The effect of PMSG dose on the incidence of chromosomal anomalies in mouse embryos fertilized *in vitro*

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Summary. Chromosomal analysis of first-cleavage mouse embryos obtained by fertilization of TO eggs with TO spermatozoa *in vitro* revealed that the incidence of polyploidy was proportional to the dose of PMSG given to the donor females. The dose-response was shown to be linear, with the level of polyploidy rising from 8.0% with a PMSG dose of 1.5 i.u./female to 20.8% with 10.0 i.u./female. A total of 64 polyploid embryos was found among the 452 examined and in all cases the polyploidy was the result of polyspermy or fertilization by diploid spermatozoa.

Introduction

The induction of ovulation in mammals by administration of exogenous gonadotrophin may be responsible for an increased incidence of chromosomal abnormalities in the resulting embryos. Fujimoto, Pahlavan & Dukelow (1974) and Fujimoto, Passantino & Koenczoel (1975) noted slight, but statistically insignificant, increases in chromosomal abnormalities in rabbit blastocysts and intratubal embryos obtained after superovulation, when compared with a control group. Boué & Boué (1973) found a significant increase in the level of trisomy among abortuses from women who had been induced to ovulate when compared with a sample of untreated women.

Experiments with mouse embryos *in vivo* have shown that a low level of chromosomal anomalies normally occurs. In studies of first-cleavage mouse embryos, Donahue (1972a) found an incidence of 1.8% aneuploidy and 1.5% polyploidy, while Kaufman (1973) found 8.3% to be aneuploid and 4.1% polyploid. We found a significantly greater incidence of triploidy in mouse morulae fertilized *in vitro* (12.8%) than in those fertilized *in vivo* (2.7%) (Fraser, Zanellotti, Paton & Drury, 1976), and this *in-vitro* fertilization technique was therefore adopted to study the effects of gonadotrophin dosage on chromosomal anomalies in the mouse. However, because a rather low proportion (33%) of analysable embryos was obtained from morulae (Fraser *et al.*, 1976), in the present study we used first-cleavage embryos which yield a high proportion of scoreable preparations. Only the PMSG dose was varied because the work of Shaver (1970) had indicated that variation in HCG dosage apparently did not affect the chromosome constitution of rabbit embryos.

Materials and Methods

Fertilization in vitro

Outbred TO mice, 2–4 months old, were obtained from the Specific Pathogen Free breeding unit of the Clinical Research Centre. Virgin females were induced to ovulate by an i.p. injection of PMSG (Gestyl: Organon) followed 48 h later by 5.0 i.u. HCG (Pregnyl: Organon). The PMSG was diluted in distilled water so that animals received 1.5, 4.5, 7.5 or 10.0 i.u. in a volume of 0.1 ml and at least two different levels of PMSG stimulation were examined in each experiment.

The methods and media used for fertilization *in vitro* were as described by Fraser & Drury (1975). After a 20-min dispersal period, epididymal sperm suspensions were diluted to a concentration of 2×10^6 spermatozoa/ml. Unfertilized eggs, obtained 13 h after the HCG injection, were released

directly into the sperm suspensions and incubated at 37°C in an atmosphere of 5% O₂, 5% CO₂, 90% N₂ for 6 h. Eggs were then washed twice in culture medium containing 4 mg bovine serum albumin (Armour)/ml and transferred to droplets of culture medium containing 10⁻⁵ mM-vinblastine sulphate (Velbe; Lilly). After overnight culture in this medium, the eggs were fixed and stained according to the method of Tarkowski (1966).

Initially, all eggs (i.e. with and without a second polar body) were cultured in Velbe-containing medium. However, because those eggs lacking a second polar body proved to be unfertilized when examined after fixation, in most experiments only eggs possessing a polar body were analysed. To verify that this did not select against potential digynic triploids which would not have had second polar bodies, the eggs which appeared to be unfertilized were cultured in Velbe-free medium. Such eggs were never observed to cleave and because experimentally produced digynic triploid TO embryos cleave normally (L. R. Fraser, unpublished observation), it was presumed that this class of embryos was absent from this study.

Fertilization in vivo

Virgin TO females, 2–4 months old, were injected i.p. with either a low dose (1.5 i.u.) or a high dose (7.5 i.u.) of PMSG and, approximately 48 h later, with a dose of 5.0 i.u. HCG. The HCG was injected 12–13 h before the estimated time of natural ovulation and the females were paired with TO males. Females which had a vaginal plug the following day were killed in the afternoon and 1-cell, fertilized eggs were recovered from the oviducts. When necessary, eggs were transferred to culture medium containing 2 mg hyaluronidase (bovine testes, Type I: Sigma)/ml to remove cumulus cells still attached to the zona pellucida. All eggs were washed twice, transferred to droplets of culture medium containing 10⁻⁵ mM-Velbe, cultured overnight and then fixed and stained as above.

