Effect of Tamoxifen and Raloxifene on the Proliferative Activity of the Breast Epithelium in Premenopausal Women*

Miliana T. Lucato[1], Ruffo Freitas-Junior[1], Marise A. R. Moreira[1], Júlio R. M. Bernardes-Junior[2], Sebastião A. Pinto[1], Regis R. Paulinelli[1] and Leonardo R. Soares[1]

[1]Teaching Hospital, Federal University of Goiás, Goiânia, Brazil. [2]Breast Clinic, Hospital Santa Casa de Misericórdia, Goiânia, Brazil.

ABSTRACT

OBJECTIVES: To compare the effects of tamoxifen and raloxifene on the proliferative activity of normal breast tissue in premenopausal women as measured by Ki-67/MIB-1 expression.

STUDY DESIGN: A total of 48 women with benign breast nodules and a recommendation for surgical removal of the lesion took part in this study. They were randomized to use tamoxifen or raloxifene for 22 days, after which they were submitted to surgery. During the surgical procedure, a 1-cm fragment of normal breast tissue was removed to study Ki-67 expression.

RESULTS: The mean percentage ratios between immunolabeled and non-labeled cells were 2.02 ± 1.09 and 3.13 ± 3.23 for the tamoxifen and raloxifene groups, respectively. There was no statistically significant difference between the tamoxifen ($n = 16$) and raloxifene ($n = 14$) groups in relation to the immunohistochemical analysis of Ki-67 ($P = 0.205$).

CONCLUSION: The results of this study showed no difference between tamoxifen and raloxifene with respect to the potential of these drugs to reduce the proliferative activity of the normal breast epithelium in premenopausal women.

KEYWORDS: breast epithelium, breast, tamoxifen, raloxifene, Ki-67, premenopausal women

CITATION: Lucato et al. Effect of Tamoxifen and Raloxifene on the Proliferative Activity of the Breast Epithelium in Premenopausal Women*. *Clinical Medicine Insights: Oncology* 2015:9 25–30 doi: 10.4137/CMO.S22456.

RECEIVED: December 02, 2014. **RESUBMITTED:** January 26, 2015. **ACCEPTED FOR PUBLICATION:** January 31, 2015.

ACADEMIC EDITOR: William C. S. Cho, Editor in Chief

TYPE: Original Research

FUNDING: Authors disclose no funding sources.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: ruffojr@terra.com.br

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Introduction

Breast cancer, the most common form of cancer affecting women worldwide, is considered a public health issue of global magnitude.[1–4] The primary prevention of breast cancer has emerged as a real possibility for reducing its incidence,[5–8] thus minimizing both treatment-related costs[9] and the psychological harm caused by this disease.[10]

According to the American Society of Clinical Oncology's guidelines, a five-year course of tamoxifen may reduce the risk of estrogen receptor (ER)-positive breast cancer in women over 35 years of age at an increased risk of breast cancer. In the case of postmenopausal women, raloxifene and exemestane may also reduce the risk of breast cancer.[6] More recently, the results from the IBIS-II (International Breast cancer Intervention Study II) trial showed that anastrozole effectively reduces the incidence of breast cancer in high-risk postmenopausal women.[7]

The head-to-head comparison of tamoxifen and raloxifene in the STAR (Study of Tamoxifen and Raloxifene) trial showed that both drugs represent good preventive options for postmenopausal women with an increased risk of breast cancer. After an extended follow-up period, raloxifene was found to be less toxic; however, its efficacy in preventing invasive breast cancer in postmenopausal women was 76% of that provided by tamoxifen.[11]

Tamoxifen and raloxifene can reduce progesterone and ER alpha expression to a similar extent in the non-neoplastic breast tissue of women of reproductive age.[12] The capacity of both tamoxifen and raloxifene to reduce the proliferative activity of the normal breast epithelium in premenopausal women has also been well established in previous studies.[13–15] Nevertheless, a direct comparison of the proliferative activity of these two compounds in the normal breast epithelium and their potential use as chemoprevention in premenopausal women are issues that remain to be determined.

