Superovulation does not affect the endocrine activity nor increase susceptibility to carcinogenesis of uterine and mammary glands of female offspring in mice

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Abstract

Purpose To evaluate the dual effects of superovulation on the endocrine activity and susceptibility to carcinogenesis of uterine and mammary glands of female offspring in mice
Method The mice were superovulated. The relative uterine weight, ER α protein expression, and endocrine activity of female offspring (F1 generation and F2 generation) were measured. Furthermore, proliferative lesion of uterine and mammary glands of female offspring (F1 generation and F2 generation) was assessed by histopathologic examinations.
Results There were no significant differences in relative uterine weight, ER α protein expression, incidence of proliferative lesion in mammary glands, and incidence of atypical hyperplasia, adenocarcinoma, and squamous metaplasia in uterine among the offspring (F1 generation and F2 generation) in

each group. Likewise, there were no significant intergroup differences in the serum levels of sex related hormones.

Conclusions No significant alterations were found in the endocrine activity and susceptibility to carcinogenesis of uterine and mammary glands of female offspring in mice produced by superovulated oocytes compared with those of naturally conceived offspring.

Keywords Superovulation · Female · Offspring · Endocrine activity · Carcinogenesis

Capsule Effects of superovulation on the endocrine activity and susceptibility to carcinogenesis of offspring

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Introduction

The use of assisted reproductive technologies (ARTs) for the treatment of human subfertility/infertility contributes 2–4 % of all children born in developed countries [1]. However, the safety of these technologies has yet to be fully evaluated. Accumulating evidence indicates that ART children are at increased risk for intrauterine growth retardation, premature birth, low birth weight, early adulthood clinical depression, binge drinking, and genomic imprinting disorders [2–4].

Superovulation, or ovarian stimulation, is an ART procedure that enables increased oocyte production. It has been common practice to treat infertility in humans. Gonadotrophins are commonly used for superovulation in animals and in humans, for example to increase the number of oocytes that can be retrieved for in vitro fertilization, thus improving the overall chance for successful fertilization and pregnancy. The use of high dose of gonadotrophins has recently caused much debate surrounding their effects on oocyte maturation [5, 6]. Because imprint acquisition has been shown to occur relatively late in oogenesis, the establishment of these imprints may be susceptible to exogenous hormone treatments [7–10].

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 [11]. An adverse in utero environment may profoundly influence an individual's susceptibility to disease late in childhood, adolescence and adult life [12]. Data suggest xenoestrogen exposure during pregnancy alters hormone programming so that the uterus responds abnormally to estrogen later in life [13]. It was reported that prenatal or developmental exposure to bisphenol A delayed the development of the male mammary gland and increased susceptibility to prostate carcinogenesis [14, 15]. Females exposure in utero to the synthetic estrogen, diethylstilbestrol (DES), commonly referred to as "DES daughters", have increased risks of vaginal cancer after puberty [16, 17]. Furthermore, neonatal exposure to DES in rats has been reported to affect mammary carcinogenesis [18]. Whether in utero exposure to estrogen excess induced by superovulation exerts long-term effects even transgenerational effects on the endocrine activity and susceptibility to carcinogenesis of uterine and mammary glands of female 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

Animals

Female C57BL/6 mice at 8–9 wk of age were used for ovarian stimulation. Male C57BL/6 mice at 30 wk 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 [19]. 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. Female F1 generation and F2 generation produced by superovulated oocytes as well as the naturally conceived female offspring were used for following studies. The experiments were

performed when the offspring were 10 months old; therefore, the animals are referred to as adult.

Immunohistochemistry

Female offspring ($n=30$ per group) was euthanized on estrus by CO₂ inhalation followed by decapitation, and body and uterine weights were determined. The relative uterine weight (uterine weight/body weight) was calculated. Uterines (with fluid) were fixed in formaldehyde in PBS (10 %), dehydrated in ethanol and embedded in Paraplast. To perform the immunohistochemistry for estrogen receptor alpha (ER α - 6 F11:sc-56836, Santa Cruz, 1:1000), biotinylated anti-mouse IgG (1:100) and normal horse serum were used.

