Reduction in fertilization rate *in vitro* of oocytes from immature rats induced to superovulate*

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Summary. Immature female rats (65–70 g) were injected with 4 i.u. PMSG (control) or superovulated with 8, 16, 24 or 40 i.u. PMSG and were killed 68–70 h later, shortly after the normally expected time of ovulation. Oocytes were recovered from the oviducts and inseminated *in vitro*. After 18 h oocytes were counted and classed as degenerate or 1-cell. Mean numbers of oocytes recovered were 8.2, 26.8, 50.7, 38.7 and 38.5 for each dose of PMSG respectively. The 1-cell oocytes were assessed for sperm penetration of the vitellus and pronuclear development and later for development to the 2-cell stage. Fertilization rates at the 1-cell stage were 76.8, 62.9, 53.6, 52.2 and 44.5% for the rats treated with 4, 8, 16, 24 and 40 i.u. respectively ($P < 0.001$). On average, 91% of fertilized 1-cell oocytes developed to the 2-cell stage and there was no difference between treatments in this respect. Significantly more of the unfertilized oocytes were degenerate in the rats treated with 24 or 40 i.u. PMSG (34.6 and 50.4%) than in those treated with 4, 8 or 16 i.u. (7.0, 13.9, and 7.5%) ($P < 0.001$).

When rats were killed 63–65 h after PMSG, just before the normally expected time of ovulation, some of the rats treated with 24 and 40 i.u. PMSG had partly ovulated: of the oocytes recovered from the oviducts only 12.3% (24 i.u.) and 26.6% (40 i.u.) were fertilized.

These results demonstrate that proportionately fewer oocytes recovered from superovulated rats are competent to undergo in-vitro fertilization than are oocytes recovered from control rats.

Introduction

Techniques for the induction of superovulation are in common use in the livestock industry particularly when large numbers of embryos are required for embryo transfer. However, the use of large superovulatory doses of exogenous gonadotrophin has been reported to result in a reduction in fertility in large domestic animals (Shea, Hines, Lightfoot, Ollis & Olson, 1976; Seidel, Elsdon, Nelson & Hasler, 1978; Evans & Robinson, 1980; Armstrong & Evans, 1983) and in small laboratory animals (Beaumont & Smith, 1975; Miller & Armstrong, 1981). In rats, much of the reduction in fertility is due to loss of embryos at the preimplantation stage (Miller & Armstrong, 1981) but there is also a reduction in fertilization rate when superovulated rats are mated naturally (Walton, Evans & Armstrong, 1983). However, it is unclear whether the reduction in fertilization rate is attributable to the effects of the exogenous gonadotrophin on sperm transport (Austin, 1950)

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or whether superovulated oocytes are less competent than normally ovulated oocytes to undergo fertilization. The following experiment was designed to assess the ability of superovulated oocytes to undergo fertilization in a controlled environment using an in-vitro fertilization technique.

Materials and Methods

Collection of oocytes

Immature female Sprague-Dawley rats were obtained from Charles River, St Constant, Quebec, at body weight 45–50 g. They were allowed free access to food and water. Temperature and lighting were controlled; the animals were kept on an artificial light cycle of 14 h light, 10 h darkness, with 'midnight' defined as the mid-point of the dark phase, set to occur at 10:00 h EST. This reversal of the normal lighting schedule allowed ovulation, which normally begins shortly after midnight, to take place during normal working hours, between 11:00 and 15:00 h EST. At body weight 65–70 g, the rats were injected s.c. with 4 (control dose), 8, 16, 24 or 40 (superovulatory doses) i.u. PMSG (Equinex: Ayerst, Montreal) in 0.2 ml saline (9 g NaCl/l) at 17:00 h EST. In Exp. 1 all animals were killed between 13:00 and 15:00 h EST, 68–70 h after injection and shortly after the normally expected time of ovulation on this lighting schedule; in Exp. 2 they were killed between 08:00 and 10:00 h EST, 63–65 h after injection, just before the normally expected time of ovulation for control rats. The ovaries and oviducts were dissected out in Dulbecco's phosphate-buffered saline (PBS). The swollen ampullae were torn open with fine forceps allowing the oocytes to escape in a cumulus mass. In addition, in Exp. 2 the oviducts were flushed with PBS using a 1-ml syringe and 30-gauge needle. The oocytes from each animal were transferred to a separate dish containing fertilization medium and were kept in a humidified atmosphere at 37°C with 5% CO₂ in air as the gas phase. The surrounding cumulus cells prevented assessment of oocyte normality at this stage. For the treatments with 16, 24 and 40 i.u. PMSG the oocytes from each oviduct were placed in separate dishes in an attempt to maintain numbers of oocytes per dish comparable to those from treatments with 4 and 8 i.u. PMSG.

