TAMOXIFEN EXPOSURE AFFECT GENES EXPRESSION INVOLVED IN MOUSE OVARY DEVELOPMENT DURING SEX DETERMINATION

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Introduction

Developmental exposure to hormone-like chemicals induces morphological, functional, and behavioral anomalies throughout the lifetime in mammalian. Among the hormone-like chemicals, tamoxifen is receiving increased attention due to extensively clinical application. Tamoxifen is a kind of drugs used for women suffered from breast cancer by inhibiting estrogen. During the last decade, studies on the effect of tamoxifen–induced recombination suggested tamoxifen effectively modify genes in the mouse embryo [2-3]. Reptile eggs exposure to tamoxifen has been shown to induce sex reversal of male to female even at low doses, such as 0.14 ppm [1]. In mammals, hormone is crucial for the development and sex differentiation and determination. Tamoxifen, as a kind of hormone-like compound has been used for decades. However, relatively little research has been conducted in the mammals to elucidate its pathogenic mechanism as an antiestrogen induced female masculinization in perinatal and adult period. Female masculinization suggested tamoxifen exposure might affect sex differentiation and steroid production. To test this hypothesis, two complementary approaches were used in this study, (i) using fetal mouse gonad cultures and (ii) using mice to evaluate the longterm consequences of fetal and adult exposure *in vivo*. The purpose of the present study was to investigate the effect of perinatal and adult exposure to tamoxifen on the development and function of the ovary and the expression of key genes involved in sex determination and stability.

Materials and methods

Animals

ICR mice were purchased from Dalian Medical University (Dalian, China). The animals were acclimatized to the laboratory for 1 week and maintained in humidity (30-40%) and temperature (24 ℃) controlled room with a 12 h light, 12 h dark cycle. The animals were given access to food and water *ad libitum*.

Experimental paradigm

Tamoxifen was dissolved in corn oil at a concentration of 10 mg/ml. The adult female mice were treated with tamoxifen at doses of 75 and 225 mg/kg body weight. Corn oil alone served as the control. The female and male mice were injected tamoxifen intraperitoneally for 5 consecutive days. Five days after the last injection, ovaries and testis from the mice were removed, weighed, rapidly frozen and stored in liquid nitrogen or fixed in paraformaldehyde. Blood was collected and serum was separated by centrifugation and stored at -20 ℃.

Gonad culture and treatments

Gonad/mesonephros complexes were dissected from 12.5 dpc (days post coitum) and 13.5 dpc. Gonads were placed on Millicell CM filters floating on 0.5 ml of culture medium (Dulbecco's Modified Eagle Medium, Invitrogen, USA), 3% fetal bovine serum as described as described by Smith et al [4]. Three to five gonad/ mesonephros complexes were placed on each filter and the gonad cultures were incubated at 37℃ and 5% CO_2. For the tamoxifen treatment experiments, gonads were cultured for 3 d with 1.0 μM and 0.1 μM tamoxifen or in the presence of a vehicle control. The culture medium was refreshed every 2 d. At the end of the culture, gonads were fixed in formalin. For RNA analysis, a pool of at least three ovaries and testes were stored at liquid nitrogen. The entire media samples were kept at -20 ℃ until use for progesterone.

Determination of hormone by RIA

Testosterone, progesterone, estradiol and gonnadotrophin concentrations were measured using a specific RIA assay as described [5].

RNA extraction, reverse transcription and qPCR analysis

Total RNA was extracted using the RNeasy Plus mini-Kit (Qiagen, Courtaboeuf, France) including DNase I treatment and 200 ng samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's instructions. Real-time quantitative PCR (Q-PCRs) was performed using the SYBR green real-time PCR kit (Applied Biosystems, Courtaboeuf, France). Each sample was measured in duplicate in two independent experiments. Results were analyzed by using the delta–delta Ct method with the use of ACTB as a normalized control. The PCR products were separated on a 1.5 % agarose gel stained with ethidium bromide and analyzed by scanning densitometry.

