulus-free oocytes. Any abnormality of cumulus-free oocytes could result from ageing, as they had presumably ovulated in response to LH present in the injected PMSG (Murphy *et al.*, 1984). These prematurely ovulated eggs would be too old to be fertilized (Gates, 1971). Moreover, it has been shown that treatment with gonadotrophins produces genetic defects in the ova *in vivo* (Luckett and Mukherjee, 1986) and *in vitro* (Maudlin and Frazer, 1977).

However, the incidence of atresia among antral follicles is significantly reduced after injection of PMSG. Two hypotheses have been suggested: PMSG either prevents some normal follicles becoming atretic or it rescues some atretic follicles so that they revert to a normal state (Peters *et al.*, 1975; Braw and Tsafirri, 1980; Moniaux *et al.*, 1984).

Although little is known about the mechanisms that produce abnormal ova, attempts have been made to find methods to decrease the embryonic loss. It was found that administration of gonadotrophins *i.p.* increases the proportion of abnormal

ova more than if they are administered *s.c.* (Lehtonen and Kankondi, 1987). Attempts were made to synchronize the administration of gonadotrophins with the innate oestrous cycle by timing the administration of hCG to coincide with the endogenous LH surge, but these attempts did not result in better embryo survival (Beaumont and Smith, 1975).

Farm animals are induced to superovulate at a known stage of their oestrous cycle to maximize superovulation yields (Sreenan and Gosling, 1977; Rajamahendran *et al.*, 1987) but in routine laboratory studies randomly selected mice are used.

The present study was undertaken to determine the most favourable stage of the mouse oestrous cycle for obtaining the highest number of viable embryos after PMSG or hCG treatment. The developmental capacity of oocytes after injection of PMSG at different stages of the oestrous cycle was examined. It was shown that in DD mice the proportion of abnormal embryos was highest after PMSG injection at dioestrus. PMSG injection at pro-oestrus, oestrus and metoestrus resulted in a large yield of viable embryos; the greatest number of viable oocytes synchronized with normal development was found after PMSG injection at oestrus.

Materials and Methods

Animals

Mature females (8–10 weeks old) of the inbred DD/HeJcgn (DD), C57BL/6JYlcgn and CBA/CaLaJcgn (CBA) strains and outbred ICR mice were tested to find a strain with a high PMSG-induced ovulation rate. Mice were kept in small groups (4–6 mice in one cage) under the natural photoperiod (during the study, the light period was from about 08:00 h to 20:00 h).

Stages of the oestrous cycle

Vaginal smears of DD mice were taken daily for 10 days to establish the pattern of the oestrous cycle for each animal

Received 10 February 1994.

before PMSG administration. Vaginal smears were taken by spatular sampling from the mucosa of vaginal walls between 12:00 h and 13:00 h. The stages of the cycle were classified as follows: oestrus, cornified cells only; metoestrus, cornified cells and leucocytes; dioestrus, leucocytes and mucous cells; pro-oestrus, nucleated cells or nucleated cells and cornified cells.

Induction of superovulation

DD females were given an injection of 10 iu PMSG (Pokrovsky Plant of Biopreparations, Pokrovsky) s.c. at 15:00 h and 10 iu hCG (Moscow Plant of Endocrine Preparations, Moscow) 48 h later to induce superovulation. The doses had been determined as optimal in a separate study (Amstislavsky *et al.*, 1991).

Experimental procedure

Treated females were individually caged overnight with males of the same strain and were examined for vaginal plugs on the next morning. The superovulated oocytes were analysed from females that were kept with a male but did not mate. Oocytes were recovered 20–22 h after hCG injection by dissecting the oviducts in medium 199 (Scientific Institute of Viral Infections, Sverdlovsk). Oocytes were characterized as cumulus-enclosed or cumulus-free. Hyaluronidase (Reanal, Budapest) was used to remove cumulus cells (Brinster, 1971). Females with vaginal plugs were analysed on day 3 of pregnancy (the day on which a vaginal plug was found was designated as day 1). Each oviduct was separated from the uterine horn at the uterotubal junction and then flushed with medium 199. Material flushed from the tubes was examined under an inverted Wild Leitz microscope ($\times 120$). Oocytes (and embryos on day 3) were counted and classified according to their stage of development. Oocytes recovered from unmated females were evaluated as fragmented, without a polar body, with a forming polar body (pear-shaped oocytes) and with a formed polar body. Embryos were considered to be normal if they had four or more blastomeres. Two-cell ova found on day 3 were considered as having arrested cleavage. Fragmented, degenerated and cleavage-arrested ova were considered abnormal. Untreated females with spontaneous ovulation were used as controls.