Analysis of preparations

Because a high incidence of polyploidy was obtained (see 'Results'), attempts were made to determine whether this was due to supernumerary male or female nuclei. Preliminary experiments were carried out using exactly the same methods but with males having a T6 marker chromosome to distinguish the male complement. It was clear from the analysis of 504 spreads that the sperm-derived chromosomes were less condensed than those derived from the egg (Pl. 1, Fig. 1). This differential condensation was also apparent in the present study and has previously been noted by McGaughey & Chang (1971) and Donahue (1972a).

Results

Polyploidy

Polyploidy was the more frequently detected anomaly in this study. Of the 536 embryos examined after in-vitro fertilization, 452 yielded scoreable preparations (84%); 64 were polyploid (14.2%) and of these 60 were triploid (Pl. 1, Fig. 2; Pl. 2, Fig. 4) and 4 were tetraploid (Pl. 2, Fig. 3). In comparison, 4 out of 228 (1.8%) embryos fertilized *in vivo* were found to be triploid. The results are presented in Table 1.

The control data from individual experiments were tested for heterogeneity and were shown to be homogeneous within hormone doses; these data have therefore been pooled for analysis. A χ^2 test for heterogeneity also showed that the proportions of polyploid embryos were homogeneous within the two in-vitro groups of 1.5 and 7.5 i.u. PMSG. A straightforward comparison was therefore made between these in-vivo and in-vitro group totals; there was no significant difference between the two groups at 1.5 i.u., but at 7.5 i.u. there was a highly significant difference ($\chi^2 = 21.5$, $P < 0.001$). This result with TO eggs and TO spermatozoa confirms that obtained with morulae from (C57BL/10 × CBA)F₁ eggs and TO spermatozoa (Fraser *et al.*, 1976).

The proportion of polyploid embryos increased as the dose of PMSG increased. Because a simple regression analysis of these proportions was not statistically sound due to the variation between

Fig. 1. First-cleavage diploid mouse embryo fertilized *in vitro* showing differential condensation of male and female chromosomes; the T6 marker chromosome is indicated by the arrow. ♂ = male complement; ♀ = female complement; pb = second polar body.

Fig. 2. First-cleavage mouse embryo fertilized *in vitro* showing a triploid chromosome complement produced by dispermy.

Fig. 3. First-cleavage mouse embryo fertilized *in vitro* showing a tetraploid chromosome complement produced by trispermy. Note the differential condensation of the three male complements, compared with that of the female chromosomes.

Fig. 4. First-cleavage mouse embryo fertilized *in vitro* showing a triploid chromosome complement produced by a diploid spermatozoon.

Table 1. Incidence of polyploidy in first-cleavage mouse embryos of the TO strain, fertilized *in vitro* and *in vivo*, after administration of different doses of PMSG to donor females

	PMSG dosage (i.u.)											
	1.5			4.5			7.5			10.0		
	N	n	%	N	n	%	N	n	%	N	n	%
<i>In vitro</i>												
1	41	2	4.9				36	3	8.3			
2	24	1	4.2				18	3	16.6			
3	15	1	6.6	25	1	4.0	33	6	18.2			
4				22	1	4.5	18	4	22.2			
5	15	2	13.3	11	1	9.1	14	2	14.3	10	3	30.0
6				28	12	42.8				6	3	50.0
7				12	1	8.3	16	2	12.5	18	1	5.5
8	18	3	16.7	26	2	7.7	8	2	25.0	38	8	21.1
Totals	113	9	8.0	124	18	14.5	143	22	15.4	72	15	20.8
<i>In vivo</i>												
Totals	61	3	4.9				167	1	0.6			

N = No. of embryos examined; n = no. of 3n embryos.

experiments within dosage groups, a series of dose-response curves were fitted by computer to each of the 8 experimental groups, using transformed data. Doses were transformed to $\log_{10}$ and the proportions of polyploid embryos were weighted to allow for the differing numbers in each proportion, a logistic transformation being applied to normalize the binomial data (Cox, 1970). A test for parallelism showed that the slopes of the lines did not differ significantly ($\chi^2 = 8.7$, d.f. 13). The response curves had a common slope of 1.1762 ± 0.5722 , indicating a significant positive regression ($P = 0.05 - 0.01$). It was therefore concluded that there was a significant response in terms of incidence of polyploidy related to PMSG dose.