Uterine transverse sections (6 μ m) were dewaxed using toluene and hydrated using decreasing concentrations of ethanol, followed by antigen recovery with citrate buffer (0.01 M, pH 6.0 - microwave, 650 watt, high temperature, 3 min $\times$ 5 min) [20]. After PBS wash, the slides were incubated for 10 min with blocking solution to avoid unspecific reactions. Then, sections were incubated overnight at 4 °C with primary antibody.

The sections were incubated for 1 h at room temperature with appropriate secondary biotinylated antibody (Vector Laboratories, Inc.). Then, slides were incubated with ABC solution (1:400) for 1 h at room temperature (VECTASTAIN ABC KIT, PK 4000, Vector laboratories, Inc.) to evidence the immunocomplex formed by secondary antibody linked to the primary antibody.

Peroxidase activity was revealed using diaminobenzidine solution (DAB) (Vector Laboratories, Inc.) for 10 min at room temperature. The sections were washed and counterstained with toluidine blue, dehydrated by applying increasing concentrations of ethanol, cleared by xylene and coverslipped with Permount. For the steroid receptors, sections were analyzed under light microscope and the evaluation was performed by semi-quantitative scale, according to intensity of staining at luminal and glandular epithelium, stroma and myometrium.

The result of immunohistochemical staining for ER α was evaluated semiquantitatively based on Guerra et al. [21]: overstained (diffusion of the reaction product and/or non-specific background), strong (black or dark brown), light (medium brown), very light staining (usually restricted to a smaller number of stained cells) or no visible staining.

Histopathologic assessment of proliferative lesions in the uterus and mammary glands

The organs ($n=30$ per group) were fixed in 10 % neutral buffered formalin. Tissues were routinely processed and stained with hematoxylin and eosin for histopathologic examination. Preneoplastic lesions were classified into three degrees of atypical hyperplasia (slight, moderate, or severe) and adenocarcinomas according to a previous study [22]. Slight/moderate

Table 1 The relative uterine weight (uterine weight/body weight), ER α protein expression, and histopathologic findings of the uterus observed in mice

	F1 generation of control group	F1 generation of superovulation group	F2 generation of control group	F2 generation of superovulation group
No. of animals examined	50	50	50	50
The relative uterine weight (%)	0.88 $\pm$ 0.06	0.86 $\pm$ 0.04	0.89 $\pm$ 0.09	0.86 $\pm$ 0.08
ER α intensity	134.6 $\pm$ 18.9	132.8 $\pm$ 16.4	136.4 $\pm$ 14.2	138.3 $\pm$ 15.2
Atypical hyperplasia	12 (24 %)	15 (30 %)	14 (28 %)	16 (32 %)
Slight	9	10	12	14
Moderate	3	4	2	2
Severe	0	1	0	0
Cystic endometrial hyperplasia	8 (16 %)	10 (20 %)	9 (18 %)	12 (24 %)
adenocarcinoma	0	0	0	0
Squamous metaplasia	4 (8 %)	5 (10 %)	3 (6 %)	4 (8 %)
Adenomyosis	5 (8 %)	2 (4 %)*	6 (12 %)	4 (8 %)
Disappearance of lumina	0	0	0	0

*: Significantly different from F1 generation of control group

endometrial hyperplasias refers increased numbers of glands with slightly/moderately atypical cells in focal and/or diffuse areas of the endometrium. Severe hyperplasias were composed of irregular proliferation of atypical glands, which were characterized by crowding, stratification, and irregularity of cells with back-to-back disposition. Lesions composed of glandular-structured epithelial cells with atypia showing invasive proliferation to the muscle layer or serosa were diagnosed as endometrial adenocarcinomas. Histological slides were observed blindly, under a light microscope, by two independent veterinary pathologists.

