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ORIGINAL ARTICLE

Ki-67 antigen expression in the mammary epithelium of female rats in persistent estrus treated with anastrozole

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Abstract

Objectives: To evaluate Ki-67 antigen expression in mammary epithelium of female rats in persistent estrus treated with anastrozole.
Materials and methods: Twenty-eight Wistar-Hanover female rats in persistent estrus induced by subcutaneous injection of 1.25 mg of testosterone propionate in the second day of life were randomly divided into two groups, control and experimental, with 14 animals each. The animals of control group received only the vehicle (propyleneglycol) and the animals of group experimental received 0.125 mg daily of anastrozole by gavage during 28 days. After 28 days of treatment, all animals were sacrificed and the first pair of abdominal-inguinal mammary glands was removed and fixed in 10% buffered formalin to investigate Ki-67 antigen expression by immunohistochemistry.
Results: The mean percentage of Ki-67-stained nuclei per 500 cells in the mammary epithelium was 76.97 ± 0.76 and 14.44 ± 2.02 [mean ± standard error of the mean (SEM)] in the control and experimental groups, respectively ($p < 0.0001$).
Conclusions: Anastrozole treatment significantly reduced Ki-67 expression in the mammary epithelium of rats in persistent estrus.

Keywords

Anastrozole, aromatase inhibitor, Ki-67, persistent estrus, rat

History

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Introduction

Selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) are drugs that have attracted the most attention in the endocrine treatment of breast cancer, whether treatment is early, adjuvant, metastatic or chemopreventive [1]. Tamoxifen, a first-generation SERM was the first drug approved by the FDA of the United States for endocrine treatment of breast cancer and subsequently for breast cancer chemoprevention, showing a decrease in the risk of invasive cancer in about 49% of women at high-risk for breast cancer [2]. However, tamoxifen has adverse effects due to its partially estrogenic action. Endometrial carcinoma is one of the effects produced by this drug [3,4]. That is why alternative endocrine treatments have been sought. Some studies have shown the role of drugs that inhibit aromatase, preventing estrogen synthesis in the endocrine treatment of hormone-sensitive breast cancer [5,6].

Aromatase inhibitors have emerged as alternative hormone therapies for the treatment of hormone-sensitive breast cancer in postmenopausal women [7]. Of the three generations of existing AIs, the third generation compounds are the most widely recommended drugs. These agents cause a highly specific inhibition of the aromatase enzyme and have less adverse effects, in comparison to previous generations of these drugs [7]. Anastrozole, a third-generation AI, was approved for the first-line treatment of hormone receptor-positive early breast cancer in postmenopausal women and also in metastatic breast cancer [6,7]. It is associated with a greater activity, better general tolerability, longer disease-free survival [7–10] and lower rates of breast cancer recurrence, when compared to tamoxifen [11]. Only one study showed that anastrozole could be used not only for treatment but also for chemoprevention, nevertheless until now none of the AIs was approved for chemoprevention of breast cancer [12–14]. Overall, little is known on the effects of anastrozole on the cell proliferation of normal human mammary epithelium. Nevertheless, there are ethical limitations in studying the direct effects of anastrozole in human normal breast. Therefore, experimental models have been sought, despite the recognition that there are limitations of extrapolating results of animals to humans [15,16].

A rat in persistent estrus is an animal model under constant estrogen stimulation that imitates polycystic ovary syndrome. This animal model has a higher concentration of ducts and alveoli, mimicking dense breasts which in humans have a higher risk for breast cancer [17] and interesting for the study of the effects of tamoxifen and AIs [16,18,19]. AIs are effective in the suppression of aromatase enzyme activity and decrease circulating levels of estrogens to nearly undetectable levels in postmenopausal women. For this reason, AIs are primarily used in the breast cancer treatment of postmenopausal women. However, several experimental methods determined the key role of *in situ* estrogen production for breast cancer growth. AIs would block this *in situ*

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estradiol synthesis without changes in plasma estradiol levels because premenopausal ovaries are relatively resistant to these drugs. This concept provides the rationale for the use of AIs against breast cancer also in women with functional ovaries [20]. Therefore, the paucity of studies evaluating the direct effects of anastrozole on cell proliferation of the mammary epithelium by means of a sensitive marker such as Ki-67, has led us to develop the design of the current study.

