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EPIGENETICS

The long-term effects of superovulation on fertility and sexual behavior of male offspring in mice

Zeng-Tao Wei · Xi-Lan Lu · Gang Zhang · Jing Yu · Hua Li · Gui-Hua Jia · Jun-Tao Li · Jian-Min Zhang

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Abstract
Purpose To evaluate the long-term effects of superovulation on fertility and sexual behavior of male offspring in mice.
Method The mice were superovaluted, and the fertility of male offspring (F1 generation and F2 generation) were evaluated in terms of the percentage of plugs and pregnancies, serum testosterone concentrations, and sperm motility. Furthermore, the sexual behavior of male offspring and sex ratio (F1 generation and F2 generation) were measured.
Results There were no significant differences in the percentage of plug and pregnancies, serum testosterone concentrations, sperm motilities and sex ratio between the offspring in naturally conceived group and superovulation groups (both F1 generation and F2 generation). The sperm hyperactivity at 90 min after incubation of F1 generation in naturally conceived group were higher than that of F1 generation in superovulation group, but the differences did not reach statistical significance. The offspring produced by superovaluted oocytes (both F1 generation and F2 generation) did not exhibit significant alterations in sexual behavior.
Conclusions No significant alterations were found in fertility and sexual behavior of male offspring in mice produced by superovaluted oocytes compared with those of naturally conceived offspring.

Keywords Superovulation · Male · Offspring · Fertility · Sexual behavior

Capsule The effect of superovulation on fertility and sexual behavior of male offspring.

Z.-T. Wei · H. Li · G.-H. Jia
Department of Obstetrics and Gynecology, Jinan Central Hospital Affiliated to Shandong University, 105 Jiefang Road, Jinan, China 250000

X.-L. Lu · J.-T. Li
Department of Reproductive Medicine, Jinan Central Hospital Affiliated to Shandong University, 105 Jiefang Road, Jinan, China 250000

G. Zhang
Department of Pain Management, Jinan Central Hospital Affiliated to Shandong University, 105 Jiefang Road, Jinan, China 250000

J. Yu
Operating Room of Jinan Central Hospital Affiliated to Shandong University, 105 Jiefang Road, Jinan, China 250000

J.-M. Zhang (✉)
Department of Reproductive Medicine, Maternal and Child Health Care Center of Shandong Province, 238 Jingshi East Road, Jinan, China 250000
e-mail: jmzszyx@126.com

Introduction

The prevalence of assisted reproductive technology (ART) is rapidly increasing as effective treatment for infertile couples. However, the consequences of manipulating germ cells and early embryos are not fully known. Accumulating evidence indicates that ART children are at increased risk for intrauterine growth retardation, premature birth, low birth weight, and genomic imprinting disorders [1, 2].

Superovulation, or ovarian stimulation, is an ART procedure that enables increased oocyte production. It has been common practice to treat infertility in humans. The use of high dose of exogenous hormones has recently caused much debate surrounding their effects on oocyte maturation [3, 4]. Because imprint acquisition has been shown to occur relatively late in oogenesis, the establishment of these imprints may be susceptible to exogenous hormone treatments [5–7].

To date, investigations of the effects of superovulation on imprinting in oocytes have produced conflicting results. Anckaert et al. observed normal methylation patterns at *Snrpn*, *Peg3*, and *H19* loci, along with the *Igf2r* locus in pooled oocytes from superovulated female mice [8]. Similarly, it was reported that maternal imprint acquisition

was not affected by superovulation [9]. In contrast to above results, Sato et al. found an aberrant gain of DNA methylation at the normally unmethylated *H19* locus in pooled mouse oocytes. Additionally, they found that individual human oocytes from women undergoing multiple hormone stimulations possessed aberrant imprinting at both the *PEG1* and *H19* loci [10]. Market-Velker et al. reported that superovulation alone affected genomic imprinting in blastocyst-stage embryos at four imprinted genes (*Snrpn*, *Kcnq1ot1*, *Peg3*, and *H19*) in a hormone dosage-dependent manner. A greater frequency of aberrant methylation occurs at the high hormone dosage [11]. Data support the notion that ovulation induction via exogenous hormones may cause aberrant methylation maintenance during preimplantation development [11].

It is becoming increasingly obvious that the period of time during fetal development is an important factor in the life-long health of the individual [12]. An adverse in utero environment may profoundly influence an individual's susceptibility to disease late in childhood, adolescence and adult life [13]. Jensen et al. found significantly reduced sperm counts, fewer motile sperm, and smaller testes in offspring of women who received hormone administration during pregnancy to prevent spontaneous abortion [14]. Whether in utero exposure to estrogen excess induced by ovarian stimulation exerts long-term effects or transgenerational effects on the fertility of male offspring or not? Since the first IVF-conceived baby was born in 1978, it is difficult to carry out large longitudinal studies, we therefore employ animal model to preliminarily testify the postulation.