Statistical analyses

Analysis of variance by ANOVA was used to test the effect of the stage of the oestrous cycle on the number of oocytes and 3-day-old embryos of different morphology. The developmental capacity of 3-day-old embryos after PMSG injection at different stages of the oestrous cycle was compared with analysis of Fourfold Tables (stages were tested in pairs).

Results

DD and ICR mice had 4–7 times more embryos after superovulation than did C57BL/6JYlcn and CBA mice. DD mice

Table 1. Embryo development after PMSG-induced ovulation in mature mice of different strains

Strain of mice	Number of animals	Number of embryos	Percentage of embryos with normal development
DD	78	42.0 $\pm$ 1.5	68.2
ICR	11	26.4 $\pm$ 3.3	52.2
C57BL/6JYlcn	14	7.9 $\pm$ 1.0	73.0
CBA	21	5.5 $\pm$ 1.2	85.0

Values are means $\pm$ SEM.

Fig. 1. The total number of oocytes (□) and 3-day-old embryos (■) obtained from DD mice after injection of pregnant mares' serum gonadotrophin at different stages of the oestrous cycle.

were the most sensitive to PMSG treatment (Table 1); this strain was therefore used during the remainder of the study.

DD females were administered gonadotrophins at different stages of the oestrous cycle (pro-oestrus, oestrus, metoestrus and dioestrus). The PMSG-induced ovulation rate was high at all stages tested. The total number of oocytes was greatest after injection of PMSG at oestrus; the total number of superovulated embryos on day 3 did not differ significantly within the oestrous cycle (Fig. 1).

Two groups of oocytes (cumulus-free and cumulus-enclosed) were obtained after PMSG injection at all stages of the oestrous cycle. All cumulus-free oocytes were fragmented while the morphology of the cumulus-enclosed oocytes was different (Table 2). Nearly all spontaneously ovulated oocytes in untreated females had a formed polar body. The pool of viable oocytes was most synchronized with normal development after PMSG injection at oestrus. The stage of the oestrous cycle significantly ($P < 0.05$) affected the number of oocytes with a formed polar body. No effects of the stage of the oestrous cycle on the number of fragmented cumulus-free oocytes, cumulus-enclosed abnormal oocytes, oocytes without a polar body or oocytes with a forming polar body were found.

Table 2. The morphology of ovulated oocytes in DD mice induced to superovulate at different stages of the oestrous cycle

Stage of oestrous cycle	Number of animals	Number of cumulus-free oocytes (fragmented)	Number of cumulus-enclosed oocytes			
			Abnormal	Without polar body	With forming polar body	With formed polar body
Oestrus	10	2.9 ± 1.5	4.0 ± 0.6	8.8 ± 3.5	18.7 ± 5.4	19.7 ± 6.2 ^a
Metoestrus	11	3.5 ± 1.0	7.0 ± 1.5	8.9 ± 1.4	13.9 ± 2.5	8.2 ± 2.0
Dioestrus	15	2.4 ± 0.8	7.4 ± 1.2	9.3 ± 1.3	14.2 ± 2.0	8.2 ± 1.8
Pro-oestrus	9	5.3 ± 1.4	5.4 ± 1.3	13.9 ± 4.4	13.3 ± 0.5	9.0 ± 0.4
Control	10	0	0	0.8 ± 0.4	0.1 ± 0.09	6.3 ± 0.86

Values are means ± SEM.

^aThe stage of the oestrous cycle significantly ($F = 3.03$; $P < 0.05$) affected the number of oocytes with a formed polar body.

Table 3. The development of 3-day-old embryos in DD mice induced to superovulate at different stages of the oestrous cycle

Stage of oestrous cycle	Number of females	Number of normal embryos	Number of abnormal embryos	Proportion of abnormal embryos
Oestrus	7	35.7 ± 8.5	14.3 ± 2.2	0.28 ± 0.06
Metoestrus	8	32.6 ± 3.9	11.0 ± 1.0	0.27 ± 0.05
Dioestrus	10	23.0 ± 2.2	21.0 ± 2.8 ^a	0.47 ± 0.05
Pro-oestrus	7	32.5 ± 3.6	13.2 ± 2.3	0.28 ± 0.06

Values are means ± SEM.

^aThe stages of the oestrous cycle significantly ($F = 3.96$; $P < 0.05$) affected the number of embryos with abnormal development.

The proportion of viable embryos was higher after PMSG injection at pro-oestrus, oestrus and metoestrus compared with dioestrus (Table 3). Analysis of Fourfold Tables showed significant ($P < 0.001$) differences between the ratios of normal: abnormal 3-day-old embryos in pairs: oestrus–dioestrus; metoestrus–dioestrus and pro-oestrus–dioestrus. Comparisons between oestrus, metoestrus and pro-oestrus showed no differences in the ratios of normal:abnormal embryos. Analysis of variance by ANOVA showed that the stage of the oestrous cycle significantly ($P < 0.05$) affected the number of abnormal embryos.

