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CLINICAL TRIAL

Evaluation of estrogen and progesterone receptors in non-neoplastic breast tissue of women of reproductive age exposed to tamoxifen and raloxifene: a randomized, double-blind study

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Abstract The objective of the present study was to compare the effects of tamoxifen and raloxifene in non-neoplastic breast epithelium. A randomized, double-blind study was carried out in 57 ovulatory, premenopausal women of 18–40 years of age, who had been diagnosed with fibroadenoma of the breast. The patients were divided into three groups: Group A: placebo, $n = 20$; Group B: tamoxifen 20 mg/day, $n = 21$; and Group C: raloxifene 60 mg/day, $n = 16$. The study medication was given for 22 days starting on the first day of the menstrual cycle. On the 23rd day, the fibroadenoma was removed and a sample of non-neoplastic breast tissue was collected for immunohistochemical evaluation of estrogen and progesterone receptors. Comparison between the mean percentages of stained nuclei in the three groups was performed by analysis of variance and multiple comparisons, using Tukey's method to compare pairwise means, with significance established at $P < 0.05$. Exposition to tamoxifen or raloxifene resulted in a significant and similar reduction in the mean percentage of stained nuclei for estrogen and progesterone receptors ($P < 0.0001$). Tamoxifen and raloxifene reduce progesterone and estrogen receptor alpha expression significantly and to a similar extent in the non-neoplastic breast tissue of women of reproductive age.

Keywords Non-neoplastic breast · Chemoprevention · SERMs · Estrogen receptor · Progesterone receptor

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Abbreviations

SERM	Selective estrogen receptor modulator
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
BSA/PBS	Bovine serum albumin in phosphate buffered saline

Introduction

Breast cancer is the most common form of cancer and the principal cause of death from cancer in the female population [1]. Reducing the incidence of breast cancer is a challenge, and various strategies have been applied to achieve this aim. Chemoprevention is an effective measure that consists in the use of pharmacological agents that prevent the development and progression of malignant mutant cell clones [2, 3]. Tamoxifen, a first-generation selective estrogen receptor modulator (SERM), was the first drug approved in 1998 by the United States Food and Drug Administration (FDA) for chemoprevention of the disease, approval being based on the results of clinical trials showing a reduction of 49% in the incidence of estrogen receptor positive invasive breast cancer in women considered to be of high risk [4].

Nevertheless, when used for long periods of time, tamoxifen was found to result in undesired side effects, particularly those related to proliferative activity in the endometrium such as endometrial polyps and even carcinoma. The risk of endometrial carcinoma in users of tamoxifen is estimated to be around 3–7-fold higher [4], making the identification of other SERMS an interesting proposal. In the study of tamoxifen and raloxifene (STAR) P-2 trial, raloxifene, a second-generation SERM was shown to be as effective as tamoxifen in reducing the risk of invasive breast carcinoma without, however, stimulating the endometrium [5].

The SERMS, tamoxifen and raloxifene, exert their mechanism of action principally by interacting with intranuclear estrogen receptors, acting as agonists or antagonists in accordance with the species and type of target tissue [6, 7]. In breast tissue, these drugs exert their antiestrogenic effect principally by blocking hormone receptors. When the receptors are occupied, immunohistochemical evaluation shows a reduction in their expression [8]. Nevertheless, approval of the SERMS—tamoxifen and raloxifene—for the primary prevention or chemoprevention of breast cancer in high-risk women took clinical and epidemiological studies into consideration. To the best of our knowledge, although some studies have evaluated the effects of tamoxifen on estrogen and progesterone receptors in normal and neoplastic breast tissue [8, 9], no studies have been conducted to date to compare the effects of tamoxifen and raloxifene in estrogen and progesterone receptor expression in non-neoplastic breast tissue, which led us to the development of the present study.

Patients and methods

Patients

The study involved 72 women of 18–40 years of age with fibroadenomas who were receiving care at the Mastology Clinic of the *Getúlio Vargas* Hospital, Federal University of Piauí between January and December 2008. Fifteen patients were excluded from the study because they had used the medication incorrectly or for technical problems that precluded analysis. The women included in the study were of reproductive age, had no endocrine diseases, no history of thromboembolic disease, were not pregnant, and had not been in use of oral contraception for the previous 12 months. The Internal Review Board of the Federal University of Piauí approved the study and all the patients signed an informed consent form prior to admission.

