Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice

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Summary. The effect of gonadotrophins on pre- and postimplantation development in mice was investigated by superovulating C57BL/6J/Bom females with pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) or by inducing ovulation with hCG. In both hormone treated groups, the proportion of abnormal preimplantation embryos increased compared with naturally ovulating animals. Postimplantation mortality increased and the mean number of live fetuses per pregnant mouse decreased in superovulated and hCG-treated mice compared with controls. Embryonic growth was highly retarded. Mean weight of live fetuses in superovulated and hCG-treated mice was reduced and skeletal examination revealed developmental retardation. In conclusion, superovulation as well as induction of ovulation adversely affected embryonic and fetal development.

Keywords: superovulation; ovulation induction; mortality; growth retardation; embryos; mouse

Introduction

In human *in vitro* fertilization (IVF), most clinics practise ovarian hyperstimulation, since the likelihood of pregnancy increases with the number of embryos transferred. Despite considerable progress in IVF during the last years, the pregnancy rate per embryo transferred is still only about 10% (Medical Research International, The American Fertility Society, 1991). There may be a relationship between treatment with gonadotrophins and the low pregnancy rate per embryo transferred. Some clinics prefer the natural cycle for IVF and avoid ovarian stimulation. Timing of ovulation with human chorionic gonadotrophin (hCG) is commonly used in these programmes (Paulson *et al.*, 1990).

Superovulation has been shown to adversely affect fertility in different species. For example, increased embryonic mortality has been reported in mice and rats (McLaren *et al.*, 1959; Beaumont & Smith, 1975; Miller & Armstrong, 1981). Genetic factors may be responsible for embryonic loss, as superovulation has been found to increase the proportion of chromosomal abnormalities in murine embryos (Elbling & Colot, 1985; 1987; Luckett & Mukherjee, 1986).

Factors associated with the uterine environment may also play a role. In a mouse embryo donation model, ovarian hyperstimulation impeded implantation presumably by causing adverse changes in uterine receptivity (Fossum *et al.*, 1989).

A high luteal phase oestradiol:progesterone ratio has been associated with implantation failure in mice by creating a uterine environment that suppresses embryonic metabolism (Safro *et al.*, 1990).

The effect of hCG treatment alone on fertility has not been examined. To investigate any possible effects of gonadotrophins in the offspring of mice, we investigated pre- and postimplantation development in superovulated mice and in animals treated with hCG alone to induce ovulation. Control animals ovulated spontaneously.

Materials and Methods

Animals

Female C57Bl/6J/Bom mice (2–3 months old) and B6 CBA/F1 male mice 3–12 months old) purchased from Bomholtgaard (Denmark) were used in all experiments. The animals were housed under a 12 h light:12 h dark regimen with a temperature of 23°C and a relative humidity of 44%. They were fed with a standard pellet diet (EWOS, Norsk landbrukskjemis A/S) and had free access to water.

Gonadotrophin treatment

Pregnant mares' serum gonadotrophin (PMSG, Antex, LEO) and human chorionic gonadotrophin (hCG, Physex, LEO) were used. Females for the experiments were chosen without regard to their oestrous cycle.

For assessment of preimplantation development, mice were superovulated with an i.p. injection of 10 iu PMSG in 0.1 ml 0.8% NaCl and then 48 h later with 5–10 iu hCG in 0.1 ml 0.8% NaCl. Other mice were injected with 0.1 ml 0.8% NaCl alone and then with 10 iu hCG in 0.1 ml 0.8% NaCl as ovulation induction. Controls were injected twice with 0.1 ml 0.8% NaCl.

For examination of postimplantation development, mice were superovulated with 5, 10 or 20 iu PMSG and 5 iu hCG. Other groups of mice were treated with NaCl alone followed by 5, 10 or 20 iu hCG as ovulation induction. Controls were treated as above. After the second injection each female was caged with a male and the presence of a vaginal plug the following morning verified a successful mating. This was designated as day 1 of gestation.

Embryo recovery and assessment of preimplantation development

Animals were killed by cervical dislocation on day 2 or 4 of gestation. On day 2 the oviducts and on day 4 the oviducts and uterine horns were excised and flushed with phosphate-buffered isotonic saline (PBI, Whittingham, 1974). The embryos were pooled, washed twice with PBI and morphologically classified as normal or abnormal. Embryos were then transferred to 0.05 ml medium no. 16 (Whittingham, 1971) under paraffin oil and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Further development *in vitro* until day 5 was assessed by daily morphological examination. Normal two-cell embryos on day 2 should become four- to eight-cell embryos on day 3, morula/early blastocysts on day 4 and expanded blastocysts on day 5.

Assessment of postimplantation development

Pregnant mice were killed on day 18 and the uterine contents examined. The fetuses were gently pinched with a blunt forceps to test for viability. The numbers of live fetuses, stillborns and resorptions (early and late) in each uterine horn were recorded. Each fetus was then examined for macroscopic malformations and weighed. In a series of experiments placentas were also weighed.

Assessment of the skeleton by Alizarin red staining

To examine possible deviations from normal on the skeleton, fetuses from control and hormone-treated females were stained with Alizarin red (Lorke, 1977).