Analysis of the polyploid embryos by distinction of the paternal and maternal chromosomes (see 'Methods') showed that all 64 were produced by di- or triandry. Most of the polyploids resulted from dispermy but 4 were due to fertilization by spermatozoa with a diploid complement (Pl. 2, Fig. 4); 3 of these embryos were 3n while the fourth was 4n with both haploid and diploid male chromosome complements.

Aneuploidy

Aneuploid counts were designated as 'confident' or 'uncertain' depending on the quality of the chromosome spreads produced. Great care was taken in the designation of hypodiploid embryos and all hypodiploids with an irregular conformation were classified as 'uncertain'.

Table 2. Incidence of aneuploidy in first-cleavage mouse embryos of the TO strain fertilized *in vitro* and *in vivo*

	Monosomic			Trisomic			Total aneuploid		
	C	U	C + U	C	U	C + U	C	U	C + U
<i>In vitro</i>	7 (1.1%)	3	10 (1.2%)	6 (0.9%)	0	6 (0.7%)	13 (2.0%)	3	16 (1.9%)
<i>In vivo</i>	1 (0.3%)	0	1 (0.2%)	2 (0.6%)	0	2 (0.5%)	3 (1.0%)	0	3 (0.7%)

C = 'confident' counts; 655 *in vitro*, 311 *in vivo*.U = 'uncertain' counts; 198 *in vitro*, 118 *in vivo*.

The counts from male and female nuclei were combined.

We were unable to detect any significant difference between the levels of male and female aneuploidy and the data from the two groups were therefore combined. The results in Table 2 do not show the variation among PMSG groups in the levels of aneuploidy because prior analysis showed that there was no significant effect of PMSG dose. There was clearly a greater degree of aneuploidy in the in-vitro embryos than in the in-vivo controls, but in no case was the difference statistically significant. There was no significant difference between the numbers of monosomic and trisomic aneuploids detected although there was a slight excess of monosomics in the in-vitro group, presumably due to the number of 'uncertain' counts.

Discussion

There have been conflicting reports about the effects of gonadotrophins on the embryonic development of mammals. Shaver (1970) found no indication that excess HCG given before mating adversely affected the chromosome constitution of rabbit blastocysts, and Fehchheimer & Beatty (1974) concluded that superovulation had no demonstrable effect on the incidence of heteroploidy in rabbit blastocysts. Spindle & Goldstein (1975) found that variation of the PMSG and HCG dosage did not affect blastocyst formation or subsequent growth of mouse embryos fertilized *in vivo* and cultured *in vitro*. Conversely, Elbling (1973) reported that gonadotrophin treatment resulted in significantly increased numbers of deformed mouse fetuses, although these findings have been disputed (Smith & Chrisman, 1975). Beaumont & Smith (1975) found that superovulation caused substantial pre- and post-implantation losses *in vivo* in superovulated mice when compared to an unstimulated group.

The overall level of polyploidy recorded here for TO eggs fertilized *in vitro* with TO spermatozoa was 14.2%, a value similar to that of 12.8% found for morulae obtained from (C57BL/10 × CBA) F₁ eggs fertilized *in vitro* with TO spermatozoa (Fraser *et al.*, 1976). The present work, however, has demonstrated a simple dose-response relationship between the PMSG dose and the incidence of polyploidy, mainly triploidy, detected in TO mouse eggs fertilized *in vitro*. Although these results were obtained using an artificial method of fertilization, they serve to confirm the indications given by Boué & Boué (1973) and Fujimoto *et al.* (1974, 1975) that chromosomal anomalies may be induced by superovulation.

The polyploid embryos in our in-vitro study were all polyandric. In a review of the origins of polyploidy, Beatty (1970) stated that the most important form of vertebrate polyploidy was produced by digyny, but much of his evidence related to the 'silver' strain of mice which has a high incidence of spontaneous second polar body suppression (Braden, 1957). Beatty (1974) also calculated that dispermy did not contribute at all to the incidence of polyploidy in man. Donahue (1972a, b), after studies of 1-cell mouse embryos fertilized *in vivo*, concluded that spontaneous triploidy was produced equally by dispermy and digyny. Wróblewska (1971) examined post-implantation mouse embryos from the cross CBA × CBA/T6T6 and found that 12 out of 18 triploids were apparently digynic in origin while the remaining 6 could not be assessed. Similarly, Fehchheimer & Beatty (1974) in a study of rabbit blastocysts concluded that, although dispermy could not be ruled out, digyny was the simplest explanation for triploidy. Takagi & Sasaki (1976), who studied A/He mouse embryos fertilized *in vivo* after superovulation, found that most of the triploids were digynic. The incidence of triploidy (4.5%) detected by Takagi & Sasaki (1976) in the absence of hormonal stimulation was similar to that found in the 'silver' strain (Beatty, 1970), suggesting that the A/He strain, like the 'silver' strain, is prone to failure of the second meiotic division and therefore to the increased production of digynic embryos. Despite this apparent genetic predisposition to digyny, Takagi & Sasaki (1976) still found 1.7–4.5% of the zygotes examined to be diandric in origin which corresponds well with the 0.6–4.9% triploid and diandric embryos obtained *in vivo* in the present work. Comparison with our work is complicated by the in-vitro technique employed here, but this should not have inhibited the formation of digynic embryos. Indeed, the 4 triploid embryos in the in-vivo groups were all diandric in origin, while the incidence of triploidy (1.8%) was very close to the 1.2% recorded by Donahue (1972a).

