ANIMAL EXPERIMENTATION

Investigation of Effects of Pregnant Mare Serum Gonadotropin (PMSG) on the Chromosomal Complement of CD-1 Mouse Embryos

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Purpose: The objective of this study was to examine the effect of superovulatory doses of gonadotropins on the frequency of chromosomal abnormalities of mouse embryos.

Methods: Chromosome analysis of 8- to 16-cell stage mouse embryos and zygotes was performed by a cytogenetic method.

Results: There was no significant effect of the pregnant mare serum gonadotropin (PMSG) dose on the level of aneuploidy and structural abnormalities from 8- to 16-cell-stage embryos among superovulated groups. However, a simple dose-response relationship between the PMSG dose and the incidence of polyploidy was observed, with the level of polyploidy rising from 2.9% with 10 IU PMSG to 10.5% with 15 IU PMSG. In zygote stage, the proportion of polyploid embryos also increased as the dose increased, from 1.9% in 5 IU to 6.7% in 15 IU PMSG. It was observed that the extra chromosomal set in polyploidy embryos originated by both fertilization of a diploid oocyte and dispermy.

Conclusions: These results indicate a dose-response relationship between the PMSG dose and the incidence of polyploidy in the CD-1 mouse. Both a disturbance at maturation division and an error at fertilization were the cause of polyploidy.

KEY WORDS: mouse embryos; mouse zygotes; pregnant mare serum gonadotropin; chromosomal abnormalities; triploidy.

INTRODUCTION

One of the most important questions that needs to be solved to improve the success of human in vitro fertilization (IVF) and embryo transfer systems is the low implantation rate. In human IVF, the live birth rate per embryo transferred is only about 16.8% (1), whereas the embryonic survival after fertilization in vivo is estimated to be 30% (2). The origin of the relatively high incidence of embryonic mortality after IVF remains unclear. One possibility is an increased number of chromosomal abnormalities. Cytogenetic data derived from human IVF programs indicate that the percentage of chromosomal abnormalities among unfertilized oocytes is extremely high (3–57%) (3). One should consider whether such high rates of chromosomal abnormalities in unfertilized oocytes are related to superovulation. However, the infeasibility of producing human embryos solely for research purpose prohibits any definitive correlations between superovulation and embryonic chromosomal abnormalities. As animal models are the best alternatives, we designed the mouse model to examine whether superovulation cause chromosomal abnormalities in mouse embryos.

MATERIALS AND METHODS

Mice

Mature female and mature, fertility-proven, male CD-1 mice (6 to 8 weeks) were obtained from Charles

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River Canada Inc (St. Constant, Quebec). Vaginal smears were taken daily until at least three consecutive 4-day estrous cycles had been observed; these mice were then selected for the study. Between 1300 and 1400 on the first day of metestrus, experimental animals were given an intraperitoneal injection of 5, 10, and 15 IU pregnant mare serum gonadotropin (PMSG; Equinex, Ayerst, Montreal, Quebec), respectively, followed 48 hr later by 5 IU hCG (Serono Canada Inc., Mississauga, Ontario). They were allowed to mate with CD-1 males after the second injection. Females with plugs were defined as the first day of pregnancy and caged separately. Spontaneously ovulating and mated females served as controls.

Chromosomal Preparation of 8- to 16-Cell Embryos

On day 3 of pregnancy, treated and control females were injected subcutaneously with 3 mg/g body weight of vinblastine sulfate (Sigma Chemical Co., St. Louis, MO) for 4 hr prior to sacrifice to obtain metaphase from 8- to 16-cell-stage embryos. Embryos with an abnormal appearance (fragmented embryos) were not used for chromosome preparation due to lack of metaphase. Therefore, only embryos displaying a normal appearance were used for this study. The zona pellucida was removed by incubation in 0.5% Pronase (4) (Sigma Chemical Co.) in Ham's F-10 culture medium (GIBCO, Grand Island, NY). When the zona pellucida was completely digested, embryos were removed individually using a finely drawn-out Pasteur pipette (internal diameter of approximately 0.2 mm) and transferred into a microdroplet containing 50 μ l of 1% sodium citrate. Blastomeres were separated mechanically by aspiration with a finely drawn Pasteur pipette during the hypotonic treatment.

The blastomeres from each embryo were drawn up in a minimal amount of hypotonic solution into a fine glass Pasteur pipette containing fixative solution A (distilled water, acetic acid, and ethanol, 5:1:4) in the tip of the pipette and placed in the center of a clean slide. Three drops of fixative B (ethanol and acetic acid, 3:1) were expelled onto the blastomeres and the drying of this fixative was hastened by placing the slide on a warming plate at 37°C. The slide, with the blastomeres, was then immersed in fresh fixative B for 2 min. A maximum of five embryos was placed on the slide and the blastomeres from each embryo were separated on a slide in different areas.