Materials and methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Animals

Female C57BL/6 mice at 8–9 week of age were used for ovarian stimulation. Male C57BL/6 mice at 30 week of age were used for mating. Mice were kept under pathogen-free condition with a 30–60 % humidity, a temperature ranging from 21 to 24 °C, a light cycle of 12 h light: 12 h darkness, and were given free access to sterile food and water. The Animal Care Committee of Shandong University approved all the experimental procedures carried out in the study.

Ovarian stimulation

The protocol was carried out as described previously [11]. The female mice were randomly divided into two groups (n=50 per group): controlled group and ovarian stimulation group (stimulation with 10 IU Pregnant Mare's Serum Gonadotropin, Intervet Canada). Administered of Pregnant Mare's Serum Gonadotropin was followed by the same dose of Human Serum Chorionic gonadotropin (hCG, Intervet Canada) 40–44 h later. Female were mated with males, and pregnancy was determined by the presence of a vaginal plug the following morning. The offspring of F0 generation were F1 generation, and the offspring of F1 generation were F2 generation. Male F1 generation and F2 generation produced by superovaluted oocytes as well as the naturally conceived male offspring were used for following studies. The experiments were performed when the offspring were 30 weeks old; therefore, the animals are referred to as adult. All the mice had not been used for any other study.

Comparison of the fetal development, sex ratio, fertility, and reproduction of offspring in mice

Individually housed male C57BL/6 mice at 30 week of age (n=50 per group) were cohabited with three female mice for a period of 5 days, after which males and females were separated. Every day during the cohabitation, females were examined for plugs as evidence of mating. On the 13th days of gestation, female mice were humanely killed with CO_2/O_2 and the uterine horns were opened. The percentages of resorption, percentages of live fetus, fetus weight, and sex ratio were registered for the different groups. Live fetuses were humanely killed after examination [15]. The aforementioned fertility study was repeated three times with different male mice.

Fasted serum testosterone measurements

Male mice (n=50 per group) were anesthetized. Whole blood sampled from the retro-orbital venous sinus was collected in BD Microtainer Serum Separator Tubes (Becton, Dickinson, and Company, Suwanee, GA), allowed to clot at room temperature, and centrifuged for serum harvest. Serum testosterone was determined using a radioimmunoassay kit obtained from Linco Research (St. Charles, Missouri) on an Apex Gamma Counter (ICN Micromedic Systems, Inc., Huntsville, AL).

Cauda epididymal sperm count measurements

Male mice (n=50 per group) were anesthetized. The testis, epididymis, and vas deferens were immediately removed. The testis was dissected from the epididymis and fat. Subsequently, the epididymis and vas deferens were dissected away from the fat. In a 12-well plate, the epididymis and vas deferens from each animal were placed in a well containing 1.0 ml of M2 buffer. Using a watch glass and tweezers, any remaining fat and blood vessels were removed from both the

epididymis and vas deferens. The epididymis was then cut at the junction between the corpus and cauda epididymis, and the cauda was placed into a well with 1.0 ml of M2 buffer. Several cuts were made in the cauda epididymis with scissors, and sperm was gently pressed. Sperm was also expressed from the vas deferens in a separate well and then removed from the plate. The pressed sperm from the cauda epididymis was then collected in an Eppendorf tube. Using a hemocytometer (15 μl per side), sperm counts were determined as number of sperm per microliter [16].

Sperm motility and progressive measurements

Quantitative parameters of sperm motility were determined by computer assisted sperm analysis (CASA). Sperm from the cauda epididymis were incubated in 1.0 ml of M2 medium at room temperature for 30 min and loaded into CASA assay chambers (Hamilton Thorne Research, Beverly, MA). Sperm tracks (1.5 s, 30 frames) were captured at 60 Hz and analyzed using HTMIVOS Sperm Analyzer software (version 12.2 L; Hamilton Thorne). The parameters during measurements were: minimum contrast, 30; minimum cell size, 4 pixels; straightness threshold, 50.0 %; path velocity cutoff, 10.0 μm/s; progressive minimum path velocity, 50.0 μm/s; static head size, 0.13 to 2.43; static head intensity, 0.10 to 1.52; and static elongation, 5 to 80 [16].

To prepare the hyperactivated sperm, sperm were allowed to disperse into 1.0 ml PBS for 5 min at 37 °C. After a brief low-speed centrifugation (100×*g* for 15 s at room temperature) to remove debris, the sperm were centrifuged at 500×*g* for 8 min at 4 °C and resuspended in 2 ml of M16 medium, and the aliquots were either processed as below or incubated at 37 °C in 5 % CO_2/95 % air for 1.5 h. Hyperactivated sperm were measured using the following sorting parameters: track speed >170 μm/s, amplitude of lateral head displacement >9.0 μm/s, and linearity <30 % [16].