Objective

The objective of this study was to compare the effects of raloxifene (60 mg/day) and tamoxifen (20 mg/day) on the

*This study was conducted within the Mastology Research Network, Goiânia, Goiás.

proliferative activity of the normal human breast epithelium during the luteal phase of the menstrual cycle as measured by the immunohistochemical expression of Ki-67/MIB–1.

Patients and Methods

A total of 48 women with benign breast nodules receiving care at the breast clinic of the Department of Obstetrics and Gynecology, Federal University of Goiás' Teaching Hospital between November 2002 and October 2004 were included in this study. The institute's internal review board approved the study protocol. The procedures to be performed were explained to the patients, and all their queries were clarified. All patients who agreed to take part in the study signed an informed consent form. It was conducted under the principles of the Helsinki Declaration.

A detailed medical history was obtained, including information on the women's characteristics with respect to their menstrual cycle, parity, lactation, and their use of hormonal drugs. The diagnosis of benign breast lesion was reached by conducting a physical examination, breast ultrasonography, and fine-needle aspiration puncture.

To be included in the study, patients had to be between 15 and 40 years of age, with benign breast nodules and an indication or desire to have the lesion removed. In addition, the women's menstrual cycles had to have been regular (28 ± 3 days) over the previous six months. Women in use of hormonal contraceptives, those who had been pregnant in the 12 months preceding the initial study visit, those with a known allergy to raloxifene or tamoxifen, those with a confirmed diagnosis of breast cancer, and those who had undergone hormonal manipulation within the previous 12 months were excluded from participating in the study.

The drugs used in this study (tamoxifen 20 mg and raloxifene 60 mg) were obtained through a partnership with the Federal University of Goiás' Foundation for the Support of Research (FUNAPE). A pharmacist was in charge of the randomization process and of masking the medication to be used in the study. The medication was distributed in identical, sealed, white, non-transparent vials, identified only by a number ranging from 1 to 48 affixed to the exterior surface of the vials, corresponding to the enrollment number of each of the patients participating in the study.

The randomization list containing the key to the code numbers was kept by the pharmacist responsible for the blinding procedure. This pharmacist had no contact whatsoever with the investigators or with the patients. The study was double-blind with respect to the medication (tamoxifen or raloxifene). The drugs were handed over to the patients in sequential order as they were admitted to the study. After receiving the vial of tablets, the patients initiated use of the medication assigned to them on the first day of their next menstrual cycle, taking one tablet per day for 22 days.

After concluding the course of medication prescribed in the study, the patients were operated on between the 23rd and 28th days of the menstrual cycle to resect the nodule. At the same time, a fragment of breast tissue measuring approximately 1 cm and with no macroscopic alterations was removed from a site 1 cm from the nodule. The surgical procedures were performed at the University Teaching Hospital.

On the day of surgery, a blood sample was collected to measure progesterone and estradiol levels. Progesterone levels >3.0 ng/mL were considered indicative of the luteal phase of the menstrual cycle.[16]

After the nodule and the fragment of normal breast tissue were excised, the specimens were appropriately identified, fixed in 10% formalin, and sent to the Department of Pathology and Imaging at the Goiânia Teaching Hospital. Subsequently, these specimens were submitted to histological and immunohistochemical evaluations. First, the material was dehydrated in ethanol, cleared in xylene, and immersed in liquid paraffin. The paraffin tissue blocks were cut in a microtome to obtain 3-µm-thick histological sections. These slides were stained using hematoxylin and eosin (HE).

Two pathologists evaluated the breast parenchyma to confirm normal histological findings, and only then was the specimen submitted to immunohistochemical evaluation.

