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Genotoxic and embryotoxic effects of gonadotropin hyperstimulated ovulation on murine oocytes, preimplantation embryos and term fetuses

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Summary. - Compared to spontaneous ovulation, gonadotropin hyperstimulated ovulation (superovulation) in mice resulted in a fourfold increase in the number of preimplantation embryos three days post coitum 50% of which will die before term. Both *in vitro* development of embryos during the preimplantation period and transfer of morulae from superovulated females to pseudopregnant untreated foster mothers indicate that the prenatal loss occurring shortly before implantation up to term is due to maternal factors rather than to direct hormonal effects on oocytes or early embryos. Indeed, no genotoxic events could be observed in 4-cell to blastocyst stage embryos from superovulated female mice as revealed by the chromosomal aberration test and the sister chromatid exchange assay. Chromosome analysis of the pronuclei from mouse zygotes showed an increased rate of aberrations in oocyte derived nuclei after superovulation in comparison to spontaneous ovulation. The present data suggest that aberrant murine oocytes may be fertilized, but they do not survive the first cleavage stages. The result is discussed with respect to the high incidence of chromosomal abnormalities found in human oocytes after gonadotropin-hyperstimulated ovulation.

Key words: chromosomal aberrations, sister chromatid exchanges, embryolethality, teratogenicity, gonadotropin, mouse.

Riassunto *(Effetti tossici e genotossici in oociti, embrioni preimpianto e feti a termine di topo provocati da superovulazione indotta da gonadotropine).* - A confronto con l'ovulazione spontanea, l'ovulazione indotta da gonadotropine (superovulazione) risulta in un incremento di quattro volte del numero di embrioni preimpianto, il 50% dei quali muore prima del termine della gravidanza. Sia lo sviluppo *in vitro* di questi embrioni durante il periodo preimpianto che il trapianto di embrioni superovulati a femmine pseudogravide non trattate indicano che questa elevata mortalità prenatale è dovuta a fattori materni piuttosto che ad un effetto diretto degli ormoni sugli oociti e sugli embrioni precoci. In embrioni superovulati, dallo stadio di 4 cellule fino allo stadio di blastocisti, non è stato possibile evidenziare eventi genotossici sia attraverso lo studio delle aberrazioni cromosomiche sia col saggio del sister chromatide exchange. L'analisi di cromosomi di pronuclei di zigoti ha evidenziato un aumentato tasso di aberrazioni in zigoti ottenuti mediante superovulazione rispetto a quelli di ovulazioni spontanee. Questi risultati suggeriscono che oociti aberranti possono essere fecondati, ma non sopravvivono dopo la prima divisione. I risultati sono discussi in relazione anche all'alta incidenza di anomalie cromosomiche trovate in oociti umani dopo superovulazioni stimolate con gonadotropine.

Parole chiave: aberrazioni cromosomiche, sister chromatid exchanges, embrio-letalità, teratogenicità, gonadotropine, topo.

Introduction

Hormone-hyperstimulated maturation of follicles and induction of ovulation is economically essential in animal breeding to increase the offspring number. Furthermore, in human infertility treatment hormonal induction of ovulation is a routine procedure. For this purpose several sex hormones are at hand, among which gonadotropins are the most important. Unfortunately, in laboratory animals, e.g. the mouse, gonadotropins are not reported to act in a beneficial way only, but seem to cause chromosomal abnormalities in preimplantation embryos [1, 2] as well as fetal death and malformations [3, 4]. However, since the halflife of human chorionic gonadotropin (hCG) was found to be 54 h in the serum of the mouse [5] a direct action even on cleavage stage embryos is unlikely, whereas oocytes from the dictyate stage on and zygotes may be affected. In reproductive toxicology defects in later embryogenesis has been partly traced back to damaged germ cells [6, 7]. Therefore, we have studied possible effects of gonadotropins from oocyte maturation through fertilisation and preimplantation development up to term in the mouse. The results of the mouse study are discussed in relation to data obtained with human oocytes and embryos.

Materials and methods

Animal housing and treatment schedule

Mice of the strains "NMRI-Kisslegg" and "Swiss Cpb:SE" (BGA, Berlin, Germany) were kept under 12 h light/dark cycle and 55% humidity at a temperature of 20-22 °C. Food (Altromine 1324 diet, Altrogge, Germany) and tap water were given *ad libitum*. Nulliparous female mice were superovulated by intraperitoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG) and 48 h later 5 IU human chorionic gonadotropin (hCG). Both hormones were generous gifts from Dr. W. Elger (Schering AG, Berlin, Germany).

On the morning after an overnight mating period (3 females were caged with 1 male) females with a vaginal plug were separated and used for the experiments. This day was designated day 0 of pregnancy. Animals serving as controls were kept and mated under the same conditions.

Endpoints determined

1. At term. - On day 17 of pregnancy at least 15 pregnant females per group were sacrificed and their uteri prepared for the determination of the number of implantation sites as well as dead and living fetuses. Furthermore, living fetuses were weighted and inspected for gross malformations and retardations. Skeletal abnormalities were examined after staining of the fetuses with Alizarin Red S as reported earlier by Lorke [8].