The slides were aged overnight at 60°C. G-bands were produced by treatment with 2 $\times$ SSC (saline

sodium citrate; 0.3 M NaCl, 0.03 M trisodium citrate) at 60°C for 2 hr, followed by 0.25% (w/v) trypsin (GIBCO Laboratories, Life Technologies, Inc., Grand Island, NY) at 4°C for 2 min. The slides were then stained with 5% (v/v) Giemsa (Gurr's R66 Improved; BDH Chemicals, UK) for 2 min (5).

Chromosomal Preparation of Zygotes

Zygotes from day 1 of pregnancy were transferred to Ham's F-10 culture medium containing 0.2% hyaluronidase (Ovine Type II; Sigma Chemical Co.) to remove cumulus cells still attached to the zona pellucida for 2 min. Zygotes were washed twice in Ham's F-10 medium and transferred to droplets of culture medium containing 10^{-5} mM vinblastine sulfate. The medium used to culture the zygotes was Ham's F-10 medium supplemented with 10% calf serum (GIBCO) and 100 IU/ml penicillin (GIBCO). The culture system consisted of microdroplets of medium under sterile, equilibrated mineral oil (Aldrich Chemical Company, Milwaukee, WI) in a 10 $\times$ 35-mm petri dish. The mineral oil was equilibrated with the culture medium. Between 5 and 10 zygotes were injected into each microdroplet. The zygotes were in culture medium containing 10^{-5} mM vinblastine sulfate and cultured in a humidified 5% CO₂ incubator at 37°C overnight. The hypotonic treatment and fixative methods were the same as described above.

The metaphase spreads were counted and examined for chromosomal abnormalities using a Nikon microscope ($\times$ 1000). Only embryos with an unambiguous number of chromosomes were used for calculations of the frequency of chromosome abnormalities. One or two metaphases were available for analysis from each embryo. Embryos with chromosome numbers less than 38 were discarded since these might have arisen through faulty preparation. The metaphase spreads with abnormal chromosome numbers or structural abnormalities were photographed.

Statistical Analysis

The exact trend test was used for dose-response analysis. Differences were considered significant at $P < 0.05$. A Bonferroni correction factor was applied for the subgroup comparisons.

RESULTS

Eight- to 16-Cell-Stage Embryos

The results of chromosome analysis of mouse embryos at the 8- to 16-cell stage are presented in Table I and Fig. 1.

Aneuploidy. The frequency of hyperdiploid embryos was 2.9, 2.2, 2.5, and 3.5% of the analyzed embryos in the control and 5, 10, and 15 IU PMSG-treated groups, respectively. Theoretically, nondisjunction should produce an equal number of embryos with 39 and 41 chromosomes. From Table I, it is obvious that the frequency of embryos with 39 chromosomes exceeds the frequency of embryos with 41 chromosomes. This may be related to the artifact caused by fixation procedure. A conservative estimate of the nondisjunction rate can be obtained by doubling the hyperdiploid rate, namely, 5.8, 4.4, 5.0, and 7.0% in the four groups. There was no significant effect of PMSG dose in the levels of hyperdiploidy among the four groups when applying the exact trend test ($P > 0.05$).

Polyploidy. There was no polyploidy found in the control and 5 IU PMSG-treated groups. However, in the 10 IU PMSG treated group, 9 (2.9%) polyploidy were found in 317 analyzed embryos, including 4 (1.3%) triploid (Fig. 2) and 5 (1.6%) tetraploid. In the 15 IU PMSG-treated group, 15 (10.5%) polyploidy embryos were found in 144 analyzed embryos, including 6 (4.2%) triploid and 9 (6.3%) tetraploid. The proportion of polyploid embryos increased as the dose of PMSG increased ($P < 0.001$).

Structural Abnormalities. A small fragment chromosome appeared in two embryos (1%) in the spontaneously ovulating group and in five embryos (1.6%) in the 10 IU PMSG-treated group. This fragment was not related to any chromosomes in the same metaphase and was considered as a chromosomal marker.

Fig. 1. A karyotype from a 12-cell-stage embryo with diploidy, 40, XY. Inset shows metaphase spread. Original magnification, $\times 40$.