Hormone assays

Serum samples ($n=30$ per group) obtained after decapitation were stored at -80°C until assay. The serum

concentration of follicle-stimulating hormone (FSH), luteinizing hormone (LH), inhibin, estradiol-17 β (E_2), progesterone (P_4), and prolactin (PRL) were determined using double-antibody radioimmunoassay and ^{125}I -labeled radio-ligands. National Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were used for rat FSH, LH, and PRL (NIAMDD, NIH, Bethesda, MD, USA) with anti-rat LH-S-11, anti-rat FSH-S-11 and anti-rat PRL-S-9 sera, as described previously [23]. P_4 and E_2 were measured using the anti-sera against P_4 (GDN 337) and E_2 (GDN 244) as described by Takahashi et al. [24]. Iodinated 32-kDa bovine inhibin and a rabbit antibody against bovine inhibin (TNDH-1) were used for measurement of immunoreactive serum inhibin, as described previously [25].

Table 2 Histopathologic findings of the mammary glands observed in mice

	F1 generation of control group	F1 generation of superovulation group	F2 generation of control group	F2 generation of superovulation group
No. of animals examined	50	50	50	50
Increased milk secretion	14 (28 %)	20 (40 %)*	13 (26 %)	15 (30 %)
Slight	12	14	10	12
Moderate	2	5	1	3
Severe	1	1	2	0
Atypical hyperplasia	2 (4 %)	3 (6 %)	1 (2 %)	2 (4 %)
Lobular hyperplasia	0	0	1 (2 %)	0
Ductal hyperplasia	0	0	0	1 (2 %)
Adenoma	0	1 (2 %)	0	0
Fibroadenoma	0	0	0	0
Oxyphilic cells	0	0	0	0

*: Significantly different from F1 generation of control group

Fig. 1 **A** Normal morphologic endometrial. **B** Endometrial hyperplasias were composed of irregular proliferation of atypical glands, which were characterized by crowding, stratification, and irregularity of cells with back-to-back disposition (magnification $\times 200$)

Statistical analysis

The proliferative lesions of uterus and mammary glands in each group were compared using chi-square test analysis. The mean relative uterine weight and the serum levels of sex related hormones were analyzed first using Bartlett's test (significance level: 1 %) to examine homogeneous of variance. If the variance was determined to be homogeneous, a Dennett's test (significance level: 5 %, indices are shown at both $P < 0.05$ and $P < 0.01$, two tailed test) was conducted. However, if the variance was heterogeneous, a Dennett's multiple comparison test (significance level: 5 %, indices are shown at both $P < 0.05$ and $P < 0.01$, two=tailed) was applied. 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

Uterus weight and ER α protein expression

No significant differences were found in the mean relative uterine weight and ER α protein expression of F1 generation between ovarian stimulation group and

naturally conceived group ($P > 0.05$). Likewise, there were no significant differences in the mean relative uterine weight and ER α protein expression of F2 generation between ovarian stimulation group and naturally conceived group ($P > 0.05$) (Table 1).

Histopathologic assessment of proliferative lesions in the uterus and mammary glands

No significant differences were observed in the incidence of uterine atypical hyperplasia, adenocarcinoma, and squamous metaplasia of F1 generation between control group and superovulation group ($P > 0.05$). There was a significant decrease in the incidence of adenomyosis of F1 generation produced by superovulated oocytes ($P < 0.05$).

There were no significant differences in the incidence of uterine atypical hyperplasia, adenocarcinoma, squamous metaplasia, and adenomyosis of F2 generation in control group in comparison to those in superovulation group ($P > 0.05$). In the mammary glands, although increased milk secretion was frequently observed in F1 generation produced by superovulated oocytes, the incidence and severity were similar among the groups ($P > 0.05$) (Table 2) (Figs. 1-2 and 3).

Fig. 2 **A** Normal morphologic mammary glands. **B**: Breast ductal hyperplasias were composed of irregular proliferation of atypical glands, which were characterized by crowding, stratification, and irregularity of cells with back-to-back disposition (magnification $\times 200$)

Fig. 3 Endometrial in the uterine muscle (→) (magnification $\times 200$)

Hormone assays

There were no significant intergroup differences in the serum levels of sex related hormones of the F1 generation in control group in comparison to that in superovulation group ($P>0.05$). Similarly, no significant differences were observed in the serum levels of sex related hormones of F2 generation between control group and superovulation group ($P>0.05$) (Table 3).