Study design

The patients were randomized into three groups according to the treatment received: Group A: placebo, $n = 20$; Group B: tamoxifen, $n = 21$; and Group C: raloxifene, $n = 16$. Tamoxifen (20 mg/day) and raloxifene (60 mg/day) were administered orally for 22 days starting on the first day of the menstrual cycle. The groups were considered homogenous with respect to age, tumor size, parity, and age at menarche (Table 1). The women were submitted to excisional biopsy of the fibroadenoma on the 23rd day of the menstrual cycle at which time serum progesterone was also measured for confirmation of ovulation (progesterone > 6.5 ng/ml). A fragment of breast tissue with no macroscopic abnormalities and containing no adipose tissue was extracted from the area adjacent to the fibroadenoma for evaluation of estrogen and progesterone receptors.

Table 1 Characteristics of the patients

	A (Control) $n = 20$	B (Tamoxifen) $n = 21$	C (Raloxifene) $n = 16$	*P* Value
Age (years)				
Mean	28.30	25.71	25.94	0.206
Menarche age (years)				
Mean	13.450	13.286	13.000	0.424
Size of fibroadenoma (cm)				
Mean	2.365	2.233	2.038	0.720
Parity percentage				
Yes	55.00	48.00	44.00	0.787
No	45.00	52.00	56.00	

Immunohistochemical determination of estrogen and progesterone receptors

The samples of non-neoplastic breast tissue were fixed in buffered formalin for a period of 12–24 h and then cut into 3 μm-thick sections. Next, they were processed and stained with hematoxylin and eosin for confirmation of non-neoplastic breast parenchyma. The slides were treated with organosilane and deparaffinized with xylol at a temperature of 60°C for 15 min and at room temperature for another 15 min. The histological slides were then hydrated in decreasing concentrations of alcohol (100, 95, 80, and 70%) for 30 s each and washed in tap and distilled water. Antigen recovery was then performed using steam (a pressure cooker at 100°C), immersing the slides in sodium citrate buffer 10 mmol, pH 6.0, for 4 min after reaching boiling point. The slides were later cooled at room temperature for 20 min and washed in tap and distilled water. Endogenous peroxidase activity was blocked using 3% oxygenated water in methanol (3% commercial solution) in two immersions of 10 min each. They were then washed in tap and distilled water and in 0.01 M phosphate buffered saline (PBS) at pH 7.4–7.6. They were then incubated overnight at 4°C in a humidified chamber following addition of the monoclonal antibody NCL-ER-6F11, class IgG1, specific for estrogen receptor alpha, obtained from Novocastra Laboratories Ltd. As recommended, the monoclonal antibody was diluted at 1:40–1:80 in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (BSA/PBS). Another battery with serial sections from the same tissue sample was processed using the antihuman PgR 636 monoclonal antibody, subclass IgG1 kappa, specific for progesterone receptors, either receptor A or B (Dako Corporation) at the recommended dilution of 150 in BSA/PBS. They were incubated overnight at 4°C in a humidified chamber. The secondary antibody BA-2000 was added the following day at a dilution of 1/300 in PBS and the slides were incubated at 37°C for 60 min in a humidified chamber. They were then washed three times in PBS with a 4-min soak during each wash period. Amplification was made by incubating them with a Dako StreptABComplex/HRP Duet (Mouse/Rabbit) kit at a dilution of 1:500 for 40 min, after which they were washed again in PBS solution. Next, the reaction was detected using the diaminobenzidine substrate chromogen system (DAB; 60 mg%) for 3–5 min at 37°C, protected from the light, and then examined under a microscope. Counterstaining was performed with Harris hematoxylin (hematoxylin 0.5 g, absolute alcohol 5 ml, potassium alum or ammonium alum10 g, red mercuric oxide 0.25 g, and distilled water 100 ml) for 1 min, after which the slides were immersed in ammonia water (aqueous solution of ammonium hydroxide at 0.5%) and washed in tap and distilled water. Next, dehydration was performed in increasing concentrations of ethylic alcohol (50, 80, and 100%) 3 × 1 min, cleared in 3 xylol baths of 1 min each, and mounted in balsam between slide and coverslip for evaluation under an optic microscope.

Quantitative method

Two observers, who were blinded with respect to the patients' identity and had no previous knowledge of any of the cases, performed quantification. It was carried out using a light microscope (Nikon Eclipse E-400, optical microscope, Tokyo, Japan) connected to a color video camera (Samsung digital camera CHC-370 N, Seoul, Korea), which captured the image and transmitted it to a computer equipped with the Imagelab® software program, version 2.3 (Softium Informática Ltda, São Paulo, Brazil) for image analysis. For estrogen and progesterone receptor expression, a minimum of 1,000 cells were counted on each slide, whether stained by the anti-estrogen and anti-progesterone receptor antibody or not, using a magnification of 400×, beginning with the area of greatest estrogen and progesterone receptor expression. In each case, the percentage of stained cells was obtained from the ratio between the number of cells with stained nuclei and the total number of cells multiplied by 100 (label index).

