Is estradiol mandatory for an adequate follicular and embryo development? A mouse model using aromatase inhibitor (anastrozole)

Muhammad Fatum · Yi Gyo · Prus Diana ·
Neri Laufer · Alex Simon

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Abstract *Background:* Although high levels of estradiol are found in the follicular fluid, little is known about its necessity for adequate follicular growth, oocyte maturation and embryo development. Arimidex (anastrozole) is a potent aromatase inhibitor capable to induce an in-vivo milieu deprived of estradiol. This study uses a mouse model applying Arimidex to create an in-vivo system lacking of estradiol, in order to explore whether this gonadal steroid hormone is mandatory for folliculogenesis followed by normal fertilization and embryo development.

Methods: Experiment 1: Immature C57 Black female mice, aged 3–4 weeks were superovulated by 5 IU PMSG given intraperitoneally. A study group (9 mice) was concomitantly injected with 0.1 mg of Arimidex intraperitoneally given the morning day before PMSG, the morning day of PMSG injection and the following two days. The control group (8 mice) was similarly injected with normal saline. Estradiol (E2) and progesterone (P) serum levels were tested 48 hours after PMSG and the ovaries of each mouse blindly examined by a pathologist to evaluate follicular development. Experiment 2: 48 h after PMSG superovulation, hCG (7.5 IU) was injected intraperitoneally, followed by mating. The study group was treated with Arimidex 0.1 mg intraperitoneally daily from a day prior to PMSG injection to the day of sacrifice. The control group was treated similarly by normal saline. Forty-two hours after mating blood was withdrawn for E2 and P levels followed by tubal dissection. Embryos of 2–4 cells were cultured in-vitro

and the development to the morula, blastocyst and hatching blastocyst stages were examined 24, 42, and 48 h later.

Results: Experiment 1: A significant reduction of E2 levels was achieved in the Arimidex group in comparison to control group (126.3 ± 104.8 and 1910 ± 960 pmol/L, respectively; $p < 0.0001$). Nevertheless, the two groups did not differ by the mean number of follicles (27 ± 9.5 and 30.4 ± 13.0) or the distribution for antral (65% and 68.4%) and pre-antral (35% and 31.6%) follicles, respectively. Experiment 2: The reduction of estradiol during follicular phase did not hamper follicular development, in-vivo fertilization and in-vitro embryo development. Similar rates of embryo development to the morula stage (90.6% and 86%), blastocyst stage (86% and 89%) and hatching blastocyst (81% and 78%) were achieved in the Arimidex group and the control group, respectively.

Conclusions: Adequate folliculogenesis is independent of estrogen but is conditioned on gonadotropin stimulation. Moreover, depletion of estradiol in the vicinity of the oocyte did not impair its developmental potential, including its fertilization and development into morulae, blastocysts and hatching blastocysts.

Keywords Anastrozole · Arimidex · Aromatase inhibitor · Embryo · Estradiol · Folliculogenesis · In-vitro fertilization · Mouse

Introduction

The development and maturation of ovarian follicles as well as the differentiation of granulosa cells are dependent on the effect of both gonadotropins, FSH and LH [1]. FSH receptors are exclusively present in granulosa cells, while the LH receptors are present in both thecal and granulosa

M. Fatum (✉) · Y. Gyo · P. Diana · N. Laufer · A. Simon
Department of Obstetrics and Gynecology, Hadassah University
Hospital, Ein Kerem,
P.O. Box 12000, Jerusalem 91120, Israel
e-mail: fatum@md.huji.ac.il

cells. According to the “two cell, two gonadotropins theory” theca interna cells are stimulated by LH to produce aromatizable androgens. The androgens diffuse to granulosa cells and are converted to estrogens by aromatizing enzymes, the aromatase complex. Under FSH stimulation, granulosa cells undergo proliferation and differentiation, acquire LH receptors and aromatizing activity potential. Granulosa cells are the principal site for estrogen synthesis in the preovulatory follicle. Estrogens play an important intraovarian role in creating granulosa cells mitogenesis, the production of intracellular gap junctions, cAMP synthesis and further stimulation of steroidogenesis and gonadotropins receptors production.