The slides were deparaffinized in hot xylene (heated to 60 °C) for 15 minutes and then in xylene at the room temperature for a further 15 minutes. Next, the slides were progressively hydrated by immersing them in a series of decreasing concentrations of alcohol (100%, 95%, 80%, and 70%) for 30 seconds in each passage, after which the slides were washed in distilled running water.

In the following step, the antigen was retrieved by heating the slides in 10 mmol of sodium citrate buffer (pH 6.0) in a pressure cooker for five minutes. Endogenous peroxidase activity was blocked using hydrogen peroxide (6%) in three stages of 10 minutes each. Subsequently, the slides were incubated overnight at 4 °C with primary Ki-67 monoclonal antibodies (MIB-1 clone; DakoCytomation) at a dilution of 1:2,400. After this stage, the slides were washed in phosphate buffer solution (PBS), incubated with the DakoCytomation LSAB+ System-HRP kit at the recommended dilution for 30 minutes, and washed in PBS.

The immunoexpression of the Ki-67/MIB-1 antibodies was quantified by determining the number of immunolabeled cells per 1,000 cells counted. Only epithelial cells were evaluated, with the stromal component being excluded from the analysis. Using a 40× objective lens (magnification 10×), cells with nuclei showing the characteristic chestnut-brown staining were considered positive. The slides were scanned in a zigzag pattern, from right to left and from bottom to top, until 1,000 cells had been assessed. The resulting ratio between the stained and unstained cells was then multiplied by 100.

The pathologist who quantified the immunoexpression of Ki-67/MIB-1 was blinded with respect to which drug was

being evaluated. The randomization key was opened only after all slides had been assessed.

Overall, 62.5% of the 48 randomized women were included in the final analysis of the study. In all, 18 cases were excluded for the following reasons. One patient used the medication but failed to show up for surgery, one became pregnant and did not use the drug, two patients were lost after moving away, two patients stopped using the medication, and one patient was diagnosed with breast cancer. Additionally, in seven cases, the fragment of breast tissue resected was insufficient to allow immunohistochemistry to be performed, and in another four cases, it proved impossible to perform immunohistochemistry because of difficulties in fixing the histological material to the slide. The woman diagnosed with cancer presented with a ductal carcinoma in situ (13 mm) in association with a microinvasive ductal carcinoma (Bloom-Richardson Grade II) that was estrogen and progesterone receptor positive and HER2++. She was referred for treatment at the breast clinic of the University Teaching Hospital, and subsequently, underwent quadrantectomy, axillary clearance, and radiotherapy.

The SPSS software program, version 11.0.1 for Windows, was used in the statistical analysis. Student's *t*-test, analysis of variance (ANOVA), and Pearson's correlation coefficient were used to analyze the continuous variables, which were expressed as means and standard deviations (SDs). For all the tests, the level for rejection of the null hypothesis (*P*-value) was set at an alpha of 0.05.

Results

The final analysis included 30 patients, 16 in the tamoxifen group and 14 in the raloxifene group. Findings at pathology revealed that the nodules consisted of fibroadenomas, fibroadenosis, and other benign lesions. No statistically significant differences were found between Group I (tamoxifen) and Group II (raloxifene) with respect to age, age at menarche, parity, nodule size, body mass index (BMI), or progesterone or estradiol levels, as shown in Table 1. None of these variables had any significant effect on Ki-67 immunoexpression in either of the two study groups, as evaluated by Pearson's correlation coefficient (Table 2).

Mean Ki-67 expression was 2.02 ± 1.09 for the tamoxifen group and 3.13 ± 3.23 for the raloxifene group, with no statistically significant difference between the two groups ($P = 0.205$) (Fig. 1).

Of the 30 cases evaluated, progesterone levels were below 3.0 ng/mL in nine cases (30%): six in Group I and three in Group II, representing 37.5% and 21.4% of the total number of cases in each group. No statistically significant difference was found between the groups (Fig. 2). The values found for plasma estradiol levels were 355.72 ± 337.98 pg/mL and

Table 1. Comparison between the characteristics of the patients in the two study groups.

