Relationship between sperm concentration and the incidence of polyspermy in mouse embryos fertilized *in vitro*

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Summary. A dose–response relationship between the incidence of polyspermy and sperm concentration was found by analysing chromosome preparations of first-cleavage mouse embryos derived from fertilizations *in vitro* with 3 concentrations of mouse spermatozoa (2×10^4 , 2×10^6 and 1×10^7 /ml).

Although millions of spermatozoa are deposited in the mammalian female reproductive tract at the time of mating, only a small proportion (fewer than 1000/oviduct) actually reach the site of fertilization (Blandau & Odor, 1949; Braden, 1953; Braden & Austin, 1954). In contrast, studies of the relationship between mammalian sperm concentration and fertility *in vitro* have indicated that a relatively high number of spermatozoa, generally 10^5 – 10^7 /ml, must be present to ensure maximum fertility (Niwa & Chang, 1974; Talbot, Franklin & Fussell, 1974; Fraser & Drury, 1975; Tsunoda & Chang, 1975; Parkening & Chang, 1976; Wolf & Inoue, 1976). We have shown that the fertilization of mouse eggs *in vitro* results in an abnormally high incidence of polyploidy (Fraser, Zanellotti, Paton & Drury, 1976), primarily due to polyspermy (Maudlin & Fraser, 1977) and perhaps reflecting the high concentration of spermatozoa used (approximately 2×10^6 /ml). Positive correlations between sperm concentration and the incidence of polyspermy are not easily made from published data, even when the same experimental system is used. For example, Wolf & Inoue (1976) reported an incidence of 5.9% polyspermy with 2×10^5 mouse spermatozoa/ml, yet Wolf & Hamada (1976) found 25% polyspermy with 1×10^5 spermatozoa/ml. By contrast, we have shown our system to give reproducible levels of polyploidy with a concentration of approximately 2×10^6 spermatozoa/ml (12.8%, Fraser *et al.*, 1976; 15.4%, Maudlin & Fraser, 1977). The present study was designed to determine, by analysis of chromosome preparations of first cleavage embryos, whether variation in sperm concentration can alter the incidence of polyspermy in mouse embryos in a consistent way.

Methods

Egg donors were (C57BL/10 $\times$ CBA) F₁ females induced to superovulate with 7.5 i.u. PMSG (Gestyl: Organon) and, approximately 48 h later, 5 i.u. hCG (Pregnyl: Organon). Sperm donors were TO males. After a 20 min dispersal period, the suspensions of epididymal spermatozoa, prepared from 2 males with the media and methods described by Fraser & Drury (1975), were diluted to concentrations of 2×10^4 , 2×10^6 and 1×10^7 spermatozoa/ml. Unfertilized eggs obtained 13 h after the hCG injection were added to the sperm suspensions, incubated for 6 h and then washed. The eggs in the lowest sperm concentration were treated with medium containing 2 mg hyaluronidase (bovine testes, Type I: Sigma)/ml to remove cumulus cells still attached to the zona pellucida; this was unnecessary with the higher sperm concentrations. All eggs were then incubated overnight in 10^{-5} mM-vinblastine sulphate (Velbe: Lilly) and processed for chromosome analysis (Maudlin & Fraser, 1977).

Results

Of the 689 1-cell embryos examined in this experiment, 617 (89.5%) could be confidently assessed as diploid or polyploid. The male- and female-derived chromosome complements can be distinguished in 1-cell embryo preparations (Donahue, 1972; Hansmann, 1973; Brewen, Payne, Jones & Preston,

Table 1. Incidence of polyspermy in first-cleavage mouse embryos fertilized *in vitro* in three different concentrations of spermatozoa

Exp. no.	Sperm conc./ml								
	2×10^4			2×10^6			1×10^7		
	N	n	%	N	n	%	N	n	%
1	44	1	2.3	34	3	8.8	33	3	9.1
2	41	3	7.3	38	12	31.6	37	21	56.8
3	29	0	—	45	5	11.1	53	3	5.7
4	—	—	—	54	6	11.1	46	6	13.0
5	90	7	7.8	37	5	13.5	36	4	11.1
Totals	204	11	5.4	208	31	14.9	205	37	18.0

N = no. of embryos examined; n = no. of polyspermic embryos.

1975) and we used this technique to determine the origin of the polyploidy detected in the present study. Of the 88 polyploid embryos found, 85 were triploid and 3 were trispermic tetraploids. Of the 85 triploids, 2 (0.3 %) were digynic in origin, 7 (1.1 %) were fertilized by diploid spermatozoa and the remaining 76 (12.3 %) were all dispermic. The 7 diploid spermatozoa were all found among embryos in Exp. 2, 4 occurring in the 2×10^6 sperm concentration and 3 in the 1×10^7 group.