In-vitro fertilization

The method used for fertilization was basically that used by Toyoda & Chang (1974) with only minor modifications. Since all published reports of in-vitro fertilization in the rat have come from a single group of investigators, and others have failed to repeat the method successfully (see Shalgi, Dekel & Kraicer, 1979; Quigley, 1982) the detailed methodology is outlined below.

Fertilization medium

Two stock solutions were prepared using triple distilled water. Solution A comprised 6.636 g NaCl/l, 427 mg KCl/l, 302 mg CaCl₂·2H₂O/l, 194 mg KH₂PO₄/l, 172 mg MgSO₄/l, 2 mg phenol red/l. Solution B comprised 12.940 g NaHCO₃/l and 2 mg phenol red/l. The working medium was prepared 12–24 h before use as follows: a mixture of 83.35 ml Solution A, 16.28 ml Solution B and 0.37 ml sodium lactate (60% solution: Sigma Chemical Co., St Louis, Missouri, U.S.A.) was prepared. To this were added 5.5 mg sodium pyruvate (Gibco Laboratories, Grand Island, New York, U.S.A.), 100 mg dextrose, 5000 units streptomycin and 5 mg penicillin (Gibco). The solution was gassed with 5% CO₂ in air and 400 mg bovine serum albumin (Fraction V, Ref. A-9647, Sigma) were added. The medium was then sterilized using a 0.20 µm positive-pressure filter (Nalgene, Sybron/Nalge, Rochester, New York) and allowed to equilibrate in a humidified incubator at 37°C with 5% CO₂ in air as the gas phase. The resultant pH was 7.4–7.6. Mineral oil (U.S.P. Heavy, Drug Trading Co., Toronto, Canada), unsterilized, was also allowed to equilibrate for several hours in the incubator. Sterile glass dishes were used for insemination; these contained 0.4 ml medium covered thinly with oil.

Sperm suspension

Adult males of proven fertility were killed by cervical dislocation and each cauda epididymidis and vas deferens were removed aseptically from each rat. Approximately 3 cm of the epididymal duct proximal to the vas deferens, and 1–2 cm of vas deferens, were dissected free of fat and blood vessels, keeping the tissue moist with fertilization medium. The duct was freed of excess moisture and the semen was expressed with fine forceps. This semen was added to 0.6 ml medium under oil and allowed to disperse for 2–3 min. Only samples with a low proportion (<15%) of spermatozoa with prominent cytoplasmic droplets, and with a high degree of motility, were used. Fertilization dishes already containing oocytes were inseminated with 20–40 µl of the sperm suspension taken from the edge of the drop under oil. The final sperm concentration was in the order of $0.1\text{--}1 \times 10^6$ ml. In our system the disturbance to equilibrium caused by frequent incursions into the incubator at this time allowed the pH of the medium in dishes to rise to 7.8–7.9, but this later returned to 7.4–7.6 and did not appear to be detrimental to fertilization.

Examination of oocytes

Dishes containing oocytes were examined under phase-contrast with an inverted microscope at about 18 h after insemination. Oocytes were considered fertilized if they had 2 pronuclei and at least one sperm tail in the vitellus; those with >1 sperm tail in the vitellus were classed as 'polyspermic'. The remaining ova were classed as one-cell unfertilized, or degenerate if they displayed fragmentation or abnormal structure. The numbers in each class were recorded. Some dishes were returned to the incubator and examined after a further 24 h for development to the 2-cell stage.

Statistical analyses

Comparisons between groups were made using χ^2 analyses.

Results

Experiment 1. The number of oocytes recovered from rats shortly after the normally expected time of ovulation, within each treatment group, increased with increasing dose of PMSG to 16 i.u. PMSG ($\approx$ 6-fold increase) and declined slightly with higher doses of PMSG (Table 1). However, no statistical analysis has been applied because heterogeneity of variance was high and was not eliminated by appropriate transformation of the data.

Table 1. Fertilization rates of rat oocytes obtained (68–70 h after PMSG) from rats superovulated with various doses of PMSG

PMSG (i.u.)	Ova recovered/rat (means $\pm$ s.e.m.)	No. of oocytes examined	% fertilized	% polyspermic*	% developing to 2-cell*	% degenerate†
4	8.2 $\pm$ 0.3	245	76.8	6.4	91.5	7.0
8	26.8 $\pm$ 2.8	213	62.9	4.5	90.3	13.9
16	50.7 $\pm$ 7.8	343	53.6	4.9	95.0	7.5
24	38.7 $\pm$ 4.3	387	52.2	18.3	83.2	34.6
40	38.5 $\pm$ 6.9	432	44.5	6.8	93.3	50.4
			$P < 0.001$	$P < 0.001$	$P < 0.1$	$P < 0.001$

* Of those oocytes that were fertilized 18 h after insemination.