	STUDY GROUP		t-VALUE	*P*-VALUE
	Tamoxifen (MEAN ± SD)	Raloxifene (MEAN ± SD)		
Age	22.31 ± 6.07	25.29 ± 6.51	1.294	0.206
Age at menarche	12.94 ± 1.61	13.29 ± 1.73	-0.571	0.573
Parity	0.63 ± 1.02	0.86 ± 1.16	-0.580	0.566
Body mass index	20.36 ± 2.53	20.04 ± 2.17	0.979	0.713
Nodule size (mm)	21.25 ± 9.00	16.68 ± 8.43	1.431	0.163
Estradiol (pg/ml)	355.72 ± 337.98	171.02 ± 119.83	2.044	0.055
Progesterone (ng/ml)	19.62 ± 17.67	14.59 ± 8.98	0.999	0.328

Abbreviations: SD, standard deviation; t-value, Student's t-test.

Table 2. Effect of selected variables on the immunohistochemical expression of Ki-67 in normal breast cells, after the use of tamoxifen or raloxifene.

	Tamoxifen			*Raloxifene*		
	r	F	*P*-VALUE	r	F	*P*-VALUE
Age	-0.066	-0.246	0.809	0.136	0.474	0.644
Age at menarche	0.040	0.148	0.884	0.129	0.450	0.661
Parity	-0.184	-0.700	0.495	0.340	1.253	0.234
Body mass index	0.181	0.687	0.503	-0.107	0.372	0.716
Progesterone	-0.174	-0.661	0.520	-0.055	-0.191	0.852
Estradiol	-0.282	1.098	0.291	0.460	1.796	0.098

Note: r, Pearson's correlation index.

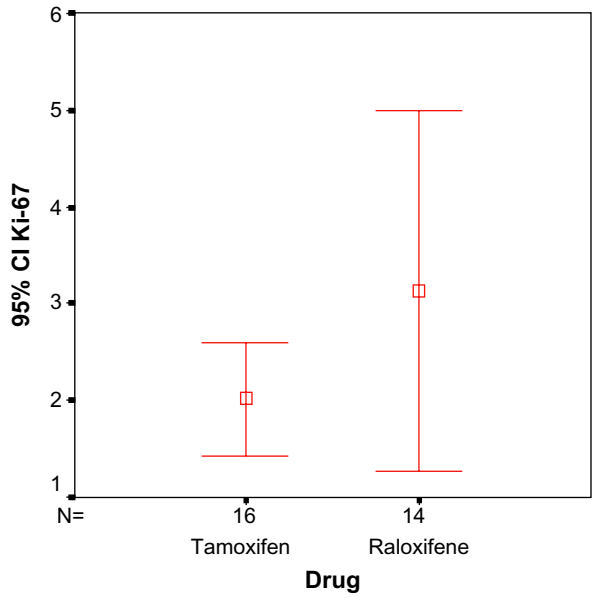

Figure 1. Comparison of Ki-67 expression in the human epithelium following the use of tamoxifen and raloxifene.

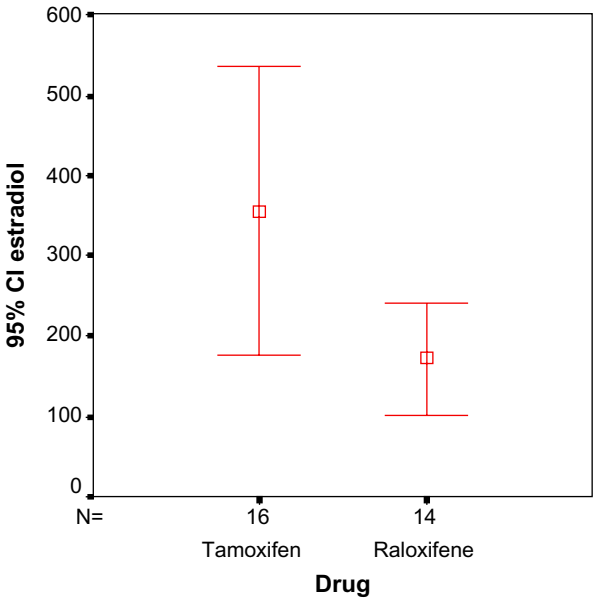

Figure 3. Comparison of the effect of tamoxifen and raloxifene on estradiol levels.