The proportions of polyspermic 1-cell embryos found in the present study are shown in Table 1. Because of the highly significant heterogeneity between experiments in the incidence of polyspermy at the two higher sperm concentrations, a simple comparison of the mean values for each group is not satisfactory. Instead, dose-response curves for each experiment were fitted by computer by using a weighted logistic analysis of the binomial proportions plotted against $\log_{10}$ of the sperm concentration. A test for parallelism showed that there was no significant difference between the slopes of the five regression lines ($\chi^2 = 11.1$; d.f. 8; $P = \text{N.S.}$); the calculated common slope (0.59 ± 0.14) value demonstrated a highly significant regression ($P = <0.001$). This analysis shows clearly that the incidence of polyspermy among mouse embryos fertilized *in vitro* may be affected by altering the concentration of spermatozoa in the suspensions used.

These differences in the incidence of polyspermy with three sperm concentrations were not reflected in differing fertilities. The fertilization levels obtained in individual experiments for each sperm concentration (calculated from the number of eggs possessing second polar bodies before incubation in 'Velbe') were 61–98 % (2×10^4 spermatozoa/ml), 71–100 % (2×10^6 spermatozoa/ml) and 79–97 % (1×10^7 spermatozoa/ml).

Of the 617 embryos scored for polyploidy, 554 (90 %) gave sufficiently well spread preparations to allow the incidence of aneuploidy to be assessed. Six aneuploid embryos, 3 monosomic and 3 trisomic, were found among the 554 scored (1.1 %).

Discussion

Studies on sperm concentration as a critical factor in the successful fertilization of mammalian eggs *in vitro* have concentrated on the effect of sperm numbers in relation to the efficiency of the technique, as measured by the resultant fertility of the eggs. By using a wide range of sperm concentrations which gave equally high levels of fertility, we have determined the incidence of polyspermy and shown a clear dose-response relationship. The fertilization obtained with the lowest sperm concentration (2×10^4 /ml) was considerably higher than previously reported (Fraser & Drury, 1975), presumably reflecting a general improvement in the fertility of TO sperm suspensions in this system (Fraser & Drury, 1976). As might have been expected, alteration of sperm concentration had no effect on the incidence of either male or female aneuploidy. All 7 diploid spermatozoa were found in a single experiment, suggesting that these aberrant spermatozoa came from an individual male producing a relatively high proportion of diploid gametes because only 2 males were used for each experiment.

We have already shown that the dose of PMSG can influence the incidence of polyspermy in mouse eggs fertilized *in vitro* (Maudlin & Fraser, 1977). Since all the eggs used in the present experiment were obtained after treatment with a single PMSG dose, any effects due to the hormone should have been the same in all 3 groups. The dose-response to sperm concentration may therefore be explained as follows. Firstly, the hormone may alter the zona pellucida so that more spermatozoa than normal can enter the perivitelline space of the unfertilized egg, thereby increasing the chances of two or more spermatozoa fusing with the vitellus. Secondly, the greater the sperm concentration the greater the probability that more than one spermatozoon will penetrate the zona. The majority of the polyspermic embryos found in the present experiment were dispermic, suggesting that the vitelline block to polyspermy was functioning but that in many eggs 2 spermatozoa were able to fertilize the egg at approximately the same time. Indeed, when 2 spermatozoa had fertilized an egg, the two sets of male chromosomes usually showed similar degrees of contraction indicating that they had penetrated the egg almost simultaneously. This hypothesis is supported by the work of Braden, Austin & David (1954) which showed that the 2 fertilizing spermatozoa in dispermic rat eggs usually entered the egg at opposite hemispheres. This suggested that sperm penetration set up a propagated reaction around the egg, leaving only a short period of time for a second spermatozoon, if present, to fuse with the egg.

After mating and fertilization *in vivo*, the incidence of polyploidy in the mouse is low, about 1% (Donahue, 1972; Ford & Evans, 1973) and this may reflect the low number of spermatozoa which reach the site of fertilization (Braden & Austin, 1954). The present work indicates that it should be possible to determine an optimal sperm concentration for fertilization *in vitro* which would yield maximal fertility and minimal polyspermy.

We thank Linda Drury and Gillian Haynes for technical assistance and Dr C. E. Ford for his interest in this work. One of us (I.M.), a visiting worker at the Clinical Research Centre, is a member of the M.R.C. External Scientific Staff.

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Received 20 May 1977