Materials and methods

Animals

The study protocol was approved by the Animal Experimentation Ethics Committee of the Federal University of Piauí (UFPI) and conducted according to ethical principles established by the Brazilian College of Animal Experimentation (COBEA). Twenty-eight Wistar-Hannover virgin rats, weighing approximately 250 g were used, originating from the Laboratory of Veterinary Science of the Federal University of Piauí. Persistent estrus was induced in all animals by subcutaneous injection of 1.25 mg of testosterone propionate in the second day of life. During the study, all rats were housed in plastic cages with a metal top (wire mesh), at a room temperature ranging from 20 °C to 25 °C and artificial light with fluorescent lamps in a light–dark cycle of 12 h light and 12 h dark with lights on between 7 and 19 h and the rats had free access to filtered water and animal food. At 90 days of life, androgenized rats were selected for the study. Rats were considered to be in persistent estrus if they exhibited occlusion of the distal third of the vagina, keratinization of the vaginal epithelium, the main characteristic of persistent estrus and also the presence of polycystic ovaries, at the time of autopsy [18,19,21]. The animals were randomly divided into two groups: Group I (control group, propylene glycol) and Group II (experimental, anastrozole) with 14 animals per group. Group I animals received 1 ml of propylene glycol, while Group II animals received 0.5 mg/kg/day, i.e. 0.125/mg/day of anastrozole diluted in 1 ml of propylene glycol by gavage during 28 days. On the 29th day, the rats were anesthetized with ketamine at a dose of 120 mg/kg associated with midazolam 8 mg/kg, by the intraperitoneal route, and then the animals were euthanized by anesthetic drug overdose. The first pair of mammary glands was removed and fixated in 10% buffered formalin for 12–24 h for immunohistochemistry analysis of the breast tissue.

Immunohistochemistry

The breast tissue was cut into 5-μm-thick sections. These sections were processed and stained with hematoxylin and eosin, for morphologic evaluation. Then the sections were dehydrated in increasing concentrations of absolute alcohol and washed with a pH 7.4 buffered solution. Immunohistochemical evaluation of marker Ki-67 was performed using a detection system combined with an antigen retrieval method. For this purpose, sections were treated with 3% hydrogen peroxide diluted in buffered solution during five minutes to block endogenous peroxide. Following antigen retrieval, the tissue samples were incubated with anti-Ki-67 mouse monoclonal primary antibody (clone MIB-5/1:100) during 16 h, overnight, in a refrigerator at approximately 4 °C. Then the samples were washed with buffered saline solution and incubated for 45 minutes with the New Link Polymer detection system. To read these reactions, all slides were treated with 3–3-diamine benzidine tetrahydrochloride solution at a concentration of 1 mg/ml of Tris-buffered saline solution and hydrogen peroxide solution for five minutes, then counterstained with Harris hematoxylin for five minutes, followed by dehydration in ethyl alcohol and xylol baths. Cells were considered positive for immunochemical expression of Ki-67 antigen when the nucleus stained a brownish color.

Quantitative method

Quantification was performed by two independent observers who were blinded to the groups where the rats belonged. Evaluation was made by using an optical microscope connected to a digital color video camera, SCC-131 model, which captured the image and transmitted it to a computer equipped with the Imagelab software program, version 2.3 for image analysis (SOFTIUM Informatics LTDA, São Paulo, Brazil). To count cells positive and negative for Ki-67 expression, a magnification of ×400 was used. At least 500 cells of breast epithelium were counted per slide, in random fields, beginning at the area of highest concentration of nuclei with Ki-67 expression, using the Imagelab®, Image Processing and Analysis Software (SOFTIUM Informatics LTDA, São Paulo, Brazil). In each case, the percentage of stained cells was obtained by the ratio between the number of cells with stained nuclei and the total number of cells, which was then multiplied by 100.

Statistical analysis

Data obtained were submitted to statistical analysis, using Student's *t*-test for two independent samples and the Mann–Whitney nonparametric test ($p < 0.05$).

Results

Under light microscopy, the concentration of cells expressing Ki-67 was greater in the animals of Group I (control) compared to those of Group II (anastrozole) (Figure 1). Quantitative analysis showed the mean percentage of Ki-67 stained nuclei in the mammary epithelium of rats in persistent estrus per 500 cells was 76.97 ± 0.76 (mean ± standard error [SE] of the mean) and 14.44 ± 2.02, in groups I (control) and II (anastrozole), respectively, $p < 0.0001$ (Table 1). Figure 2 clearly shows the difference between the mean percentage of nuclei stained with Ki-67 in control and experimental groups.

Discussion

A study of the effects of drugs on normal human breast tissue is difficult for ethical reasons and this has led us to use an experimental model, despite the limitations of extrapolation of the results. Therefore, we chose a rat model in persistent estrus, which is under continuous estrogen production mimicking polycystic ovary syndrome. The mammary gland of these animals is under constant estrogen stimulation and is similar to breasts with increased density, mimicking a situation of breast cancer risk, being an optimal tissue to study the effect of drugs with antiestrogenic action or blockers of estrogen synthesis by aromatase inhibition, such as anastrozole [16,18,19].