Hormone assays

Oestradiol and progesterone concentrations in serum on day 2 and 4 of gestation were analysed. Blood was collected by an incision in the groin while the animals were under anaesthesia after i.p. injection of 0.5 ml per 10 g body weight Dormicum:Hypnorm (Midazolam/Fentanyl/Fluanison, Ullevål Apotek, Oslo, Norway). Oestradiol and progesterone analyses were performed as previously described (Fosså *et al.*, 1976, Torjesen & Aakvaag, 1976).

Statistical analysis

The data were analysed by two sample-test or χ^2 test using Minitab software, PA, USA. Differences were considered significant at $P < 0.05$.

Results

Assessment of preimplantation development

Thirty-two mice were killed on day 2 and 25 mice on day 4 to examine the effect of gonadotrophins on preimplantation development.

On day 2 (Table 1) superovulation with PMSG and hCG increased the mean number of embryos per mouse, but considerably decreased the proportion of morphologically normal embryos compared with controls. Embryos with abnormal blastomeres or fragmentation, empty zonae and unfertilized/uncleaved oocytes were considered abnormal (Fig. 1). Further development *in vitro* of normal embryos on day 2 to normal blastocysts on day 5 was also impaired. Treatment with hCG alone had no effect on the proportion of embryos appearing normal on day 2; but further development *in vitro* to day 5 was affected.

Table 1. Effects of pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) on preimplantation development of day 2 embryos in mice^a

Treatment	Total number of embryos	Mean number per mouse	Day 2 Number of normal embryos (%)	Day 5 Number of blastocysts (%)
Control	71	7.1	57 (80)	54 (95)
10 iu PMSG + 5-10 iu hCG	179	16.2	100 (55) ^b	82 (82) ^c
NaCl + 5-10 iu hCG	73	6.6	57 (78)	36 (63) ^b

^aMice were killed on day 2 of gestation. Embryos that appeared normal were cultured *in vitro* until day 5.

^b $P < 0.005$ versus control. ^c $P < 0.05$ versus control.

Fig. 1. Embryos from hormone-treated mice. Mice were killed on day 2 of gestation. The oviducts were flushed and the embryos collected and cultured *in vitro* until day 5 (blastocyst stage). (a) Day 2: normal and abnormal embryos from superovulated mice; (b) day 5: blastocysts and abnormal embryos from superovulated mice; (c) day 5: blastocysts and abnormal embryos from hCG-treated mice.

On day 4 of gestation the proportion of morphologically abnormal embryos increased in both hormone treated groups, but further *in vitro* development to normal blastocysts was not affected (Table 2).

Table 2. Effects of pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) on preimplantation development of day 4 embryos in mice^a

Treatment	Total number of embryos	Mean number per mouse	Day 4 Number of normal embryos (%)	Day 5 Number of blastocysts (%)
Control	43	7.1	36 (83)	33 (92)
10 iu PMSG + 5–10 iu hCG	116	12.8	60 (51) ^b	52 (86)
NaCl + 5–10 iu hCG	21	2.1	12 (57) ^c	11 (91)

^aMice were killed on day 4 of gestation and embryos appearing normal were cultured *in vitro* until day 5.

^b $P < 0.005$ versus control. ^c $P < 0.025$ versus control.

There was no difference in serum oestradiol concentrations on day 2 and 4 in superovulated mice compared with controls. However, serum progesterone ($\text{mmol l}^{-1} \pm \text{SEM}$) was increased on day 2 from 39 ± 7 to 173 ± 22 and on day 4 from 50 ± 6 to 113 ± 15 ($P < 0.005$). Treatment with hCG alone did not affect serum concentration of oestradiol or progesterone.

Assessment of postimplantation development

To examine the effect of gonadotrophins on postimplantation development, 152 mice were killed on day 18 of gestation (Table 3). When superovulated mated females were killed, the animals often did not have resorption sites, stillborns or live fetuses. Only 42% of the animals were pregnant compared with 91% of controls. The mean number of live fetuses per pregnant mouse was lower not only in the hCG-treated group, but also in the superovulated group compared with naturally ovulating animals.

Table 3. Effects of pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) on postimplantation development in mice^a

Treatment	Number of mice pregnant	Offspring		
	Number of mice with vaginal plug positive (%)	Mean number of live fetuses per pregnant mouse ^b	Resorptions + stillborns (%)	Mean weight (g) of live fetus ^b
Control	31/34 (91)	7.7 ± 0.3	22 (8)	0.98 ± 0.008
5–10 iu PMSG + 5 iu hCG	41/97 (42)	4.9 ± 0.6^c	151 (43) ^c	0.61 ± 0.013^c
NaCl + 5–20 iu hCG	15/21 (71)	3.9 ± 0.9	31 (35) ^c	0.50 ± 0.019^c

^aMice were killed on day 18 of gestation after superovulation with PMSG and hCG or after injection with hCG as ovulation induction.

^bValues are means $\pm$ SEM.

^c $P < 0.001$ versus control.