171.02 ± 119.83 pg/mL for Groups I and II, respectively ($P = 0.055$) (Fig. 3).

Discussion

Various studies involving selective estrogen receptor modulators (SERMs) have been conducted. On the one hand, tamoxifen has been shown in previous studies to reduce the risk of breast cancer.[5,7,8,11,17] On the other hand, another SERM, raloxifene, evaluated in studies for the prevention and treatment of osteoporosis also plays an important role in the prevention of breast cancer in postmenopausal women.[7,11,18–20]

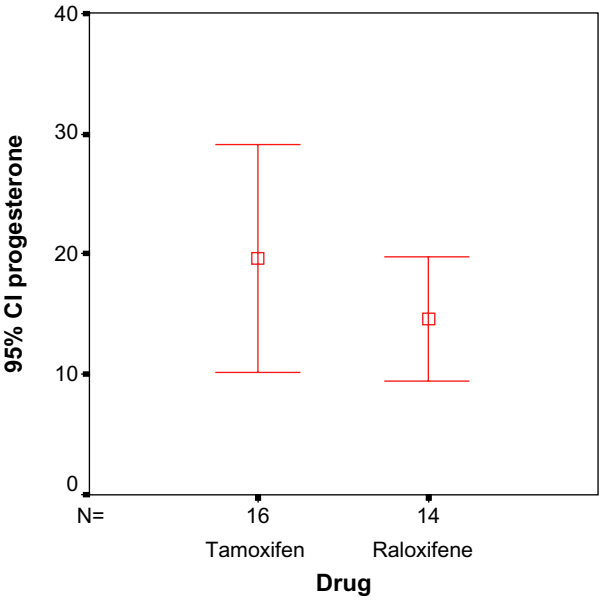

Figure 2. Comparison of the effect of tamoxifen and raloxifene on progesterone levels.

The advantages and disadvantages of raloxifene have been compared with those of tamoxifen for the prevention of breast cancer in postmenopausal women.[11,19] The superior benefits of tamoxifen over raloxifene in reducing the risk of breast cancer come at a cost: more cases of endometrial cancer, more hysterectomies, more thromboembolic events, and more cataracts.[8,11] Although these toxicities could even be considered acceptable for the treatment of breast cancer, they may represent a barrier to the use of tamoxifen as chemoprevention for that condition.[8,11]

Ki-67/MIB-1 antibodies constitute a good marker of the proliferative activity of the breast epithelium, since Ki-67 antigens are expressed in all the phases of the cell cycle with the exception of G0. In the G0 phase, the cells do not form part of the replication process, thus making it possible to have a very reliable measurement of the cell growth fraction.[21,22] These antibodies enable studies of cell proliferation to be conducted in paraffin-embedded material, with results showing a reaction pattern similar to that initially described for Ki-67 antibodies in frozen material.[21,22]

Ki-67 antibodies are used as a predicting factor for treatment response when evaluating different neoadjuvant endocrine therapies for breast cancer such as tamoxifen and aromatase inhibitors.[23–25] The capacity of these drugs to reduce tumor cell proliferation, as measured by Ki-67 expression, is considered to be a good marker of the efficacy of the agents studied.[23,26,27] On the other hand, failure to reduce Ki-67 expression in patients with hormone receptor-positive tumors is associated with a lack of response to tamoxifen and an increased risk of breast cancer recurrence.[23,24]