2. In preimplantation embryos. - On day 3 of pregnancy 25 pregnant females per group were sacrificed. Morulae and early blastocysts were flushed from the uteri and counted. Fifty preimplantation embryos per group were cultured for 96 h in medium NCTC supplemented with 10% fetal calf serum [9]. At the end of the culture period the formation of an inner cell mass (ICM) served as a parameter for evaluating the developmental capacity of the embryos *in vitro*.

In addition, 4- and 8-cell embryos as well as morulae and blastocysts obtained from NMRI mice were cultured for 24 h in the presence of 10^{-8} M Bromodeoxyuridine (BrdU). Preparation of the embryos on slides [10] and staining according to the Fluorescence-Plus-Giemsa technique by Perry and Wolff [11] enable the evaluation of sister chromatid exchanges [12]. Sister chromatid exchange (SCE) is a very sensitive parameter for genotoxicity testing. Furthermore, the chromosome aberration test was carried out on the same slides.

3. In zygotes. - On the morning of day 0 of gestation 25 females per dose group and mouse strain were sacrificed. Zygotes were obtained from the oviducts, counted and cultured for 6 h in the presence of colcemid for analysis of the pronuclei chromosomes. Since the chromatin of the two pronuclei - male and female - decondenses asynchronously, the female one is always distinguishable from the delayed and more diffuse appearing male pronucleus. Per data point 100 zygotes could be analysed successfully.

Statistics

The numbers of zygotes, preimplantation embryos, implantation sites, and term fetuses were compared using the Wilcoxon-test ($p \leq 0.05$). Student's t-test ($p \leq 0.001$) was applied for comparison of the mean values of SCE per metaphase and the chi square-test ($p \leq 0.01$) for statistical analysis of data from the chromosome aberration test and the *in vitro* development studies. Data are presented as mean ± standard deviation and compared within a strain and not between the two strains.

Results

Loss of embryos during postimplantation development

Embryo-fetal death and developmental disruptions during the postimplantation period were studied in the mouse strains Swiss and NMRI. After spontaneous ovulation no significant difference between the number of implantations and the number of viable term fetuses could be detected. Following superovulation, however, 24% of the Swiss and 15% NMRI implantations were lost up to term, while the number of implantations was increased by more than 100% in both strains compared to spontaneous ovulation (Table 1).

At term, living NMRI fetuses did not show any signs of malformation or retardation. An incidence of 9% open eyelids in the hormon treated animals (controls: 3%) and 5% cleft palates (control: 1%) as well as a high rate of 23% fetuses showing general signs of retardation like delayed ossification and low birth weight (controls: 0) were found exclusively in the Swiss mouse strain.

Loss of embryos during implantation

To determine embryonic loss around implantation the number of preimplantation embryos at the morulae and early blastocyst stage flushed from the uteri at day 3 of gestation was compared to the number of implantation sites at term. While no significant loss could be observed after spontaneous ovulation, 38% (NMRI) and 35% (Swiss) of the day 3 embryos from superovulated females failed to implant (Table 1).

In order to test the developmental potential of embryos derived from superovulated females, preimplantation mouse embryos flushed from the uteri of superovulated and spontaneous ovulated females at day 3 of gestation were grown *in vitro* for 96 h. All morulae and early

Table 1. - Prenatal loss in the mouse strains NMRI and Swiss after spontaneous ovulation and superovulation by gonadotropins

	Spontaneous ovulation		Superovulation	
	NMRI	Swiss	NMRI	Swiss
Zygotes/female	10.5 ± 2.3	10.5 ± 3.1	49.0 ± 15.9	41.3 ± 20.8
loss (% of zygotes)	*(a)*	*(a)*	25.3	21.9
Morulae/female	9.9 ± 2.1	9.9 ± 2.9	36.6 ± 15.0	32.2 ± 17.7
loss (% of zygotes)	*(a)*	*(a)*	28.6	27.1
Implantation sites/f	10.9 ± 2.3	10.0 ± 3.7	22.6 ± 7.8	21.0 ± 10.4
loss (% of zygotes)	*(a)*	*(a)*	7.1	12.1
Living term fetuses/f	9.9 ± 2.6	9.0 ± 3.8	19.1 ± 6.9	16.0 ± 10.3
% of zygotes	*(a)*	*(a)*	39	39

(a) Values not different (Wilcoxon test, $p \leq 0.05$).

Table 2. - Chromosomal abnormalities in zygotes of the mouse strain NMRI after spontaneous ovulation and superovulation

Female pronuclei with	Spontaneous	Superovulated
Structural aberrations		
1 chromatid break	0	3%
2 chromatid breaks	0	2% (5/100)
Numerical aberrations		
21 chromosomes	0	3%
22 chromosomes	0	2% (5/100)
Genome aberrations		
dispermy	0	5% (5/100)
Zygotes in total (no. = 100 zygotes per group)	0	15%

blastocysts developed into expanded blastocysts and about 55% of the embryos from spontaneously ovulated as well as from superovulated females formed an inner cell mass (data not shown). Thus, no differences in developmental potential could be detected between embryos from spontaneously ovulated and superovulated females.