Histopathology and immunohistochemistry

For histopathological assay, organs and gonads were fixed in 4% paraformaldehyde overnight, dehydrated via a series of alcohol and xylene treatment, and embedded with paraffin under vacuum. 7 μm sections were cut on an RM2155 rotary microtome (Leica, Nusslock, Germany), and stained with hematoxylin and eosin. Commercial antibodies, including AMH and SOX9, were used in immunostaining. Stained slides were examined with Olympus CX31 microscope. Images were captured by Canon A640 camera. At least three pairs of control and tamoxifen-induced gonads were analyzed.

Statistical analysis

Results were expressed as the mean ± S.E. Statistical differences were determined using One-Way ANOVA followed by t-test for samples from the same littermate or different littermates respectively.

Results and discussion

Effect of tamoxifen on the morphology of embryo gonad

The morphology of embryo XX gonads was not markedly changed after administrating tamoxifen to pregnant mice (Fig.1). Tamoxifen is frequently associated with a fluctuation in progesterone, testosterone and LH concentrations, which are important hormone secreted during gonad development and involved in masculinization, in different *in vivo* and *in vitro* models. Gonads were incubated in the absence or presence of tamoxifen (0.1mM to 1 mM) for 72 h. The results included the consequences of tamoxifen stimulation on progesterone and testosterone. Medium progesterone concentrations of the gonad exposure to tamoxifen for culturing 3 d reduced with no significance because of a large variation (data not shown). However, the concentrations of progesterone and LH in mouse serum were reduced in adult female group by intraperitoneal injection of tamoxifen ($P<0.001$). Testosterone concentrations in mouse serum and in medium did not exhibit significant change in the present study (data not shown). To assess whether ovary growth was affected after exposure to tamoxifen, body weight and organ coefficient of ovary and uterus were measured. Significant differences were observed among the experimental groups, where the bilateral ovaries and uterus weight decreased in the tamoxifen-treated animals (Table 1).

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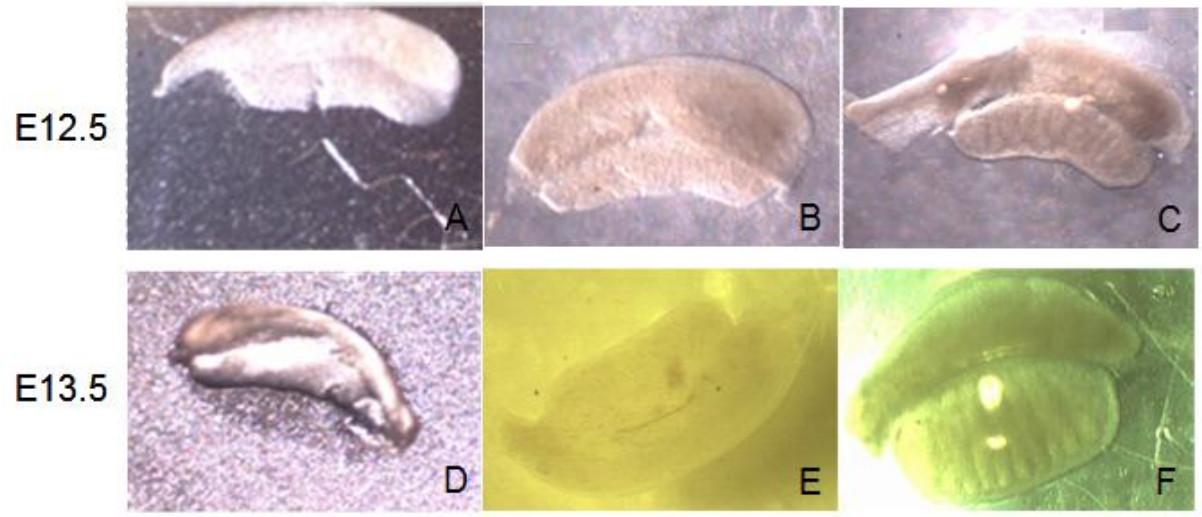