Some studies have been conducted on the effect of tamoxifen and raloxifene on breast tissue proliferation.[13,14] Sousa et al evaluated Ki-67 expression after the use of tamoxifen at doses

of 10 and 20 mg. The Ki-67 values found were 4.5% and 3.2% for the doses of 10 and 20 mg, respectively, while the drug was found to significantly reduce the proliferative activity of the breast epithelium compared to placebo ($P < 0.001$).[13] In another study, Lopes et al compared raloxifene at a dose of 60 mg/day with placebo over a period of 22 days and reported that raloxifene significantly decreased the proliferative activity of the normal breast epithelium ($P < 0.001$), with a mean of 1.2 (±1.0) stained cells per 500 cells counted.[28]

The IMPACT (Immediate Preoperative Anastrozole, Tamoxifen, or Combined With Tamoxifen) trial evaluated Ki-67 expression after the use of anastrozole and tamoxifen, either alone or in conjunction, after 2 and 12 weeks of treatment. These data indicate that short-term changes in proliferation in the neoadjuvant setting may be able to predict outcome during adjuvant use of these same treatments.[26,27] Therefore, the findings of the present study with the use of tamoxifen and raloxifene for 22 days are comparable with the results of previous studies.[26–28]

In the present sample, randomization resulted in two homogenous and comparable groups, as reflected by the fact that there were no statistically significant differences between the groups with respect to the control variables: the patients' age ($P = 0.206$), age at menarche ($P = 0.573$), parity ($P = 0.566$), BMI ($P = 0.713$), and nodule size ($P = 0.163$). In addition, none of the abovementioned variables was found to affect Ki-67 expression in either of the two groups.

The results of the present study are comparable to findings reported from previous studies,[13–15] with no differences being found between Group I (tamoxifen) and Group II (raloxifene) ($P = 0.205$) with respect to the proliferative activity of the human breast epithelium as evaluated by Ki-67/MIB-1 expression. These results indicate that both drugs represent good options for reducing the proliferative activity of the normal breast epithelium, and they may be equally effective in this respect. Therefore, raloxifene may play a role in breast cancer prevention in premenopausal women.

Mean progesterone levels following use of the medication were 19.62 ± 17.67 ng/mL and 14.59 ± 8.98 ng/mL in Group I (tamoxifen) and Group II (raloxifene), respectively, with no statistically significant difference between the groups ($P = 0.328$). However, with regard to plasma estradiol levels, there was a tendency toward lower mean values in the women using raloxifene compared to those using tamoxifen, although this difference was not statistically significant. Two possible explanations for this are that tamoxifen is able to block ERs more effectively, thus increasing the release of estradiol, or that raloxifene tends to reduce circulating estradiol levels. Indeed, the latter possibility gains ground from the fact that estrogen acts as a breast cancer promotion factor. This leads us to speculate on the possibility of reducing the incidence of breast cancer by decreasing circulating estradiol levels. In addition, the effect resulting from the interaction between raloxifene and ERs should be taken into account.

The absence of a placebo control group may be considered a limitation of the present study; however, since the model used has been previously tested with success,[14,15] a placebo group was not believed to be necessary in the present study.

In conclusion, there was no difference in the proliferative activity of the normal breast epithelium following the use of tamoxifen or raloxifene for a 22-day period. This finding suggests that raloxifene may play a role in breast cancer prevention in premenopausal women. Further randomized clinical trials should be conducted with this population.

Ethical Standards

This study was conducted in accordance with the current Brazilian ethical regulations.

Author Contributions

Conceived and designed the experiments: MTL, RFJ, JRMBJ. Analyzed the data: MTL, RRP, MARM, SAP. Wrote the first draft of the manuscript: MTL. Contributed to the writing of the manuscript: LRS, RFJ. Agree with manuscript results and conclusions: MARM, RFJ, LRS, RRP. Jointly developed the structure and arguments for the paper: MTL, RFJ. Made critical revisions and approved final version: MTL, RFJ, MARM, SAP, JRMBJ, RRP, LRS. All authors reviewed and approved of the final manuscript.

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