Effect of tamoxifen on ovarian reserve: A randomized controlled assessor-blind trial in a mouse model

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Abstract

Objective: To determine whether tamoxifen (TMX) exposure causes a permanent decrease in ovarian reserve.

Material and Methods: A randomized controlled assessor-blind trial including 30 adult female inbred BALB/C mice. Fifteen mice in the TMX group were given a single 0.1-mg dose of TMX intraperitoneally. Fifteen mice in the control group were given a single dose of the vehicle at the same volume intraperitoneally. Two cycles later, blood samples were collected for determination of anti-Müllerian hormone (AMH) levels, and the mice were sacrificed. After gonadectomy, ovarian size was measured, and follicles were counted under light microscopy.

Results: Median serum AMH levels were 6.53 and 6.14 ng/ml in the control and TMX groups, respectively (p=0.03). Ovarian size was significantly decreased in the TMX group. While the number of primordial (9 vs 8), primary (6 vs 3), and secondary (4.5 vs 5) follicles were similar, there were significantly fewer preantral (11.5 vs 6, p<0.01) and antral (2 vs 1, p: 0.03) follicles, as well as corpora lutea (6 vs 3, p: 0.04), in the TMX group than in the control group. The number of atretic (2.5 vs 5, p: 0.048) follicles was increased in the TMX group.

Conclusion: Tamoxifen administration leads to arrested growth of gonadotropin-sensitive follicles, while insensitive follicles can remain unaffected. TMX is merely an endocrine disruptor, and it does not cause a decrease in primordial follicle pool. (J Turk Ger Gynecol Assoc 2014; 15: 228-32) **Key words:** Tamoxifen, anti-Müllerian hormone, ovarian reserve, folliculogenesis, antral follicle count

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Introduction

The incidence of premenopausal breast cancer is 15.1 per 100,000 woman-years for white women under 40 years of age (1). As such, it is the most common malignancy among women of reproductive age (1). While surgery is the mainstay of treatment, adjuvant chemotherapy significantly improves the survival of women with breast cancer. The 15-year survival rate of breast cancer patients under age 40 is increased by 6.1% if they receive chemotherapy (2).

However, chemotherapy can be gonadotoxic and impair the reproductive potential of women who survive the disease. The extent of gonadal damage inflicted by chemotherapy depends on several factors, including the age and pre-treatment ovarian reserve status of the patient, as well as the type, dose, and duration of the chemotherapy regimen.

Tamoxifen (TMX) is one of the commonly used agents for adjuvant chemotherapy following breast cancer. Compared to placebo, TMX results in a 13% and 15% reduction in the risk of recurrence and breast cancer mortality, respectively (3). While the gonadotoxicity of some chemotherapeutics, such as alkylating agents, is well established, there is limited information regarding whether TMX is harmful to the ovaries.

In the present study, we evaluated ovarian reserve, as assessed by serum anti-Müllerian hormone (AMH) levels and follicle counts, before and after TMX administration in a mouse model.

Material and Methods

The study protocol was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care Committee of Uludağ University (no: 2012-04/08) (4).

Animals and experimental protocol

Thirty 8-week-old female inbred BALB/C mice weighing 25-30 g were housed in ambient temperature of 20-24°C and humidity of 60%-70%. The lab had a 12-h light and dark cycle. Mice had access to chow and water *ad libitum*. The mice were randomly allocated to two groups. Fifteen mice in the TMX group were given a single 0.1-mg dose of TMX (100 μ L TMX in vehicle consisting of 10% ethanol and 90% corn oil) with an intraperitoneal injection. Fifteen mice in the control group were given a single dose of the vehicle at the same volume intraperitoneally.

Mice were sacrificed by ether inhalation after two estrus cycles. Ovaries were removed and fixed in Bouin's solution for 3-4 hours and embedded in paraffin blocks. Blood samples were collected by intracardiac catheterization following anesthesia. Blood samples were immediately centrifuged at 3000 r/min for 30 minutes at room temperature, and the supernatant was collected for cryostorage at -20°C until analysis.

Follicle counts

Paraffin-embedded ovarian tissues were sectioned at 4-5 μ m. The largest cross-section was used for Crossmann's triple staining in order to count the follicles in each developmental stage under light microscopy (5). The surface area of this section was measured in order to give a surrogate measure for ovarian size. A digital camera (Sony DSC-F717, Tokyo, Japan) attached to a Nikon 4S-2 Alphaphot light microscope (Nikon 4S-2 Alphaphot, Tokyo, Japan) and Scion-Image software (Scion Corporation, Boonsboro, Maryland, U.S.) were used for the measurements. The image analysis system was calibrated using a stage micrometer.