Discussion

The different strains of 8–10-week-old mice produced different numbers of embryos following PMSG/hCG treatment. DD and ICR mice showed high superovulation yields, while C57BL/6 JYlcn and CBA mice showed low superovulation yields. These differences may be due to different concentrations of endogenous hormones in females (Goto *et al.*, 1987) or to a different responsiveness of the ovaries to exogenous gonadotrophins (Spearow, 1988a, b), which could influence the number of eggs superovulated.

In agreement with previous studies (Edwards *et al.*, 1963) we noted that the total number of superovulated oocytes and embryos on day 3 did not vary significantly during the cycle. It is known that preantral follicles that can mature (100–149 µm in diameter) grow faster than any other follicles

(Numazawa and Kawashima, 1982). Their growth rate is not significantly influenced by the stage in the oestrous cycle (Pedersen, 1970). Presumably, the total number of these competent follicles present in the ovary of the treated females could determine the total number of eggs superovulated.

Two groups of oocytes (cumulus-free and cumulus-enclosed) were present after PMSG injection at all stages of the oestrous cycle in DD mice. Fragmented, cumulus-free oocytes could result from ageing, as they had presumably been ovulated because of an LH-like effect of PMSG (Gates, 1971). Fully developed follicles are present in late pro-oestrus in mouse and rat ovaries (Pedersen, 1970; Richards, 1978), which may explain why we observed a higher proportion of cumulus-free oocytes after an injection of PMSG during pro-oestrus.

The different morphology of cumulus-enclosed oocytes showed that oocytes at different developmental stages (i.e. at different ages) could be induced to ovulate. The age of an oocyte is known to be of great importance for its developmental capacity (Martin and Terranova, 1982). The ageing of oocytes leads to a decreased fertilization rate (Evans and Armstrong, 1984), to an increased incidence of polyspermy (Odor and Blandau, 1956), to changes in some organelles (Bedford, 1971) and to a decreased RNA concentration in oocytes (Bogomolova *et al.*, 1981). Thus, the different developmental stages (different ages) of superovulated oocytes could explain the difference in their developmental capacity. Under normal conditions the extrusion of the first polar body in the mouse occurs before the egg is released from the follicle (Bedford, 1971; Hogan *et al.*, 1986). The oocytes with a formed

polar body therefore ovulated before they were too old. The best developmental capacity might thus be expected among the pool of oocytes that ovulated with a formed polar body, as these are synchronized with normal development. The highest proportion of oocytes of this type was found after PMSG injection at oestrus.

The developmental capacity of oocytes depends upon the size of the follicles from which the oocytes were isolated, but not strictly upon the size of the oocyte (Eppig *et al.*, 1992). Eppig and Schroeder (1989) showed that oocytes from pre-antral follicles of mice can complete growth and acquire full developmental competence *in vitro*. So we could expect normal development of oocytes of different morphology. To estimate the developmental capacity of oocytes of different morphology, the number of ovulated oocytes and the number of viable embryos on day 3 were compared (Tables 2, 3). These data suggest that normal development occurs not only in oocytes with a formed polar body but also in oocytes with a forming polar body and those without a polar body. The oocytes with a forming polar body do not seem to be too old to be viable.

There are two possible explanations of how oocytes without a polar body may develop normally. First, in rats, and often in mice, the first polar body disappears soon after its extrusion (Bedford, 1971). The development of oocytes without a polar body could be ahead of that of others, and such oocytes may have lost their polar body before their morphology was assessed. Second, the oocyte is released from the follicles 11–13 h after hCG injection in gonadotrophin-treated mice (Gates, 1971); that is, competent oocytes ovulate 59–61 h after PMSG injection. However, under normal conditions preantral follicles develop to ovulating follicles within two cycles (Numazawa and Kawashima, 1982). Hence, it is possible that not all oocytes have time to complete the first meiotic division before ovulation, and that some oocytes without a polar body could have a chance to complete the first meiotic division in the ampulla of the Fallopian tube and to be fertilized later than others.

Superovulation yielded a significantly higher proportion of viable embryos after PMSG injection at pro-oestrus, met-oestrus and oestrus compared with PMSG injection at dioestrus. After PMSG injection at dioestrus, 47.7% of 3-day-old embryos were abnormal, which is in agreement with the result of Beaumont and Smith (1975), who found that 44% of ova were lost before implantation after inducing superovulation with exogenous PMSG at dioestrus. In rodents, the pool of committed growing follicles is greatest at oestrus as a result of the pro-oestrus surge of endogenous FSH (Pedersen, 1970; Richards, 1978). It seems that synchronizing PMSG treatment with such an endogenous hormonal event does provide optimal conditions for the development of superovulated oocytes.

This work was supported in part by a grant from the Russian State Program 'Frontiers in Genetics'.

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