In addition, there are several important extragonadal effects of the elevated estradiol levels during the follicular phase, i.e. the build-up of receptive endometrium, an important process preceding implantation, the production of the cervical mucus and pituitary priming for the mid-cycle LH surge.

At the follicle level, there is a growing body of evidence that estrogens have paracrine and autocrine functions at the cellular compartments of the developing follicle and the oocyte.

It is well established that estrogen alpha-receptor is present in the human oocyte [2]. Recently, with the isolation of the estrogen beta-receptor and its presence in the human granulosa cells, the old debate about the local effects and the importance of estradiol in the growing follicle was reopened.

The expression of both alpha and beta receptors in the mouse ovary was shown in several studies, revealing a distinct pattern of distribution of both estrogen receptors. Estrogen alpha-receptors are highly expressed in the interstitial/thecal compartment, whereas estrogen beta receptors expression is limited to granulosa cells of growing follicles [3, 4].

Although high levels of estradiol are found in the follicular fluid, little is known about its necessity for adequate follicular growth, oocyte maturation and embryo development.

Arimidex (Anastrozole) is a potent highly selective aromatase inhibitor lacking any other intrinsic hormonal activity that when used results in a selective decrease in estrogen synthesis.

In the present study, we apply Arimidex in a mouse model in order to study the importance of estradiol in folliculogenesis, oocyte maturation and in-vivo ovulation. In addition, the effects of hypoestrogenic environment on embryo development into morulae, blastocysts and hatching blastocysts are studied in-vitro.

Materials and methods

Animals

Immature C57Black female mice aged 3–4 weeks weighing 20–25 gm and mature 6–8 month old C57 Black male mice

of a previously proved fertility were used. The animals were housed in air-conditioned quarters that were illuminated between 8:00 AM and 10:00 PM and pellet food and water were always available.

To induce superovulation, 5 IU of pregnant mare's serum gonadotropin (PMSG, Gestyl; Organon, Oss, The Netherlands) was injected intraperitoneally at 16.00 PM (day 1) followed by 7.5 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis Missouri) given 48 hours later (day 3). After hCG administration, each female mouse was caged with a single male mouse and left overnight. Mating was confirmed by the presence of a vaginal plug on the following morning (day 4).

Medication

Arimidex (Anastrozole) an achiral benzyltriazole derivative is a potent, highly selective aromatase inhibitor with no intrinsic hormonal activities [5]. One tablet of the drug (1 mg) was crushed and grinded to a powder to be suspended in 2 ml of normal saline. As needed, 0.2 ml of the suspension (0.1 mg) was injected intraperitoneally to the female mice. The control groups were similarly injected by normal saline as appropriate.

Experiment 1: Is estradiol mandatory for normal folliculogenesis in-vivo?

Immature C57 Black female mice aged 3–4 weeks were superovulated as previously mentioned. The study group included nine mice. This group was concomitantly injected with 0.1 mg of Arimidex intraperitoneally given in the morning day (8 AM) before PMSG, the morning day of PMSG injection and for the following two days. The control group included eight mice and was similarly injected with 0.2 ml of normal saline.

Six to eight hours after the last Arimidex injection, the mice were anesthetized with Ketamin (Ketalar; Parke-Davis, UK) 100 mg/kg and Xylazine 2% veterinary (Vitamed LTD, Bat Yam, Israel) 5 mg/kg given intraperitoneally. Blood samples were withdrawn from the inferior vena cava for estradiol and progesterone levels. The ovaries of each mouse were dissected and sent for histological examination. Ovaries were fixed in formaldehyde solution embedded in paraffin wax and cut serially into 5 μ m sections. These were stained with hematoxylin and eosin and both ovaries of each mouse were blindly examined by an experienced gynecopathologist to evaluate follicular development and search for any morphologic changes in its histologic appearance, if present.