Table I. Chromosome Analysis of 8- to 16-Cell-Stage Mouse Embryos from Spontaneously Ovulating (SO), 5, 10, and 15 IU PMSG Superovulated CD-1 Mice^a

Group	Diploidy	Aneuploidy*		Polyploidy**		Structural abnormalities marker*
		Hyper	Hypo	Tri	Tetra	
SO	180/211 (85.7%)	6 (2.9%)	16 (7.6%)			2 (1%)
5 IU	190/225 (84.4%)	5 (2.2%)	21 (9.3%)			
10 IU	245/317 (77.3%)	8 (2.5%)	31 (9.8%)	4 (1.3%)	5 (1.6%)	5 (1.6%)
15 IU	102/144 (70.8%)	5 (3.5%)	16 (11.1%)	6 (4.2%)	9 (6.3%)	

^a F exact trend test. Hyper, hyperploidy; hypo, hypoploidy; tri, triploidy; tetra, tetraploidy.

* $P > 0.05$.

** $P < 0.001$.

Fig. 2. A karyotype from an eight-cell-stage embryo with triploidy, 58, XY, -16, -X or Y. Inset shows metaphase spread. Original magnification, $\times 40$.

Zygotes

The numbers of zygotes available for cytogenetic analysis were 126, 108, 106, and 120 in the spontaneously ovulating and 5, 10, and 15 IU PMSG-treated female mice, respectively (Table II).

Aneuploidy and structural abnormalities were difficult to judge because of overlap chromosomes prepared from the zygotes. Only polyploidy was detected in this study. The origin and gametic source of the polyploid embryos were determined on the basis of differential condensation of maternally and paternally derived chromosomes. Male pronuclei showed longer chromosomes with a stronger tendency to overlap, whereas female chromosomes were shorter and usually showed less overlap (Fig. 3).

No polyploidy was found in 126 embryos examined in the untreated group. In comparison, 15 (4.5%) in

superovulated groups were found to be polyploid (14 triploidy and 1 tetraploidy). The proportion of polyploid embryos increased as the dose of PMSG increased ($P < 0.001$). In the 5 IU PMSG-treated group, two polyploid embryos observed were of maternal origin (Fig. 4). In the 10 IU PMSG-treated group, five polyploid embryos were present, with two of maternal and three of paternal origin. A total of eight polyploid embryos was found in the 15 IU PMSG group including two with triploidy of maternal origin, five triploidy of paternal origin, and one tetraploid embryo. The tetraploid embryo showed two sets of haploidy of maternal origin and two sets of haploidy of paternal origin. The number of diandric triploid and digynic triploid embryos in each group was too low to allow for statistical analysis. However, the proportion of polyploidy detected in superovulated groups

Table II. Incidence and Origin of Polyploidy in Mouse Zygotes from Spontaneously Ovulating (SO) and 5, 10, and 15 IU PMSG Superovulated CD-1 Mice

Group	Number of embryos examined	Triploidy		Tetraploidy (%)	Total (%)*
		Diploid oocyte (%)	Dispermy (%)		
SO	126	0	0	0	
5 IU	108	2 (1.9)	0		2 (1.9)
10 IU	106	2 (1.9)	3 (2.8)		5 (4.7)
15 IU	120	2 (1.7)	5 (4.2)	1 (D,D) ^a (0.8)	8 (6.7)
Total	460	6 (1.3)	8 (1.8)	1 (2.2)	15 (3.3)

^a D,D—diploid oocyte, dispermy.* *F* exact trend test, *P* < 0.001. **Fig. 3.** First-cleavage diploid mouse embryo fertilized in vivo showing differential condensation of male and female chromosomes. Original magnification, $\times 1000$.

Fig. 4. First-cleavage mouse embryo fertilized in vivo showing a triploid chromosome complement derived by a diploid oocyte and a haploid sperm. Original magnification, $\times 1000$.

was increased by the same trend as in 8- to 16-cell-stage embryos ($P < 0.001$).

DISCUSSION

Embryonic chromosomal aberrations are recognized as one of the primary causes of reproductive failure. However, it has not yet been determined if superovulation adversely affects the chromosome constitution of either released oocytes or embryos in humans (6).

Fujimoto *et al.* (7) studied rabbit embryos produced by superovulation treatment where 8.3% of blastocysts showed chromosomal abnormalities, including mosaics, aneuploidy, and triploidy. No abnormalities were detected in 36 blastocysts in the control group. In contrast, Sengoku and Dukelow (8) did not find a difference in the incidence of chromosomal abnormalities (aneuploidy) in 8- to 16-cell-stage hamster

embryos resulting from superovulation and natural cycles (2.2 vs 1.2%). Moreover, a high variation of the incidence of aneuploidy was noted between different strains of superovulated mice; it was higher in inbred strains than in random-bred mice from early implanted embryos (9).