Statistical analysis

Parity was compared in the three groups using Pearson's chi-square test for homogeneity of distribution [10]. The quantitative variables (age, age at menarche, and the size of the fibroadenoma) were compared in the three groups using the Kruskal–Wallis non-parametric test [11]. Next, the medians of each group were compared in pairs using the Mann–Whitney test [11] and the Bonferroni method [12]. The mean percentages of stained nuclei in the three groups were compared using analysis of variance and multiple comparisons, using Tukey's method to compare pairwise means [12].

Results

Light microscopy showed greater concentration of stained nuclei for estrogen and progesterone receptors in the placebo control group compared to the group exposed to tamoxifen or raloxifene (Fig. 1). The mean percentage of stained nuclei for estrogen receptors in the control group was 49.9 ± 2.3 compared to 17.7 ± 1.7 and 22.4 ± 1.6 in the raloxifene and tamoxifen groups, respectively ($P < 0.0001$) (Table 2 and Fig. 2). There was no statistically significant difference between the mean percentages in the raloxifene and tamoxifen groups ($P = 0.3576$). The mean percentage of stained nuclei for progesterone receptors in the control group was 59.8 ± 3.7 compared to 18.1 ± 2.1 and 15.2 ± 1.4 in the raloxifene and tamoxifen groups, respectively ($P < 0.0001$) (Table 2 and Fig. 3). There was no statistically significant difference between the mean percentages of raloxifene and tamoxifen groups ($P = 0.9244$).

Discussion

Few experimental studies have been published on the effect of SERMs on non-neoplastic breast tissue. De Sousa et al. [13] confirmed that tamoxifen significantly reduced Ki-67 expression in the non-neoplastic breast epithelium of premenopausal women. De Lima et al. [8] showed that at doses of 5, 10, and 20 mg/day, tamoxifen significantly reduced the positivity of estrogen and progesterone receptors in the non-neoplastic breast epithelium of premenopausal women, while more recently, Da Silva et al. [14] showed a reduction in cell proliferation in the non-neoplastic breast following Ki-67 protein evaluation in premenopausal women exposed to raloxifene. To the best of our knowledge, the present study was the first to compare the effects of tamoxifen and raloxifene on estrogen and progesterone receptor expression in non-neoplastic breast tissue.

The present findings show that both tamoxifen and raloxifene significantly reduce the mean percentage of stained nuclei for estrogen and progesterone receptors in

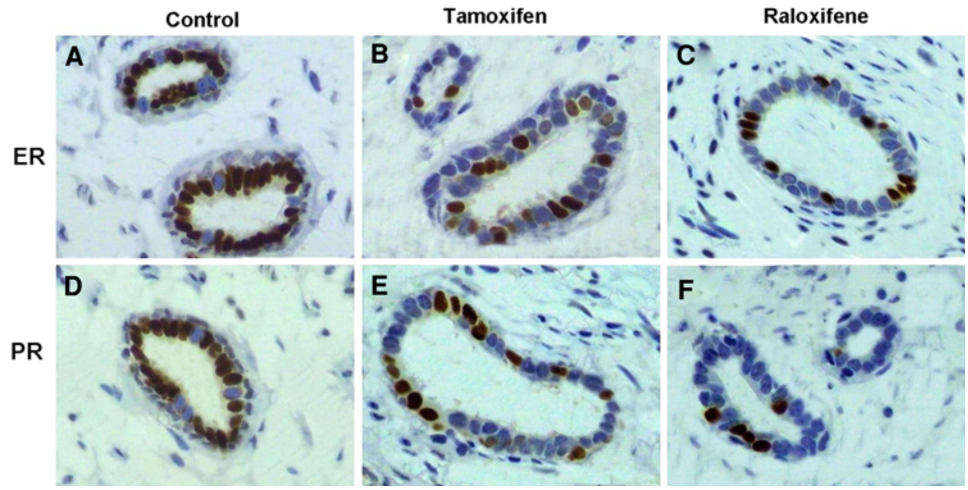

Fig. 1 Photomicrography of histological section of a portion of breast lobule, showing innumerous brown-stained nuclei with strong reactivity to estrogen and progesterone receptors in Group A (placebo) compared to sparse brown-stained nuclei in Groups treated with tamoxifen (B) and raloxifene (C)

Table 2 Mean percentage of stained nuclei of estrogen receptor and progesterone receptor by group

Variable	Group	*n*	Mean	SE mean	Minimum	Median	Maximum
Estrogen receptor	Control	19	49.9	2.3	36.0	49.0	73.3
	Raloxifene	16	17.7	1.7	6.3	19.0	32.7
	Tamoxifen	21	22.4	1.6	7.5	21.5	34.5
Progesterone receptor	Control	19	59.8	3.7	27.2	58.0	85.2
	Raloxifene	16	18.1	2.1	43.0	17.1	36.8
	Tamoxifen	21	15.2	1.4	3.3	15.9	27.1