Fig.1. Whole-mount light field microscopic images of the genital ridge.
A, D: 0 mg/kg·BW, XX gonad; B, E: 75 mg/kg·BW, XX gonad; C, F: XY gonad

Table 1: Body weight and organ coefficient of ovary and uterus in female mice exposed to corn oil or tamoxifen

	Control vehicle	Tamoxifen	
		75 mg/kg·BW	225 mg/kg·BW
Female mice	n=9	n=8	n=9
Body weight at necropsy (g)	35.65±1.43	36.43±1.32	38.38±0.76
Uterus (g/ kg·BW)	5.33±0.38	4.73±0.21	4.64±0.32
Bilateral ovaries (g/ kg·BW)	0.64±0.02	0.35±0.04[a]	0.39±0.05[a]

Effect of the exposure to tamoxifen on the reproductive organs was analyzed using One-Way ANOVA. All data are expressed as means ± S.E. a, P<0.001 vs. control group

Testicular phenotype in ovary exposure to tamoxifen

Vascular formation in mammalian gonad occurred in a sex-specific manner. At 12.5 dpc, the XY gonad develops a distinct male-specific vasculature, which includes the development of a large coelomic vessel and formation of testis cords [6]. In the male, a blood vessel forms at the coelomic surface of the testis that is not seen in the ovary. The results suggested the presence of coelomic blood vessel in tamoxifen treated ovaries stained with an antibody to Pecam (platelet endothelial cell adhesion molecule). The Pecam expression in the ovary exposure to tamoxifen was not different to the control (data not shown). Most vascular markers such as Pecam label endothelial cells in both XX and XY gonads. However, sex-specific markers such as *Jag1* and platelet-derived growth factor receptor alpha (*Pdgfr*-α) are normally associated with the coelomic blood vessel in the XY gonad but are not found in the XX gonad [7]. We performed RT-PCR and whole mount in situ hybridization on *Jag1* and *Pdfgr*-α, and the results showed Jag1 and *Pdgfr*-α expressed in XY gonad and testis, not in XX gonad and ovaries. However, *Jag 1*and *Pdfgr*-α expressed in the group of exposure to tamoxifen (data not shown).

SOX9 and AMH expression after exposure to tamoxifen

Because tamoxifen influences hormone production, we have tested the effects of the treatment on ovaries and testis in the absence (control) or presence of tamoxifen. Control group showed no AMH and SOX9 expression, which are the Sertoli cell markers, whereas testis and ovaries displayed strong SOX9 and AMH expression similar to testis throughout the entire ovary by tamoxifen treatment (Fig. 2, SOX9 not shown). In agreement with the expression of SOX9 and AMH, tamoxifen exposure in adult altered the shape and structure of the follicles. Granulosa cells were almost broken and number of oocytes decreased, coincident with the high levels of AMH in the ovaries. These data showed that tamoxifen induced ovaries alterations or defunctionalization (Fig. 2-3).

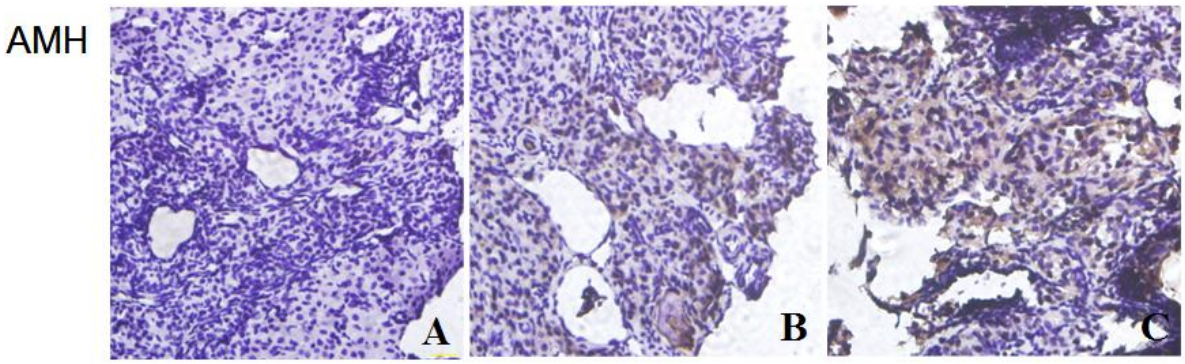

Fig.2. Immunohistochemical staining of AMH on PND 7 w ovaries following continued exposure to tamoxifen for 5 d. A: 0 mg/kg·BW; B: 225 mg/kg·BW; C: Testis