In both groups of hormone-treated mice embryonic mortality was high. The proportion of resorptions and stillborns was 43% in superovulated mice and 35% in hCG-treated mice compared with 8% in controls. Furthermore, embryonic growth was highly retarded (Fig. 2). Mean weight of

Fig. 2. (a) Mean weight of the live fetuses on day 18 of gestation from superovulated mice was considerably reduced compared with controls. (b) Skeletal examination revealed delayed ossification as a sign of developmental retardation. Bar: 0.23 cm.

the live fetuses in superovulated and hCG-treated mice was reduced by 38% and 51%, respectively compared with controls.

There was a negative correlation between postimplantation development and hormone doses used, but this was not statistically significant.

Besides the striking difference in fetal weight observed, skeletal examination revealed developmental retardation of the skeleton in fetuses from hormone-treated females. Signs of retardation were lack of metatarsal ossification centres in the extremities and incomplete development of skull bones and distinctly larger fontanelles and wider cranial sutures. In general, ossification of all bones was delayed compared with control fetuses (Fig. 2). There was no difference between treated and control groups in other types of macroscopic malformations. Only one malformed fetus was obtained in each group. The malformations were anencephaly and umbilical hernia in treated and control animals, respectively.

The mean weight of placentas ($n = 93$) was not reduced in either group of hormone-treated mice compared with controls.

Discussion

Adverse effects of superovulation on embryo development have previously been reported (Beaumont & Smith, 1975; Miller & Armstrong, 1981). However, possible adverse effects of induction of ovulation with hCG treatment alone have not been examined.

In our experiments, administration of gonadotrophins was not synchronized with the oestrous cycle of the animal. However, the adverse effect of gonadotrophins presumably cannot be attributed to asynchrony, since an earlier study has shown that superovulation after synchronization also increased embryonic loss (Beaumont & Smith, 1975). The high proportion of abnormal embryos on day 2 of gestation in the superovulated group was partly due to a high number of uncleaved oocytes. It is known that superovulation may result in two different sets of oocytes (Stern & Schuetz, 1970); an initial set ovulated within 20 h after the PMSG injection and a second set ovulated in response to the administration of hCG 48 h after the PMSG injection.

Preimplantation embryonic loss occurred earlier, mainly before day 2, in superovulated mice compared with hCG-treated mice. Whether this difference reflects different factors responsible for the embryonic loss cannot be determined from these experiments.

The adverse effect of gonadotrophins may be a result of impaired oocyte quality, factors in the maternal environment or a combination of both. Genetic factors may be responsible for impaired oocyte quality as shown by increased sister chromatid exchanges in pre- and postimplantation embryos in mice after superovulation (Elbling & Colot, 1985, 1987). The proportion of chromosomally abnormal embryos was found to be significantly higher in the superovulated group of inbred mice than in their spontaneously ovulating counterparts, but such effects were not observed in random bred mice (Luckett & Mukherjee, 1986). It can be assumed that different strains of mice respond in a nonuniform way to superovulation.

A high luteal phase oestradiol:progesterone ratio has been found to create a uterine luminal environment that suppresses embryonic metabolism and inhibits implantation (Safro *et al.*, 1990). In our experiments the oestradiol:progesterone ratios on days 2 and 4 were decreased in superovulated mice. This change in concentration of ovarian steroid hormones may also affect embryonic development. However, the serum hormone concentrations were not changed in animals treated with hCG alone.

As in previous reports with superovulation (McLaren *et al.*, 1959; Beaumont & Smith, 1975), we found that superovulation as well as hCG alone as an inducer of ovulation increased the postimplantation loss. Both the proportion of resorptions and of stillborns were increased. Consequently, the losses must have occurred at different stages of pregnancy.

The intrauterine growth retardation of live fetuses on day 18 of gestation was highly significant in both groups of hormone-treated animals. In superovulated mice, this effect on fetal growth has been attributed to overcrowding in the uterus resulting in fetal competition for limited uterine flow and hence nutrient or oxygen supply or both (Evans *et al.*, 1981). In our study, overcrowding in the uterus presumably was not the reason for the low body weight of live fetuses. On the contrary, the number of implantations was reduced in hCG-treated mice compared with controls. Furthermore, in hormone-treated mice intrauterine growth retardation appeared to occur irrespective of the number of implantations. Fetal placenta weight, as a measure of maternal nutrients and oxygen, was not decreased.

Superovulation in mice has been found to induce malformations such as forelimb defects and to a smaller extent central nervous system anomalies (Elbling, 1973, 1975). In our experiments, delayed skeletal ossification was seen in the extremities and cranium, and there was one case of anencephaly in a fetus of the hormone-treated group.

Superovulation with PMSG and hCG and ovulation induction with hCG alone adversely affected the embryonic and fetal development of C57Bl/6J/Bom $\times$ B6CBA/F1 mice. A cascade of embryonic failure of varying degrees of severity was seen. It is not known whether impaired oocyte quality or factors in the maternal environment, or a combination of both, are responsible for this adverse effect.

We thank P. Torjussen, Aker Hospital, Oslo, Norway, for kindly performing the hormone assays.

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Received 14 October 1991