Experiment 2: Is estrogen deprived microenvironment in-vivo affect embryo development in-vitro?

Mice were superovulated similarly by 5 IU PMSG given intraperitoneally.

Forty-eight hours later, 7.5 IU hCG was injected intraperitoneally followed by overnight mating. The study group (15 mice) was treated with Arimidex 0.1 mg injected intraperitoneally daily from a day prior to PMSG injection, in a similar manner to that scheduled in the first experiment, to the day of sacrifice. The control group (13 mice) was treated similarly by normal saline. Forty-two hours after mating (day 5), mice were anesthetized and blood was withdrawn for E2 and P levels followed by microscopic tubal dissection. Tubal dissection took place in human tubal fluid medium (HTF) supplemented with HEPES buffer and enriched with 0.4% bovine serum albumin (BSA, Sigma Chemicals Co., St. Louis, MO). Embryos of 2–4 cells were isolated and transferred for culture in P1 medium (Irvine Scientific, Santa Ana, CA, USA). Embryos were incubated at 37°C under a 5% CO₂, 5% O₂ and 90% N₂ environment and examined for the development of morulae after 24–26 h of culture. Morulae were transferred to blastocyst medium (Blastocyst medium, Irvine scientific, California, USA) and examined for the development to the blastocyst stage and hatching blastocyst after additional 18 and 24 h of incubation, respectively.

Statistics

Data were analyzed using the χ^2 test or Student's *t*-test as appropriate and *P* value of < 0.05 was considered as statistically significant. Statistical analyses were performed using a standard computer program of Microsoft Excel for Windows. Results are expressed in mean $\pm$ Standard deviation (SD).

Results

Experiment 1

As demonstrated in Table 1, a significant reduction of estradiol levels was achieved in the Arimidex group in comparison to the control group, 126.3 $\pm$ 104.8 pmol/L (mean $\pm$ SD) and 1910 $\pm$ 960 pmol/L, respectively (*P* < 0.0001). This indicates the efficacy of Arimidex suspension (0.1 mg intraperitoneally) used in this experiment to lower the follicular estradiol levels in the study group. Despite the significant difference in estradiol levels during the follicular phase, the two groups did not differ by the mean total number of developing follicles (27 $\pm$ 9.5 and 30.4 $\pm$ 13) nor by the distribution for antral (65% and 68.4%) and preantral (35% and 31.6%) follicles, for the study and control groups respectively (Table 1).

Table 1 Steroids serum levels and follicular distribution in the pre-ovulatory period in the Arimidex and control groups

	Arimidex group (<i>n</i> = 9)	Control group (<i>n</i> = 8)	<i>P</i> value
Estradiol (pmol/L)	126.3 $\pm$ 104.8	1910 $\pm$ 960	< 0.0001
Progesterone (nmol/L)	10.2 $\pm$ 11.7	44.4 $\pm$ 19.4	< 0.0001
Total no. of follicles	27 $\pm$ 9.5	30.4 $\pm$ 13	NS
Antral (%)	65%	68.4%	NS
Preantral (%)	35%	31.6%	NS

Figure 1(A) and (B) show the histologic appearance of ovaries of the study group (A) and the control group (B). As demonstrated, both ovaries show similar number and distribution of growing follicles. No other morphologic changes, i.e., atretic or degenerative follicles, were noticed in both groups. Within the use of PMSG that posses also some hCG activity, luteinization may took place and explains the progesterone production during the follicular phase in both groups (Table 1).