In Djungarian hamsters, aneuploidy in oocytes was directly correlated with the hormonal dose injected (10). According to the results, Hansmann (11) proposed that failure of the endocrine control of oocyte maturation cause an altered meiotic division leading to nondisjunction. However, this hypothesis was not supported by our study.

In the present study, the incidence of aneuploidy in three superovulated groups was 4.4, 5.0, and 7.0%, respectively, and was not significantly increased as the PMSG dose increased ($P > 0.05$). These data are in agreement with the results of Maudlin and Fraser (12), who reported that there were no significant effects of

PMSG on the incidence of aneuploidy in mouse zygotes.

In contrast to aneuploidy, gonadotropin stimulation seems to increase the incidence of polyploidy. In this present study, no polyploidy was found in the spontaneously ovulated group and 5 IU PMSG-treated group. The incidence of polyploidy recorded for 8- to 16-cell-stage embryos was 2.9 and 10.5% in the 10 and 15 IU PMSG groups, respectively, showing a dose-response relationship between the PMSG dose and the incidence of polyploidy ($P < 0.001$). These results serve to confirm the suggestion given by Boué and Boué (13) and Maudlin and Fraser (12) that polyploidy may be induced by superovulation.

Polyploidy found in this study was of both maternal and paternal derivation. The digyny occurring after superovulation could be due to suppression of either the first or the second polar body. One mouse strain (NMR/Han) responds to gonadotropins by ovulating a significant number of diploid oocytes most often arrested at metaphase I (14). In a A/He mouse strain, the frequency of digynic triploid embryos was significantly higher in the superovulated group than in the control group. The major cause of digyny was a defect of the second meiotic division (15). The arrest of meiosis at either metaphase I or metaphase II may be caused by interference of the spindle apparatus or a synchronous chromosome separation of all bivalents in oocytes subjected to superovulation.

The response to PMSG demonstrated in this study by an increase in diandric polyploidy suggests that the hormone affects not only oocyte nuclear events but also extranuclear events. These might involve failure of cortical granule (CG) exocytosis in immature oocytes and altered CG distribution in aged oocytes. During superovulation by administration of gonadotropins, multiple waves of premature or asynchronous ovulations are often observed in several species. If more than one ovulation wave occurs with gonadotropin stimulation, asynchronous nuclear maturation of oocytes may occur. This may result in oocytes of various stage of maturity including immaturity and over-maturity, which can be a source of chromosome imbalance (16).

Polyspermy and digyny were reported to be increased significantly due to delayed fertilization in the rabbit (17) and mouse (18). Superovulation with PMSG in mice (12) and hamsters (8) led to an increase in triploidy and was believed to be the result of delayed fertilization (19). Also, an increased incidence of polyspermy has been reported in association with delayed fertilization in the rat (20). Therefore, in the superovu-

lated mice, where fertilization of oocytes is delayed, an increased incidence of polyspermy and digyny may occur.

An increase in the rate of polyspermy in embryos derived from immature oocytes compared to the controls was observed in mice (16). A significant increase in polyspermy was observed at one superovulatory dose level during in vitro fertilization in immature rats (21). In human IVF programs, the incidence of polyspermy varies between 2 and 9% (22). Trounson *et al.* (23) and Van der Ven *et al.* (24) found a high level of polyspermy (about 30%) when immature oocytes were fertilized. Polyspermy in immature oocytes can be explained by failure of cortical reaction since it has been shown that the cortical granules are still migrating to the vicinity of the plasma membrane.

In humans, most tripronuclear oocytes have altered cleavage patterns and various types of mosaicism have been observed (25). Anomalous cleavage and poor quality of embryos could result from severely unbalanced chromosome complements. Chromosomal abnormalities may be an important factor in early embryonic loss. It is plausible that the extent of chromosome imbalance might have a direct effect on early development of the embryo. Therefore, polyploidy may be partly responsible for the embryo loss at preimplantation stage.

CONCLUSIONS

The most significant finding in mouse experiments demonstrates a dose-response relationship between the PMSG dose and the incidence of polyploidy. Both a disturbance at maturation division and an error at fertilization were the cause of polyploidy. These data suggest that further research is needed to determine if superovulatory treatment in women might increase the incidence of chromosomal abnormalities which contribute to the low IVF pregnancy rate.