† Of those oocytes that were unfertilized 18 h after insemination.

The percentage of oocytes fertilized declined with increasing dose of PMSG ($P < 0.001$). Of the fertilized oocytes, a higher percentage was polyspermic in the 24 i.u. group than in the other groups ($P < 0.001$); of the polyspermic eggs only two pronuclei (1 male, 1 female) developed almost without exception. There was no significant difference between groups in the percentage of 1-cell oocytes which developed to the 2-cell stage. Of the unfertilized oocytes, an increase in the percentage which were degenerate was observed in the 24 and 40 i.u. groups ($P < 0.001$) compared with those treated with 4, 8 or 16 i.u. PMSG.

Experiment 2. When rats were killed just before the normally expected time of ovulation, oocytes were recovered from the oviducts only of rats treated with 24 and 40 i.u. PMSG (Table 2). These oocytes were largely unfertilized 1-cell or degenerate oocytes when examined after insemination and fertilization attempts.

Table 2. Recovery and fertilization rates of oocytes obtained (63–65 h after PMSG) from superovulated rats before the normally expected time of ovulation

PMSG (i.u.)	No. of rats		No. of oocytes			
	Treated	Ovulating	Recovered	Fertilized	1-cell unfertilized	Degenerate
4	7	0	—	—	—	—
8	4	0	—	—	—	—
16	5	0	—	—	—	—
24	7	5	81	10	52	19
40	7	5	45	12	1	32

Discussion

It is apparent from the present results that increasing the dose of PMSG used to superovulate immature rats resulted in a continuous reduction in fertilization of the oocytes shed when oocytes were recovered at the normal time of ovulation. This phenomenon is not necessarily related to the number of oocytes shed *per se* since the highest yield of ova did not result from the use of the highest dose of PMSG, as found by Ying & Meyer (1969) and Kostyk, Dropcho, Moltz & Swartwout (1978).

The high number of oocytes observed to have degenerated at time of examination for fertilization in rats treated with 24 or 40 i.u. PMSG undoubtedly accounted for much of the high degree of fertilization failure observed in these groups. It is apparent that at least some of these oocytes resulted from early ovulations, probably caused by the direct LH-like effect of large doses of PMSG (De La Lastra, Forcelledo & Serrano, 1972; Kostyk *et al.*, 1978). In an *in-vivo* study, it was shown that cumulus-enclosed oocytes obtained just after the normally expected ovulation time from immature rats treated with 40 i.u. PMSG underwent fertilization at rates comparable to those for similar oocytes obtained from rats treated with 4 i.u. (Walton & Armstrong, 1983), indicating that oocytes ovulated 66–68 h after PMSG injection are normal. In our experiments, the oocytes ovulated early do not seem to account fully for the low rate of fertilization in 24 and 40 i.u.-treated rats. However, because of the high variability in recovery of oocytes between rats, one cannot make reliable numerical comparisons between groups of rats (11 and 6 oocytes respectively were, on average, ovulated early by 24 and 40 i.u.-treated rats, whereas means of 18 and 21 oocytes, respectively, were unfertilized following the main ovulation). The possibility remains that significant numbers of abnormal oocytes were ovulated at the normal time of ovulation. Similarly, in rats treated with 8 and 16 i.u. PMSG, in which there were no early ovulations and only low numbers of degenerate oocytes observed, the fertilization rates were reduced in comparison with those in 4 i.u.-treated rats. The reasons for the decline in fertilization rate associated with

increasing ovulation rate are not clear, but increased variability in time of ovulation in relation to time of insemination, thus resulting in greater ageing of a proportion of oocytes in the rats on higher doses of PMSG, could be a contributing factor. Intrafollicular ageing of oocytes by delay of ovulation is known to impair the developmental ability of rat oocytes after fertilization (Fugo & Butcher, 1966; Martin & Terranova, 1982).

Despite the small reduction in oocyte fertilization rate in animals superovulated with moderate doses of PMSG, the vast increase in numbers of fertilizable oocytes outweighs the disadvantages. However, normal fertilizability of superovulated oocytes does not necessarily imply that normal embryo development will ensue. Nevertheless, we could not detect any differences between treatments with respect to development of 1-cell zygotes to 2-cell embryos, and only the eggs in the 24 i.u. group exhibited a high degree of polyspermy. In addition, it has been shown *in vivo* that cumulus-enclosed oocytes obtained from immature rats treated with 40 i.u. PMSG have an ability to develop to 20-day fetuses equal to that of oocytes from 4 i.u.-treated rats (Walton & Armstrong, 1983).

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