# The aromatase inhibitor anastrozole is associated with favorable embryo development and implantation markers in mice ovarian stimulation cycles

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**Objective:** To investigate the embryonic and endometrial effects of anastrozole in preimplantation and implantation phases in FSH-induced cycles in mice.
**Design:** Blind randomized study.
**Setting:** University research laboratory.
**Animal(s):** Twenty-seven mature female mice.
**Intervention(s):** Single-dose anastrozole (25 mg/kg [0.75 mg]), recombinant FSH (5 IU/mL), and hCG (5 IU/mL) (n = 9); recombinant FSH (5 IU/mL) and hCG (5 IU/mL) (n = 9); or sterile saline (1 mL) (n = 9). The morning of finding the vaginal plug was designated as day 1 of embryonic development (E1). Three mice from each group were sacrificed on E1 and embryos aspirated from uterine tubes. The rest of the mice were sacrificed on E2.5–3 and uteruses removed.
**Main Outcome Measure(s):** Embryo quality, endometrial histologic evaluation, and immunohistochemical analysis of tumor necrosis factor-$\alpha$, leukemia inhibitory factor, laminin, and collagen IV staining.
**Result(s):** Anastrozole use in FSH-induced cycles not only caused an increase in preimplantation receptivity and implantation but also supported release of implantation markers. The enhanced embryo development seen in this study would explain the higher implantation because embryo development is synchronized with endometrial development.
**Conclusion(s):** In mice, the use of anastrozole in FSH-induced cycles has a positive effect on embryo quality and implantation. This effect might be species dependent, and human studies are needed. (Fertil Steril® 2005;83: 1797–806. ©2005 by American Society for Reproductive Medicine.)

**Key Words:** Anastrozole, aromatase inhibitor, ovulation induction, implantation, mouse embryo

In infertile women, ovarian stimulation can achieve a fertility rate that is approximately equal to or in some anovulation types more than that for normal populations. Ovarian stimulation is the first choice for gaining ovulatory cycles in anovulatory conditions like polycystic ovary syndrome (1, 2). It is also used in ovulatory infertility conditions, such as endometriosis (3) and unexplained infertility (4, 5), to augment ovulation. Ovulation stimulation is also used in conjunction with assisted reproductive technologies to produce several mature follicles (controlled ovarian stimulation) (6).

Clomiphene citrate (CC) is the first-line therapy in ovulation induction. In CC failures, gonadotropins (predominantly recombinant FSH) have been used for ovarian hyperstimulation (7). Multiple follicular development, resulting in ovarian hyperstimulation syndrome and multiple pregnancies, and high treatment costs are major drawbacks of gonadotropin treatment (8–11).

Received July 23, 2004; revised and accepted January 29, 2005.
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Aromatase (a product of the CYP 19 gene) is a cytochrome P450 hemoprotein-containing enzyme complex that catalyzes the rate-limiting step in the production of estrogens: the conversion of androstenedione and T through three hydroxylation steps to estrone and $E_2$ (12). Aromatase is a good target for selective inhibition of estrogen (E) biosynthesis because it is the terminal step in biosynthetic sequence. Aromatase inhibitors have been used in clinical applications over the last 20 years (13). The lack of specificity and the unfavorable toxicity profile of first-generation aromatase inhibitors have led to research for more selective aromatase inhibitors. Third-generation aromatase inhibitors, including anastrozole and letrozole, have been approved for the treatment of postmenopausal breast cancer (14). These aromatase inhibitors have a short half-life (approximately 45 hours), few side effects, and more potency (15).

Mitwally and Casper (16, 17) have suggested aromatase inhibitor use in ovulation stimulation as an alternative to CC treatment. They have hypothesized that in ovarian stimulation, administration of aromatase inhibitors, which have a short half-life and do not possess the adverse antiestrogenic effects of CC in the early part of the

0015-0282/05/$30.00
doi:10.1016/j.fertnstert.2005.01.100

menstrual cycle, might suppress E production, release the hypothalamic–pituitary axis from E-negative feedback, and increase gonadotropin secretion, resulting in stimulation of ovarian follicles.

Despite progress in assisted reproduction technologies, the lack of control of implantation remains a major obstacle to obtaining successful pregnancies. Embryo implantation is a complex event involving apposition, followed by the adhesion of the blastocyst to the maternal endometrium, and finally the invasion of this endometrium (18).

Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) is a marker that is expressed from different layers of trophoblasts and is classified as one of the most important cytokines taking part in implantation (19). The expression of leukemia inhibitory factor (LIF) and LIF receptor (LIFR) proteins and pinopodes in endometrial samples from healthy women suggests that both molecular and structural cell changes are important in the initiation of human blastocyst implantation (20). Laminin-1, a multifunctional glycoprotein of the basement membrane, is thought to be important in embryogenesis, embryonic implantation, and placentation (21). Collagen IV and laminin reactivity increase in the basal lamina and underlying subepithelial stroma as pregnancy proceeds (22).

The present study was undertaken to determine the embryonic and endometrial effects of aromatase inhibitors in preimplantation and implantation phases of FSH-induced cycles in mice.

## MATERIALS AND METHODS

### Animals

All experiments were performed according to the institutional guidelines for animal experimentation at Celal Bayar University Faculty of Medicine (Manisa, Turkey). We obtained approval from the research ethics board of The University of Celal Bayar for the study.

Twenty-seven sexually mature female mus musculus albino (C/C) mice, weighing 30 g and 6 weeks of age, were used. The mice were kept in our laboratory for at least 3 weeks before the experiments under controlled temperature conditions and a 12-hour light/dark regimen (lights on from 7:00 AM to 7:00 PM).

The 27 mice were divided into three groups of 9 mice each. Vaginal smears were obtained from all mice to confirm that they had regular cycles for three cycles before drug administration. Mice were weighed daily. Group I received single-dose (25 mg/kg [0.75 mg]) anastrozole (Arimidex; Astra Zeneca, Istanbul, Turkey) in the proestrous phase (5:00 PM) by lavage. The following day (1:00 PM), a single dose (5 IU/mL SC) of recombinant FSH (Puregon; Organon, Istanbul, Turkey, or Gonal-F; Serono, Istanbul, Turkey) was given, and 48 hours after FSH, hCG (5 IU/mL SC) (Pregnyl; Organon or Profasi-HP; Serono) was given. Group II was not given anastrozole but received FSH (in estrous phase) and hCG as in group I. Group III (control group) received sterile saline (1 mL SC). Mice were caged one female to two fertile males on the night of hCG administration and mated. The morning of finding the vaginal plug was designated as day 1 of embryonic development (E1). Three mice from each group were sacrificed on E1, and 42 embryos were aspirated from uterine tubes with the micropipette flushing method. The rest of the mice were sacrificed on E2.5–3 (implantation period). Implantation areas were detected in uteruses of groups II and III, and six to eight embryonic sacs were macroscopically identified in group I animals. The uteruses were cut into 3–4-mm pieces so the identified implantation sites and embryos would stay in the center of each piece. Uteruses with embryo were removed and fixed in 10% formalin for 48 hours.

Samples were then washed with tap water and soaked in a series of 50%, 60%, 70%, 80%, and 90% ethanol for 30 minutes, then in 95% and 100% ethanol for 1 hour. They were held in a solution of 100% ethanol and xylene (1:1 ratio) for 30 minutes, then embedded in paraffin and held at 60°C for 1 hour to make paraffin blocks. Transverse sections (5 $\mu$m) were taken from the blocks and prepared for both histochemical and immunohistochemical staining.

### Histochemical Observation

Sections dewaxed at 60°C overnight were immersed in xylene for 1 hour, then rehydrated through a graded series of ethanol (100%, 95%, 80%, 70%, and 60%) for 2 minutes in each concentration, then washed in tap water. Sections were stained by hematoxylin-eosin according to routine protocols. Slides were mounted with Entellan UN 1866 (Merck, Darmstadt, Germany), covered with glass cover slips before viewing, and photographed under light microscopy (Olympus BX-40; Tokyo, Japan).

### Immunohistochemistry

After deparaffination at 60°C overnight, sections were held in xylene for 1 hour. After washing with serial concentrations of ethanol (95%, 80%, 70%, and 60% for 2 minutes each), the sections were washed with distilled water and phosphate-buffered saline (PBS) for 10 minutes. They were held in 2% trypsin in Tris buffer at 37°C for 15 minutes, then washed in PBS (three 5-minute washes). The limits of sections were drawn with a Dako pen (S-2002; Dako, Carpinteria, CA) and incubated in 3% hydrogen peroxidase for 15 minutes to inhibit the endogenous peroxidase activity. The tissues were then given three 5-minute washes in PBS. The primary antibodies—monoclonal anti-TNF-$\alpha$ in a 1/100 dilution (1E8-G6; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-LIF in a 1/100 dilution (SC-1336; Santa Cruz Biotechnology), polyclonal anti-laminin in a 1/100 dilution (L-9393; Sigma Chemical, St. Louis, MO), and monoclo-