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REFERENCES

1. American Fertility Society, Society for Assisted Reproductive Technology. Assisted reproductive technology in the United

- States and Canada: 1992 results generated from the American Fertility Society/Society for Assisted Reproductive Technology Registry. 1994;62:1121-1128
2. Short R: The evolution of human reproduction. *Proc R Soc London Ser B* 1986;195:3-24
  3. Pellestor F: Differential distribution of aneuploidy in human gametes according to their sex. *Hum Reprod* 1991;6:1252-1258
  4. Bacchus C, Buselmaier W: Blastomere karyotyping and transfer of chromosomally selected embryos. Implications for the production of specific animal models and human prenatal diagnosis. *Hum Genet* 1988;80:333-336
  5. Roberts CG, O'Neill C: A simplified method for fixation of human and mouse preimplantation embryos which facilitates G-banding and karyotypic analysis. *Hum Reprod* 1988;3:990-992
  6. Pellestor F: Differential distribution of aneuploidy in human gametes according to their sex. *Hum Reprod* 1991;6:1252-1258
  7. Fujimoto S, Pahlavan N, Dukelow WR: Chromosome abnormalities in rabbit preimplantation blastocysts induced by superovulation. *J Reprod Fertil* 1974;40:177-181
  8. Sengoku K, Dukelow WR: Gonadotropin effects on chromosomal normality of hamster preimplantation embryos. *Biol Reprod* 1988;38:150-155
  9. Luckett DC, Mukherjee AB: Embryonic characteristic in superovulated mouse strains. *J Hered* 1986;77:39-42
  10. Hansmann I, Jenderny J, Probek HD: Mechanism of non-disjunction: Gonadotrophin-induced aneuploidy in oocyte from *Pholopus sungorus*. *Eur J Cell Biol* 1980;22:24
  11. Hansmann I: Factors and mechanism involved in nondisjunction and X-chromosome loss. In *Cytogenetics of the Mammalian X Chromosome*, AA Sandberg (ed). New York, Alan R Liss, 1983, pp 131-170
  12. Maudlin I, Fraser LR: The effect of PMSG dose on the incidence of chromosomal anomalies in mouse embryos fertilized in vitro. *J Reprod Fertil* 1977;50:275-280
  13. Boué JG, Boué A: Increased frequency of chromosomal anomalies in abortions after induced ovulation. *Lancet* 1973;1:679-680
  14. Hansmann I, El-Nahass E: Incidence of nondisjunction in mouse oocytes. *Cytogenet Cell Genet* 1979;24:115-121
  15. Takagi N, Sasaki M: Digynic triploidy after superovulation in mice. *Nature* 1976;264:279-281
  16. Badenas J, Santalo J, Calafell JM, Estop AM, Egozcue J: Effect of the degree of maturation of mouse oocytes at fertilization: a source of chromosome imbalance. *Gamete Res* 1989;24:205-218
  17. Shaver EL, Carr DH: The chromosome complement of rabbit blastocysts in relation to the time of mating and ovulation. *Can J Genet Cytol* 1969;11:287-293
  18. Vickers AD: Delayed fertilization and chromosomal anomalies in mouse embryos. *J Reprod Fertil* 1969;20:69-76
  19. Chang MC: Digynic triploidy after superovulation. *Nature* 1977;266:382-383
  20. Odor DL, Blandau RJ: Incidence of polyspermy in normal and delayed matings in rats of the wistar strain. *Fertil Steril* 1956;7:456-467
  21. Evans G, Armstrong DT: Reduction in fertilization rate in vitro of oocytes from immature rats induced to superovulate. *J Reprod Fertil* 1984;70:131-135
  22. Pieters MHEC, Dumoulin JCM, Ignoul-Vanvuchelen RCM, Bras M, Evers JLH, Geraedts JPM: Triploidy after in vitro fertilization: Cytogenetic analysis of human zygotes and embryos. *J Assist Reprod Genet* 1992;9:68-76
  23. Trounson AO, Mohr L, Wood C, Leeton JF: Effects of delayed insemination on in vitro fertilization, culture and transfer of human embryos. *J Reprod Fertil* 1982;64:285-294
  24. Van der Ven HH, Al-Hasani S, Diedrich K, Hamerich V, Lehmann F, Krebs D: Polyspermy in vitro fertilization of human oocytes: Frequency and possible causes. In *In Vitro Fertilization and Embryo Transfer*, M Seppala, RG Edwards (eds). New York, Ann NY Acad Sci, 1985, pp 88-95
  25. Ma S, Kalousek DK, Ho Yuen B, Moon YS: The chromosome pattern of embryos derived from tripronuclear zygotes studied by cytogenetic analysis and fluorescence in situ hybridization (FISH). *Fertil Steril* 1995;63:1246-1250