The follicles were categorized as follows: primordial, an oocyte surrounded by squamous pre-granulosa cells, of which not more than one is an enlarged granulosa cell; primary, a relatively larger oocyte surrounded by at least two or more cuboidal granulosa cells; secondary (transitional), an oocyte surrounded by two to three layers of cuboidal granulosa cells; pre-antral, an oocyte surrounded by more than three layers of granulosa cells with no apparent antrum; antral, an oocyte surrounded by multiple layers of granulosa cells with an antrum; and atretic, an oocyte with a pyknotic nucleus and vacuolated cytoplasm and detached granulosa cells also showing degenerative changes and floating freely in the antrum (Figure 1) (6).

AMH measurements

A mouse AMH kit (Hangzhou Eastbiopharm Co. Ltd.; Hangzhoui, China) was used to determine plasma AMH levels using the ELISA method. The analytical sensitivity of the kit was 0.05 ng/mL, and the boundaries of analysis were 0.1-40 ng/mL.

Statistical analysis

Continuous variables were defined with median and interquartile ranges. Mann-Whitney U-test was used for comparisons between the study groups. A two-sided p value <0.05 was considered statistically significant. Statistical Package for Social Sciences v20 (IBM; Armonk, N.Y., U.S.A.) was used for analyses.

Results

While 25 mice survived during the study period, 5 mice in the control group were found dead in their cages 4 days after intraperitoneal injection. Upon laparotomy, there was no evidence of peritonitis or bowel perforation, and the cause of death remained unknown for these mice. They are not included in the comparative analysis.

Area of ovarian section

The largest cross-section of the ovaries was significantly smaller in TMX-exposed mice than in the controls (1.51, 1.09-1.84 mm^2 vs 2.12, 1.63-2.62 mm^2 , respectively, p=0.01).

Follicle counts

Follicles of all stages were observed in ovarian sections of both the control and TMX groups (Figure 2).

While the numbers of primordial, primary, and secondary follicles were similar in the two groups, there were significantly fewer preantral and antral follicles and corpora lutea present in the TMX group than in the control group. The number of atretic follicles was increased in the TMX group. The follicle counts in both groups are presented in Table 1.

Plasma levels of AMH

Median serum AMH levels were 6.53 (5.71-7.47) and 6.14 (6.06-6.52) ng/mL in the control and TMX groups, respectively (p=0.03).





Figure 1. a, b. Ovarian sections of control (a) and tamoxifen-exposed (b) mice show atretic follicles. Arrows indicate atretic follicles



Figure 2. a-d. Ovarian sections of control (a,c) and tamoxifen-exposed (b,d) mice show follicles in different stages of development. Ovarian size is reduced in tamoxifen-exposed mice

| Table 1. Follicle counts | in ovarian | specimens | from | tamoxi- |
|--------------------------|------------|-----------|------|---------|
| fen and control groups | | | | |

| Follicle type | Control (n=10) | Tamoxifen (n=15) | p value | | |
|---|-------------------|---------------------|---------|--|--|
| Primordial | 9 (6-11) | 8 (6-13) | 0.89 | | |
| Primary | 6 (3-7) | 3 (3-5) | 0.26 | | |
| Secondary | 4.5 (2.8-7.8) | 5 (4-6) | 0.68 | | |
| Preantral | 11.5 (9-16.3) | 6 (4-9) | < 0.01 | | |
| Antral | 2 (0.8-2) | 1 (1-1) | 0.03 | | |
| Corpora lutea | 6 (2-8) | 3 (1-5) | 0.04 | | |
| Atretic | 2.5 (1.5-4.5) | 5 (4-7) | 0.048 | | |
| All values are median (25th-75th percentile). | | | | | |

Discussion

Our results indicate that TMX exposure is associated with decreased ovarian size and decreased numbers of pre-antral and antral follicles in ovarian sections. However, the numbers of primordial, primary, and secondary follicles were not affected. In addition, while the number of corpora lutea decreased, the number of atretic follicles increased following TMX exposure. Finally, TMX was also associated with a significant decrease in serum AMH levels, a finding that is in keeping with the observed decrease in pre-antral and antral follicle counts, the major source of circulating AMH.