There was a significant and similar reduction of estrogen and progesterone receptors in normal breast of women treated with tamoxifen and raloxifene ($P < 0.0001$)

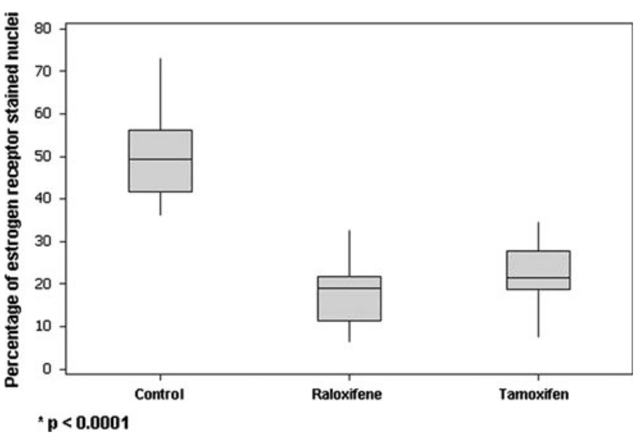

Fig. 2 Mean percentage of stained nuclei for estrogen receptors, as illustrated by the *line* in *boldface*

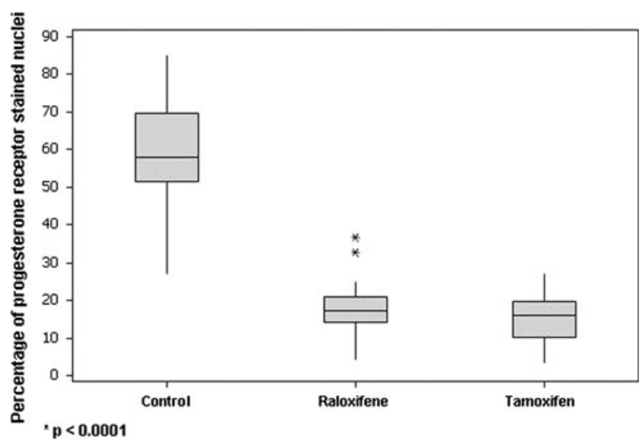

Fig. 3 Mean percentage of stained nuclei for progesterone receptors, as illustrated by the *line* in *boldface*

non-neoplastic breast tissue to a similar degree. Doses of 20 mg/day of tamoxifen and 60 mg/day of raloxifene were used, these being the doses that have been tested in clinical trials and approved for use in chemoprevention. Use of the drugs for 22 days beginning on the first day of the menstrual cycle, followed by resection of the fibroadenoma and non-neoplastic breast tissue for analysis in the luteal phase, was based on the reports that it is in this phase that mitotic activity is greatest in the breast epithelium [15, 16].

The 6F11 monoclonal estrogen receptor antibody was selected for use because the estrogenic effect through the alpha-receptor is considered to be principally responsible for proliferation in the breast epithelium [16, 17]. Evaluation of progesterone receptors was performed using the PgR 636 monoclonal antibody, which recognizes both A and B receptors [18], since the synthesis of progesterone receptors is induced by the effect of estrogen [18, 19].

The mechanism of action of SERMs is yet to be fully clarified, but their antagonistic effect on breast tissue occurs principally by occupying the estrogen receptors with high affinity and competitiveness, thus blocking estradiol binding; also, by inhibiting the binding of coactivator proteins to transcriptional activating function 2 (TAF-2) in the ligand-binding domain; and finally, by inducing the recruitment of co-repressor proteins to the estrogen receptor, which causes repression of the transcription gene [20]. Thus, estrogenic function does not occur. When the estrogen receptors in the breast tissue become occupied, their immunohistochemical expression is reduced [8], as occurred in the present study.

The updated results reported in STAR P-2 trial after a median follow-up of 81 months demonstrate that raloxifene appears to be as effective as tamoxifen in preventing primary invasive breast cancer, appearing to retain approximately 76% of tamoxifen's effectiveness. The update results also showed that raloxifene is about 78% as effective as tamoxifen in reducing the risk of non-invasive breast cancer [5]. Currently, it is recommended that tamoxifen is used to prevent breast cancer in premenopausal women and raloxifene for postmenopausal women [5, 21]. Nevertheless, our results show that tamoxifen and raloxifene reduce progesterone and estrogen receptor expression to a similar extent in the non-neoplastic breast tissue of women of reproductive age. Thus, further studies must be developed with the objective to validate the prophylactic effect of raloxifene in premenopausal women.

Conclusions

These results show that tamoxifen and raloxifene had a similar efficacy as antiestrogenic agent in non-neoplastic breast tissue of premenopausal women.

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