Sexual behavior

Fifteen days after the testectomy, male mice received testosterone propionate for testing male sexual behavior. For assessing male sexual behavior, mice received 1 mg per animal testosterone propionate and observed 24 h later under red-light illumination during the dark phase of their cycle. The females utilized for the behavioral test were previously treated with estradiol benzoate (20 μg/kg, i.p.) for inducing estrus 24 h before the test [17]. The animals were placed into the observation cage, and observed for 30 min for male sexual behavior (recognition of the receptive female and mount) [18].

Statistical analysis

The percentages of resorption, percentages of live fetus, sex ratio, percentage of plugs and pregnancies, sperm motility, and frequency of male sexual behavior were compared using chi-square test analysis. The mean fetal weight, sperm count, serum testosterone concentrations, number of mounts, and latency to the first mount were compared by analysis of variance (ANOVA). Values were considered significant when $P < 0.05$. SAS version 8.1 software (SAS Institute, Cary, NC, USA) was used for all statistical analysis.

Results

Effect of ovarian stimulation on fetal development and sex ratio

The percentages of live fetus, mean fetus weight of F1 generation in ovarian stimulation group were significantly lower or less than those in naturally conceived group respectively, and the percentage of resorption of F1 generation in ovarian stimulation group was significantly higher than that in naturally conceived group ($P < 0.05$). No significant differences were found in sex ratio of F1 generation between ovarian stimulation group and naturally conceived group ($P > 0.05$). There were no significant differences in the percentages of resorption, percentages of live fetus, mean fetus weight, and sex ratio of F2 generation between ovarian stimulation group and naturally conceived group ($P > 0.05$) (Table 1).

Effect of ovarian stimulation on copulation and fertility

No significant differences were observed in the percentage of plugs and pregnancy rates of F1 generation between control group and superovulation group ($P > 0.05$). There were no

Table 1 The percentages of resorption, percentages of live fetus, mean fetus weight, and sex ratio in each group

Group	Percentages of resorption (%)	Percentages of live fetus (%)	Mean fetus weight (g)	Sex ratio (male/female)
F1 generation of control group	2.9±0.2	95.6±8.9	158.2±12.4	1.01±0.09
F1 generation of superovulation group	15.4±0.8	79.5±9.3	121.8±16.4	0.09±0.08
F2 generation of control group	1.9±0.1	96.9±6.7	156.7±14.2	1.02±0.08
F2 generation of superovulation group	2.1±0.2	95.8±9.2	152.3±16.8	1.01±0.08

Table 2 The percentage of plug, pregnancy rates, and serum testosterone concentration

Group	Percentage of plug (%)	Pregnancy rates (%)	Serum testosterone concentration (ng/dl)
F1 generation of control group	76.8±4.8	79.1±8.6	625.8±23.8
F1 generation of superovulation group	74.6±5.6	76.9±7.9	618.9±31.6
F2 generation of control group	75.9±6.2	79.3±5.1	627.1±33.9
F2 generation of superovulation group	73.6±5.1	80.9±5.8	628.2±29.8

significant differences in the percentage of plugs and pregnancy rate in the F2 generation in control group in comparison to those in superovulation group ($P>0.05$) (Table 2).

Effect of ovarian stimulation on serum testosterone concentration

There were no significant difference in serum testosterone concentration in the F1 generation in control group in comparison to that in superovulation group ($P>0.05$). Similarly, no significant differences were observed in serum testosterone concentration of F2 generation between control group and superovulation group ($P>0.05$) (Table 2).

Effect of ovarian stimulation on sperm count, motility

No significant differences were observed in the sperm count and sperm motility of F1 generation between control group and superovulation group ($P>0.05$). Similarly, there were no significant differences in the sperm count and sperm motility of F2 generation between control group and superovulation group ($P>0.05$). The F1 generation in superovulation group exhibited a slight decrease in sperm hyperactivity at 90 min after incubation in comparison to F1 generation in control group. However, the differences did not reach statistical significance ($P>0.05$) (Table 3).

Sexual behavior

No significant differences were observed in the frequency of male sexual behavior, number of mounts, and latency to the first mount between the F1 generation in control group and that in superovulation group ($P>0.05$). Similarly, there were no significant differences in the frequency of male sexual behavior, number of mounts, and latency to the first mount between the F2 generation in control group and that in superovulation group ($P>0.05$) (Table 4).