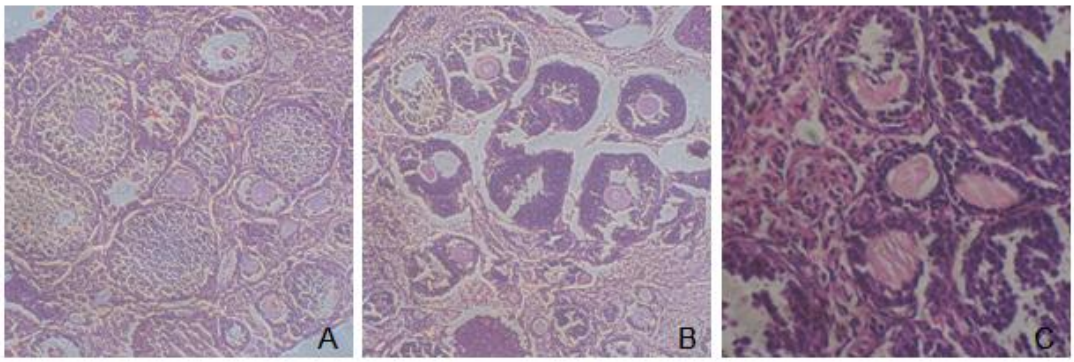

Fig.3. Light photomicrographs of the ovaries from the vehicle control (A) or from the mice administered tamoxifen at dose of 75, 225 mg/kg/BW, respectively (B, C). Hematoxylin-eosin stain, Magnification is 100×.

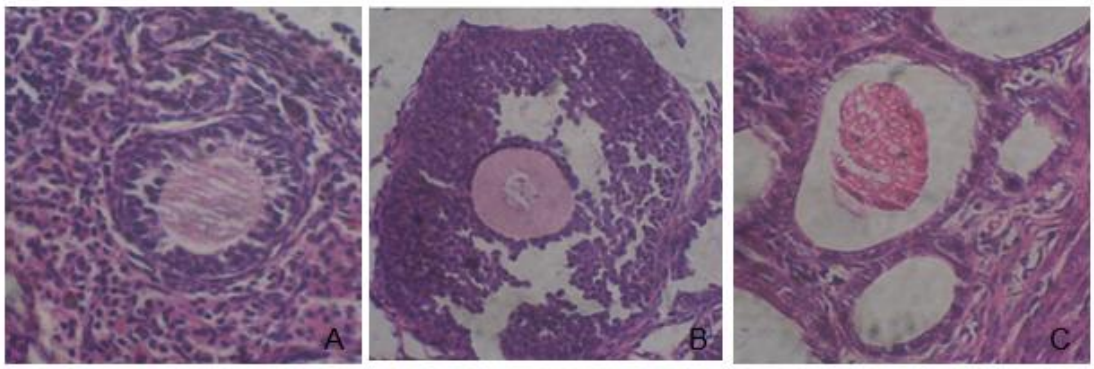

Fig.4. Light photomicrographs of the ovaries from the vehicle control (A) or from the mice administered tamoxifen at dose of 75, 225 mg/kg/BW, respectively (B, C). Hematoxylin-eosin stain, Magnification is 200×.

Our studies demonstrated that tamoxifen treatment can reduce LH and progesterone secretion. Tamoxifen induced ovary defunctionalization by AMH and SOX9 ectopically expression. In conclusion, despite the apparent efficacy of the oral tamoxifen treatment in the human patient, our data have shown some alterations in

the fetal and adult ovaries function due to tamoxifen.

Acknowledgements
This work was supported by National Nature Science Foundation of China (No.81273119).

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