Discussion

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 [26]. There are now increasing data that these responses are, at least partially, underpinned by epigenetic mechanisms. Data from mouse experiments and the in vitro production of livestock provide strong evidence that imprint establishment in late oocyte stages and reprogramming of germline genomes for somatic

development after fertilization are vulnerable to environmental cues [4]. 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 later [27]. At the same time, these individuals suffered from increased risks for obesity, coronary artery disease, accelerated cognitive aging, and schizophrenia [28]. 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 [29]. Bosquiaz et al. reported that perinatal exposure to diethylstilbestrol altered the functional differentiation of the adult rat uterus [30]. Moreover, neonatal exposure to estrogen might be a risk factor for uterine carcinogenesis [24].

In this study, there were no significant differences in relative uterine weight and ER α protein expression among the offspring in each group. Likewise, no statistical differences were noted in the incidence of uterine atypical hyperplasia, adenocarcinoma, and squamous metaplasia among the groups. It is worth mentioning that there was a significant decrease in the incidence of adenomyosis of F1 generation produced by superovulated oocytes. In the mammary glands, although increased milk secretion was frequently observed in F1 generation produced by superovulated oocytes, the incidence and severity were similar among the groups. Therefore, we concluded that superovulation did not increase tendency toward severity of proliferative lesion in uterine and mammary glands of offspring produced by superovulated oocytes in mice.

Endocrine activity is regarded as a very useful indicator of delayed adverse effects on the female reproductive tract [24]. The results of this study indicated that there were no significant intergroup differences in the serum levels of sex related hormones.

Transgenerational effects results from a mother's exposure and are inherited through successive generations in the absence of direct exposure of the offspring. Such environmental

Table 3 Serum level of sex-related hormones at 10 months of age

	F1 generation of control group	F1 generation of superovulation group	F2 generation of control group	F2 generation of superovulation group
No. of animals examined	50	50	50	50
E ₂ (pg/ml)	75.8 $\pm$ 5.8	73.1 $\pm$ 6.1	72.9 $\pm$ 5.3	75.4 $\pm$ 4.9
P ₄ (ng/ml)	15.6 $\pm$ 0.9	17.1 $\pm$ 1.1	16.8 $\pm$ 1.1	16.3 $\pm$ 1.4
FSH (ng/ml)	2.1 $\pm$ 0.1	1.9 $\pm$ 0.1	2.2 $\pm$ 0.1	2.3 $\pm$ 0.2
LH (pg/ml)	38.6 $\pm$ 2.1	37.4 $\pm$ 2.6	40.1 $\pm$ 3.9	41.8 $\pm$ 3.1
PRL (ng/ml)	2.4 $\pm$ 0.2	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1	2.3 $\pm$ 0.2
Inhibin (ng/ml)	3.9 $\pm$ 0.2	4.1 $\pm$ 0.3	3.8 $\pm$ 0.2	4.0 $\pm$ 0.3

factors can affect spermatogenesis at the level of germ and stertoli cells and the composition of seminal fluid [31], in some cases lasting dozens of generations [32]. The results of this study preliminarily proved that superovulation does not exert transgenerational effects on the endocrine activity and susceptibility to carcinogenesis of uterine and mammary glands of female offspring in mice. Nevertheless, Sato et al. found that individual human oocytes from women undergoing multiple hormone stimulations possessed aberrant imprinting at both the *PEG1* and *H19* loci [33]. 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 [19]. So, we suggest large-scale, multi-center follow-up studies of the long-term effect of superovulation on the human offspring be performed.

In summary, the data of this animal experiment preliminarily prove that no significant alterations were found in the endocrine activity and susceptibility to carcinogenesis of uterine and mammary glands of female offspring in mice produced by superovulated oocytes compared with those of naturally conceived offspring.

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

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