In the current study, rats in persistent estrus were used to evaluate the effects of anastrozole specifically on breast tissue. Anastrozole significantly reduced expression of Ki-67 antigen on the mammary epithelium of rats in the experimental group, in comparison to the control group. The drug was administered by the oral route (gavage), which despite being more difficult, it is similar to the route commonly used by women [21]. In the postmenopause, anastrozole is generally used by women at a dose of 1 mg/day, for treatment of breast cancer. Nevertheless, differences especially in absorption and metabolism, make it difficult to establish a correspondence between doses in women and in rats. This explains the wide variation in concentrations observed in different studies. In this study, we used a dose of 0.5 mg/kg daily or 0.125 mg/animal/day and this dose of

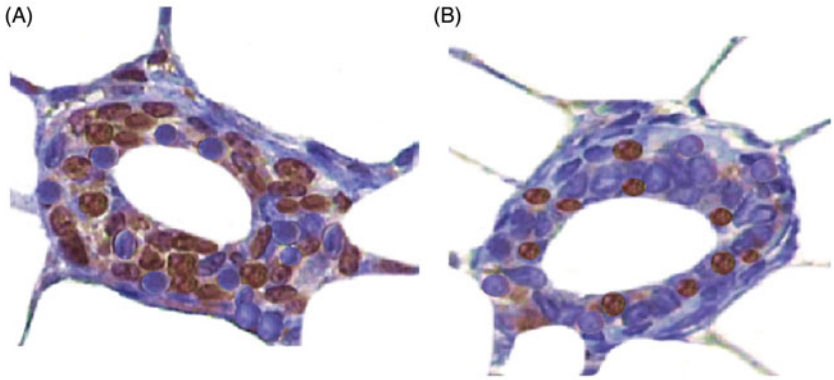

Figure 1. Photomicrography of a histological section of the mammary gland from a rat in persistent estrus. Note the presence of numerous nuclei stained brown by anti-Ki-67 antibody prior to treatment with anastrozole, control group (A), and sparsely stained nuclei post-treatment, experimental group (B) (original magnification, ×400).

Table 1. Mean percentage of Ki-67 nuclei per 500 cells in the control and experimental groups.

Group	*n*	Mean	SE
Control (I)	14	76.97	0.76
Experimental (II)	14	14.44*	2.02

*There was a statistically significant decrease in Ki-67-stained nuclei after anastrozole treatment ($p < 0.0001$).

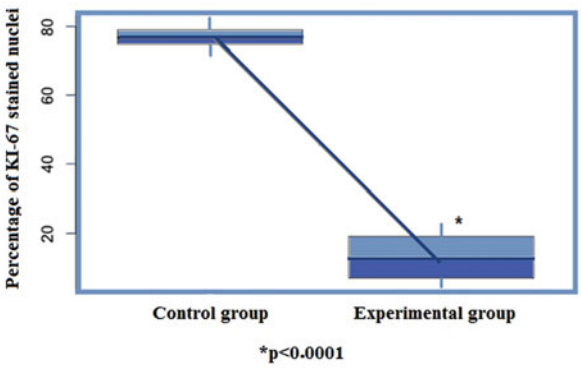

Figure 2. Box plot clearly shows the difference between the mean percentage of Ki-67 stained nuclei in the control and experimental groups.

anastrozole was based on studies by Kubatka et al. [22] and Sadlonova et al. [23].

Kubatka et al. [22] observed that an orally administered dose of 0.5 mg/kg of anastrozole in a conventional premenopausal rat model is equivalent to a daily clinical dose of Aramidex® (1 mg) given to postmenopausal women with breast cancer. Furthermore, Sadlonova et al. [23] confirmed that the oral administration of 0.5 mg/kg of anastrozole in a conventional model of premenopausal rats is similar to a daily dose of 1 mg given to women. These studies show that this dosage (0.5 mg/kg) is sufficient to produce the desired effect in this experimental model. Therefore, the dose of anastrozole used in this study is in agreement with doses used in the literature available and could mimic the dose used by these women for endocrine treatment of breast cancer.

The comparative study between both groups showed that in the control group (rats in persistent estrus) which received only propylene glycol had a high concentration of cells with proliferative activity in the mammary glands. These findings are consistent with the few studies published in the literature such as a study by de Sousa et al. [16] which observed a high proliferative activity, represented by a immunohistochemical study with KI-67 in the breast of rats in persistent estrus using propylene glycol as placebo. Also, these findings are in agreement with reports in the literature describing that rats undergone neonatal androgenic treatment, show a greater release of prolactin and continuous estrogen production, which favor cellular proliferation [19,21].

In contrast, the administration of anastrozole at a dose of 0.5 mg/kg or 0.125 mg/animal/day during 28 days, used by the experimental group, significantly decreased the mean percentage of KI-67 expression on the breast epithelium of rats in persistent estrus compared to a control group. A study reported by Kubatka et al. [22] investigated the chemopreventive effects of anastrozole at a dose of 0.5 mg/kg in breast carcinogenesis induced with N-methyl-N-nitrosurea, and histopathological analysis showed a significant reduction in the incidence of malignant tumors in the group treated with anastrozole in comparison to the control group. These findings are justified by blockade of estrogen synthesis due to aromatase inhibition [24–26]. Nevertheless, to the best of our knowledge, until now there is no report in the literature assessing the expression of Ki-67 antigen in the mammary epithelium of rats in persistent estrus treated with anastrozole. Thus, we conclude that anastrozole administered to rats in persistent estrus at a dose of 0.5 mg/kg/day, i.e. 0.125 mg/day, during 28 days significantly decreased Ki-67 antigen expression on the mammary epithelium of rats in persistent estrus.

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Declaration of interest

The authors declare that they have no competing interests.

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