Tamoxifen is a selective estrogen receptor modulator; however, its mechanism of action in different tissues is not clearly understood. Upon binding to nuclear estrogen receptors, TMX causes a conformational change of the dimeric receptor and interferes with its interactions with co-factors of estrogen receptormediated gene regulation. The type of estrogen receptor, such as ER-alpha or ER-beta, and variations in co-factors in different tissues and under different conditions are likely to play a role in the tissue-specific effects of TMX. While TMX blocks the effects of endogenous estrogen in breast tissue, it exerts estrogenic effects in the uterus, bone, and liver. It is thought to interfere with estrogen feedback at the hypothalamic and pituitary levels. However, there are limited data about the direct effects on the ovary and ovarian follicles. The effects of TMX on various organ systems, the estrous cycle, and fertility potential were investigated in a number of rodent studies (7-11). In these studies, TMX was administered orally (7, 9-11) or subcutaneously (8) for 2 to 4 weeks. The daily TMX dose ranged between 0.03 and 5 mg/kg. TMX exposure led to anovulation in the majority of rats in all but one study assessing estrous cycles-70% of TMX-exposed rats were anovulatory in the study by Donath and Nishino (8, 9, 11).

Compared to controls, both absolute and relative ovarian weight was found to be significantly decreased following TMX exposure in all but one study (7-11). Similar to our findings, Matsuda et al. (7) and Tsujioka et al. (11) noted a decrease in the number and size of corpora lutea in TMX-treated groups. Tsujioka et al. (11) reported similar numbers of small- and medium-sized follicles in TMX-treated and control groups, which was consistent with our observations. In addition to follicle counts under light microscopy, Tsujioka et al. (11) conducted immunohistochemical staining with anti-proliferating cell nuclear antigen (PCNA) antibody in order to better identify resting primordial follicles. PCNA immune-stained sections confirmed the similar numbers of small- and medium-sized follicles in TMX and control groups. Unchanged numbers of small follicles, which represent gonadotropin-insensitive primordial and primary follicles, accompanied by decreased numbers of pre-antral and antral follicles, as well as decreased numbers of corpora lutea, were observations that were common to all of the studies mentioned above (7-11). This suggests that TMX per se is not harmful for resting primordial and gonadotropin-insensitive small follicles. However, the decreased numbers of pre-antral and antral follicles and corpora lutea, accompanied by increased numbers of atretic follicles, suggest that gonadotropin stimulation of further follicular growth and ovulation is impaired with prolonged TMX exposure. This is also supported by the observation of impaired estrous cycles in the studies by Donath (8) and Tsujioka (11). In the only study in which serum gonadotropin levels were assessed, basal levels of serum luteinizing hormone, androgens, and estradiol were found to be decreased in TMX-treated rats. Preovulatory surges of LH and follicle-stimulating hormone (FSH) were also suppressed in TMX-treated rats in the same study (8). In addition, the significantly decreased pituitary weight that was observed following TMX exposure in the studies by Matsuda et al. (7) and Tsujioka et al. (11) suggested decreased gonadotropin production following extended TMX exposure.

In addition to gonadotropin deprivation, local effects of TMX on follicular estrogen receptors can contribute to follicular atresia. Although the exact mechanisms are unknown, it is widely accepted that FSH action is augmented by estradiol (12). Estradiol maximizes FSH induction of antrum formation, aromatase expression, and activity and LH receptor expression and LH activity (12-20). Whether extended TMX exposure only decreases gonadotropin production or also interferes with the stimulation of granulosa cells by existing FSH remains to be determined, but the latter could have contributed to our observations.

Overall, TMX seems to decrease gonadotropin stimulation of pre-antral and antral follicles, leading to anovulation and follicular atresia. Decreased numbers of these large follicles can lead to decreased ovarian size/weight, as observed in the present and former studies. As pre-antral and early antral follicles produce AMH, the decreased serum AMH levels observed in our study are consistent with histological observations. However, the preserved numbers of primordial, primary, and secondary follicles suggest that the resting follicle pool is unaffected by extended TMX exposure. Therefore, despite impaired growth of gonadotropin-sensitive/-dependent pre-antral and antral follicles, it is reasonable to suggest that ovarian reserve, defined as the quantity of resting follicles, remains unaffected by TMX exposure. TMX seems to be solely an endocrine disruptor, and ovaries should be able to respond adequately to gonadotropin stimulation following restoration of hypothalamo-pituitary-ovarian feedback mechanisms.

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