Fig. 1 The effect of Arimidex on ovarian histology. A. An ovary from Arimidex treated animal. B. An ovary from untreated animal. No difference in the histologic appearance with similar total number of follicles and the comparable preantral/antral distribution. Optical magnification X50

Table 2 Steroids serum levels during the luteal phase and embryo development in-vitro in the Arimidex and control groups

	Arimidex group (<i>n</i> = 15)	Control group (<i>n</i> = 13)	<i>P</i> value
Estradiol (pmol/L)	117.0 ± 32.8	200.5 ± 92.3	< 0.01
Progesterone (nmol/L)	207.9 ± 145.0	180.5 ± 110.9	NS
Total no. embryos 2–4 cells	360	335	
Morula stage (%)	326 (90.6%)	289 (86%)	NS
Blastocyst stage (%)	281 (86%)	256 (89%)	NS
Hatching blastocyst (%)	228 (81%)	199 (78%)	NS

Experiment 2

Table 2 indicates the estradiol and progesterone serum level in blood withdrawn from the inferior vena cava just before tubal dissection. The estradiol level in the post-ovulatory phase is significantly higher in the control group in comparison to that in the Arimidex group. Its level, however, is significantly reduced in comparison to that in the pre-ovulatory phase. This reflects the luteinization that took place after the exposure to hCG and the shift to progesterone production in the corpora lutea.

The postovulatory progesterone levels were similarly elevated in both groups as compared to the pre-ovulatory levels, once again, indicating that luteinization took place in the study group despite the exposure to Arimidex.

In spite of the lower levels of estradiol induced by exposure to Arimidex during the follicular phase, ovulation took place followed by adequate mating and in-vivo embryo production. In addition, when the developmental potential of these embryos was assessed in-vitro, similar rates of embryonic development into morulae (90.6% and 86%), blastocysts (86% and 89%) and hatching blastocysts (81% and 78%) were achieved in the study and control groups, respectively (Table 2).

Discussion

This study demonstrates that although a significant reduction of estradiol levels was achieved with Arimidex treatment during the follicular phase, it did not hamper follicular development, oocyte fertilization in-vivo and embryo development in-vitro. These results may indicate that, at least in mice, intraovarian estradiol plays no role in oocytes maturation or its developmental potential.

The high levels of estradiol during the follicular and the preovulatory phases are considered as the hallmark of the follicular phase. Estrogens are considered to play important roles and several pivotal activities at the ovarian follicle level. These include assisting granulosa cells proliferation and differentiation, augmentation of gonadotropin action, in-

duction of gap junctions and acceleration of steroidogenesis. In addition, estrogens have several extragonadal effects: i.e. endometrial proliferation and cervical mucus biosynthesis.

Despite the high levels of estradiol present in the follicular fluid during the follicular phase, little is known about its necessity for adequate follicular growth, oocyte maturation and subsequent embryo development. During the last years, several studies have addressed to this important issues in order to elucidate the role of intraovarian estradiol. Nevertheless, among the different primate and non-primate animal models no uniform findings were achieved concerning this matter.

To date, studies addressing to this issue can be divided into three categories: I. Genetic mutations affecting enzymes participating in estradiol biosynthesis. II. Animal models with knock-out mutations in estrogen receptors. III. Aromatase inhibitors used in animal models.

Malproduction of estradiol

There have been several case reports showing multiple follicular development with the use of exogenous gonadotropin administration in women with congenital enzymatic defects on the estrogen biosynthesis pathway [6–8]. In these women despite normal development of follicles, ovum pick up and fertilization, no pregnancy was reported in those patients. These reports indicated that, in human, folliculogenesis as well as normal fertilization may take place unrelated to estradiol production. Although the number of cycles reported is low, embryo quality might be the crucial variable associated with the IVF failure.

In two women with mutations in the aromatase gene that results in total absence of aromatase activity, large ovarian cysts resembling the polycystic ovaries syndrome (PCOS) phenotype had been described indicating that the growth of antral follicles may occur despite the absence of intraovarian estrogen biosynthesis [9, 10].

Knock out mutations in estrogen receptors

It was shown [11] that a proportion of estrogen receptor knock-out (ER KO) mice can have ovarian folliculogenesis and normal ovulation with the development of what appeared to be a functional normal corpus luteum.