Loss of embryos during preimplantation period

The incidence of embryonic loss before implantation was investigated in the two mouse strains relating the average number of zygotes shortly after fertilization to the number of morulae and blastocysts per female on day 3 p.c. In non-hormone treated mice no loss was detectable. After superovulation, however, 25% of the NMRI zygotes and 22% of the Swiss zygotes were lost up to day 3 of gestation (Table 1).

To study adverse effects of the hormonal treatment, chromosomes from zygotes as well as morulae and blastocysts of the NMRI strain were analysed. No signs of numerical or structural aberrations could be detected in all pronuclei derived from the males. In female pronuclei as well no aberrations were found after spontaneous ovulation. However, after hyperstimulated ovulation a variety of chromosomal abnormalities was found in the female chromosome sets (Table 2).

The persistence of these adverse effects which could be detected shortly after fertilisation was tested: on days 2 and 3 of gestation none of the surviving embryos from superovulated females was found to be chromosomally aberrant. Moreover, the SCE-frequencies of 4- and 8-cell embryos as well as morulae and blastocysts from superovulated females were not different from control values (Table 3).

Discussion

Loss of embryos during postimplantation period

Frequency of postimplantational embryonic loss in superovulated females is significantly higher (NMRI = 15%; Swiss = 24%) than in spontaneously ovulated females (10%). Nevertheless, gonadotropin treatment lead to a higher litter size. However, individually different reactions to hormonal treatment are evident particularly in Swiss mice as indicated by large standard deviations.

Table 3. - Chromosome aberrations and sister chromatid exchanges (SCE) in early embryos of the mouse strain NMRI after spontaneous ovulation and superovulation

	Spontaneous	Superovulated
Zygotes		
aberrations (no. = 100)	0	15
4- and 8-cell embryos		
aberrations (no. = 100 cells)	0	0
SCE/metaphase (no. = 25 cells)	14.7 ± 4.1	14.1 ± 4.2
Morulae and blastocysts		
aberrations (no. = 100 cells)	0	0
SCE/metaphase (no. = 25 cells)	14.0 ± 3.1	15.9 ± 5.4

Results of a previous study in mice indicate that embryonic death and developmental retardations which occur during postimplantation development after ovulation induction with gonadotropins, are predominantly due to hormone effects on the uterine environment rather than to direct effects on the embryo [13].

Loss of embryos during implantation

Our results are in good accordance with previous reports on normal development *in vitro* of preimplantation mouse embryos that were derived from gonadotropin exposed oocytes [14] and also *in vivo* up to term after transfer of such embryos to untreated foster mothers [13]. Thus, embryos do not seem to be affected by the hormons directly, but maternal factors must be responsible for the implantation failure of more than one third of day 3 embryos. The loss of embryos could be related to the abnormal high numbers of zygotes and early embryos per mother animal after hormonal treatment.

Loss of embryos during preimplantation period

From the data of the present study on mice it cannot be excluded that gonadotropins are able to interfere with DNA of maturating oocytes resulting in structural aberrations and also with the function of the meiotic spindle which finally leads to aneuploidy. In addition, gonadotropin treatment seems to change the zona pellucida as indicated by an increased rate of polyspermy. However, exposure of oocytes to gonadotropin did not exhibit a persisting genotoxic influence on cleavage stage embryos as revealed by genotoxicity tests on surviving day 2 and day 3 embryos. Zygotes bearing chromosome abnormalities after hormonal treatment were completely eliminated up to day two of pregnancy. In NMRI mice the frequency of the loss of preimplantation embryos is corresponding well with the rate of chromosomally aberrant zygotes. Although some of the polyploid zygotes could possibly have been deregulated to normal diploid embryos as observed after human IVF [15], abnormalities on the chromosomal level are evidently the main cause for embryo loss in early murine pregnancy after superovulation with gonadotropins.

Relation to human data

Data from animal experiments seem to be of particular importance, since gonadotropin stimulated maturation of oocytes and ovulation is widely applied for treatment of human infertility. One part of the present findings agrees well with the observations published by several authors, that a high rate of chromosomal abnormalities was found in unfertilized human oocytes as well as zygotes in the course of IVF trials [16, 17]. In contrast to the observations of the present and previous experiments on mice, chromosomal abnormalities were also found in cleavage stage human embryos [18]. Even in human abortions an increased rate of chromosomal abnormalities has been reported after hormonal stimulated ovulation compared to spontaneous ovulation [19]. The persistence of these abnormalities may indicate an even greater risk for defects generated by gonadotropins during the complex process of oocyte maturation at the chromosomal level in human embryos than in murine embryos. In our experiments the rate of malformations at term was not enhanced markedly. Only a strong increase in developmental retardations was found in the more sensitive Swiss strain. In humans, however, the induction of malformation by gonadotropins is still controversial and open to question. Recently published data of follow up studies in humans point to an increased risk of neural tube defects and the transposition of the great vessels among children born after IVF [20-22]. In these cases, oocyte maturation and ovulation was stimulated using different hormones among which gonadotropins play a dominant role.

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