Discussion

Genome imprinting is a phenomenon leading to parent-of-origin-specific monoallelic expression of genes in diploid cells. For most genes, both the paternal and maternal alleles are actively transcribed. In contrast, a small number of so-called "imprinted genes" are expressed from only the paternal or from only the maternal allele [19]. A balanced expression of imprinted genes is indeed essential for normal embryo development, placental differentiation and pre- and postnatal growth, but also for normal neurobehavioral processes and metabolism [2, 20]. Data from mouse experiments and the in vitro production of livestock provide strong evidence that imprint establishment in late oocyte stages and reprogramming of the two germline genomes for somatic development after fertilization are vulnerable to environmental cues [20]. During early life, an organism is able to adjust its phenotypic development in response to environmental cues. There are now increasing data that these responses are, at least partially, underpinned by epigenetic mechanisms. A mismatch between the early life environments may lead to inappropriate early life-course epigenomic changes that manifest in late life as increased vulnerability to disease [21]. For example, individuals who were conceived during a famine period showed methylation changes in the imprinted growth factor *IGF2* and other medically relevant genes more than 60 years late [22–24]. At the same time, these individuals suffered from increased risks for obesity, coronary artery disease, accelerated cognitive aging, and schizophrenia [25, 26]. Srinivasan

Table 3 The sperm count, sperm motility, and sperm hyperactivity at 90 min after incubation in each group

Group	Sperm count ($\times 10^5$/mL)	Sperm motility (%)	Sperm hyperactivity at 90 min after incubation (%)
F1 generation of control group	2.65±0.12	67.5±4.6	9.2±0.8
F1 generation of superovulation group	2.63±0.13	68.9±4.3	8.8±0.6
F2 generation of control group	2.66±0.11	69.3±5.1	9.1±0.9
F2 generation of superovulation group	2.67±0.16	67.9±5.8	8.9±0.8

Table 4 Frequency of male sexual behavior, number of mounts, and latency to the first mount (seconds) during 30 min of observation in each group

Group	Frequency (%)	Number of mounts	Latency to the first mount (s)
F1 generation of control group	99	36.5±2.4	191.5±13.4
F1 generation of superovulation group	100	35.2±2.1	193.8±11.8
F2 generation of control group	100	37.5±2.8	187.1±9.4
F2 generation of superovulation group	100	38.2±2.9	189.5±11.4

et al. reported that maternal high-fat diet consumption results in fetal malprogramming predisposing to the onset of metabolic syndrome-like phenotype in adulthood [27].

Prenatal exposure to androgen excess can lead to, as adults, disrupted ovarian cycles and abnormalities of early follicle development that mimic those observed in women with polycystic ovary syndrome [28–31]. It was reported that environmental factors can affect spermatogenesis at the level of germ and Stertoli cells and the composition of seminal fluid [32, 33]. It raises the concern that in utero exposure to estrogen excess exerts everlasting effect on male reproductive function.

In this study, the percentages of live fetus, mean fetus weight of F1 generation in ovarian stimulation group were significantly less or lower than those in naturally conceived group, and the percentages of resorption of F1 generation in ovarian stimulation group were significantly higher than those in naturally conceived group. It has been reported that superovulation of female mice delayed embryonic and fetal development [3, 34]. The possible explanation is that superovulation increases the proportion of chromosomal abnormalities in oocytes in rats and uterine horn is incapable of nourishing large number of blastocysts tyring to implant and also to develop in to a fetus [35]. However, there were no significant differences in the percentages of resorption, percentages of live fetus, and mean fetus weight between the F2 generation in ovarian stimulation group and naturally conceived group.

The present experiment showed that there were no significant differences in the percentage of plug and pregnancies, sex ratio, serum testosterone concentrations, and sperm motilities of offspring (both F1 generation and F2 generation) between naturally conceived group and ovarian stimulation group. It is worth mentioning that the sperm hyperactivity at 90 min after incubation of naturally conceived group were higher than that in ovarian stimulation group, but the differences did not reach statistical significance. Furthermore, the offspring produced by superovaluted oocytes (both F1 generation and F2 generation) did not exhibit significant alterations in sexual behavior. The results of this study demonstrated a negative correlation between superovulation and fertility reduction or/and sexual behavior alteration of male offspring.

Transgenerational effects results from a mother's exposure and are inherited through successive generations in the absence of direct exposure of the offspring. Such environmentally induced effects have been demonstrated, in some cases lasting dozens of generations [36, 37]. The results of this study preliminarily proved that superovulation does not exert transgenerational effects on the fertility or/and sexual behavior alteration of male offspring in mice.

In summary, the data of this animal experiment preliminarily prove that ovarian stimulation does not lead to significant reduction in fertility or/and sexual behavior alteration of male offspring. However, we suggest large-scale, multi-center follow-up studies of the long-term effect of superovulation on the human male offspring fertility be performed.

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Conflict of interest The authors declare that there are no conflicts of interest.

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