In addition, in the ovaries of mice lacking estrogen receptor β (ER β -/-), follicles can be seen at all stages of development ranging from primordial to fully developed antral follicles [12]. However, when stimulated by gonadotropins, there was an increased number of antral follicles but low number of matured oocytes. Accordingly, fewer corpora lutea were observed resulting in a reduced fecundity. Taken together, these works challenged the view that estradiol has a mandatory role for folliculogenesis and ovulation in mice.

Aromatase inhibitors

There were several studies that utilized aromatase inhibitors, thus blocking the estrogen biosynthesis in animals. The results reported using different animal models were not consistent making it hard to draw a definite conclusion as to the effect of aromatase inhibitor on ovarian function. In rats, a negative correlation was observed between estrogen deficient environment and normal folliculogenesis leading to oocyte maturation [13]. Moreover, histological examination of the rat ovaries indicated a decrease in the number of the healthy follicles in the aromatase inhibitor treated group compared to controls.

In rhesus monkeys [14], the administration of aromatase inhibitor, 1,4,6-androstatrien-3,17-dione (ATD; Steraloids, Wilton, NH) during the late follicular phase of gonadotropin treated cycles resulted in 84% reduction of estradiol levels. However, there was no alteration in follicular diameters or the total number of follicles per animal relative to control values. Nevertheless, completion of oocyte meiosis to metaphase II and the in-vitro fertilization of these oocytes were retarded in the treated group compared to control animals.

In other studies using Fadrozole (CGS 1694A) to block estrogen synthesis, it was shown that normal cyclical follicular maturation as well as normal hCG/LH induced ovulation were relatively unaffected in all animals studied (hamsters, rabbits, monkeys) other than rats [15]. These findings are in an agreement with our results achieved in a mouse model. We used Arimidex (anastrozol) suspension in a dose of 0.1 mg and were able to drastically lower serum estradiol levels during the preovulatory period. Nevertheless, follicular development, oocyte fertilization in-vivo and embryo development in-vitro were not affected. In addition, despite a significant reduction of estradiol levels, the histologic examination showed no difference in follicular development or in the distribution of preantral and antral follicles between the Arimidex treated group and the control group. Our findings corroborate with a recent report by Hu et al. [16] who utilized Arimidex to investigate the effects of reduced estrogen levels on in-vitro culture of preantral mouse follicles. Their findings indicated that in-vitro mouse follicles can develop normally under very low levels of estrogen and that a local androgen increase by a factor of 100 is not atretogenic. However, significantly less oocyte were fertilized in the group cultured in the presence of Arimidex, but, once fertilized, there was no difference in embryo development. The authors concluded that in mice a pronounced estrogenic environment is not essential for in-vitro folliculogenesis. In our study, however, we did not observe any negative correlation between Arimidex administration in-vivo and fertilization rates as was manifested by the same mean number of embryos collected from mice of the two groups.

It should be mentioned that although estradiol levels were drastically reduced by Arimidex in our study, total reduction could not be achieved and thus, although low, estradiol levels might be sufficient enough to support normal folliculogenesis, adequate and healthy oocytes resulting in normal in-vivo fertilization and in-vitro embryo development. This assumption is supported by the report of Fisher et al. [17] who described the phenotype of aromatase knock-out mice to be infertile, with ovaries lacking corpora lutea. Similarly, Britt et al. [18] observed an age related degenerative changes in the ovaries of aromatase knock-out mice. Thus, by the age of one year there were no secondary or antral follicles and all primary follicles present were atretic. These findings suggest the possibility that total deprivation of estradiol may indeed interfere with ovarian function.

In summary, our study suggests that, in mice, adequate folliculogenesis is independent of estrogen but is conditioned on gonadotropin stimulation. Moreover, depletion of estradiol in the vicinity of the oocyte did not impair its developmental potential, including its in-vivo fertilization and in-vitro development into morulae, blastocysts and hatching blastocysts.

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