The response to PMSG demonstrated here by an increase in polyploidy and the polyandric nature of this polyploidy suggests that the hormone affects extra-nuclear events rather than the egg nucleus.

This might involve changes in the properties of the zona pellucida to permit entry of more than one spermatozoon or in the vitelline block to polyspermy which would normally allow only one spermatozoon to fuse with the egg (Austin, 1961). Morphological (Katzberg & Henrickx, 1966) and biochemical (Conrad, Buckley & Stambaugh, 1971; Fraser & Dandekar, 1975) changes have been noted in the zonae pellucidae of mammalian eggs obtained by superovulation. It is evident that the block to polyspermy, whether at the zona pellucida or the vitelline membrane, is less effective *in vitro* than *in vivo*, perhaps reflecting the inability of the egg to respond adequately in the presence of relatively high numbers of spermatozoa *in vitro*. Nevertheless, even though all the eggs in our study were mixed with the same concentration of spermatozoa, those eggs stimulated by lower doses of PMSG were more resistant to penetration by extra spermatozoa than those obtained with high doses of hormone.

The presence of diploid spermatozoa in 4 of the embryos examined (i.e. 1%) raises interesting questions as to the frequency of their occurrence *in vivo*. In a microdensitometric study, Carothers & Beatty (1975) found only 1 diploid spermatozoon outside their randomly chosen sample of 1000 mouse spermatozoa obtained from the 'silver' strain, specifically selected because of its high incidence of polyploidy. However, the 'silver' strain is noted for a high incidence of triploidy, mainly due to suppression of the second polar body in the egg (Braden, 1957). Stolla & Gropp (1974) also failed to find diploid spermatozoa in *M. musculus* and *M. poschiavinus* but did find a low incidence in the F_1 hybrid. There is, however, ample evidence from other mammals to show that diploid spermatozoa do occur and at quite high frequencies. For example, Sumner (1971) found 16 diploid spermatozoa in a sample of 1670 human spermatozoa (0.96%) and Carothers & Beatty (1975) reported 0.56% $2n$ spermatozoa out of 5554 human spermatozoa. In the rabbit, Beatty & Fechheimer (1972) found a mean incidence of 0.31% $2n$ spermatozoa for 3 strains of rabbit, a value later confirmed by Carothers & Beatty (1975).

There is some dispute about the fertilizing ability of diploid spermatozoa. Schindler & Mikamo (1970) agreed with Bomsel-Helmreich (1965) that there was no evidence that diploid mammalian spermatozoa were capable of successful fertilization. In man, however, it has been estimated that diploid spermatozoa account for 30% of triploid conceptions (Beatty, 1974). We have shown that diploid spermatozoa are indeed capable of fertilization in the mouse, but whether they contribute to *in-vivo* levels of triploidy cannot be determined from these experiments. Krzanowska (1974) has presented evidence that $2n$ spermatozoa may be filtered out at the uterotubal junction in the mouse.

The incidence of aneuploidy, unlike that of triploidy, did not vary with PMSG dose, and the overall levels in the present work were 1.9% *in vitro* and 0.7% *in vivo*. Comparable studies by Donahue (1972a) and Kaufman (1973) of first-cleavage mouse embryos gave 1.8% and 8.3% aneuploidy, respectively. Donahue dismissed monosomic counts as artefactual due to fixation or spreading, while Kaufman included counts of <37 chromosomes in his estimate. In the present study, hypodiploid counts were only considered to be 'confident' if the spreads showed no irregularities. The results of the present work are in close agreement with those of Donahue and suggest that his aneuploid counts may not have been artefactual. Since additional chromosomes cannot be produced artefactually, the fact that we found no significant difference between the numbers of monosomic and trisomic counts indicates that the monosomic data are reliable.

Because polyploidy is a frequent source of developmental failure in man (Carr, 1971), the present results, albeit in mice and *in vitro*, suggest that hormone treatment of women and perhaps even normal fluctuations of FSH might well increase the incidence of chromosomal anomalies in human embryos.

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