Proliferative effects of different hormone regimens on mammary glands in ovariectomized rats

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Summary

Objective: To compare the proliferative effect of different hormone regimens and estrogen receptor modulation on mammary glands in a rat model of surgical menopause.

Design: Experimental animal study.

Setting: University Hospital.

Intervention: In a rat model of surgical menopause, 78 adult Sprague Dawley female rats were ovariectomized and treated with estrogen, estrogen combined with continuous or intermittent progesterone or the estrogen receptor modulator raloxifene and their respective vehicle controls. Following intraperitoneal drug administration for 20 days, rats were perfused, mammary glands were removed, tissues were processed for immunohistochemical (Ki-67) and hematoxylin-eosin staining, and investigated under light microscope.

Main outcome measure: Histopathological examination of mammary glands and Ki-67 positive cells (proliferation index).

Results: Histological examination showed dilatation in the duct cysts and vacuolization in the epithelial cells in groups receiving progestin, either intermittent or continuous. Histological findings in the raloxifene group were no different from the control group, and the atrophic terminal ductal lobular unit in adipose tissue rich stroma was similar to postmenopausal breast.

In animals with a proliferative response, increased proliferation started and dominated in the terminal ductal lobular unit epithelium. Comparison of Ki-67 proliferation indices between groups revealed that estrogen alone or combined with intermittent progesterone yielded significantly higher Ki-67 indices compared to controls; estrogen combined with continuous progesterone also resulted in increasing the probability of proliferation, but the effect was not as pronounced as the other two groups. Raloxifene treatment, on the other hand, did not cause proliferation.

Conclusion: Estrogen alone or combined with progesterone may increase the risk of breast cancer by enhancing proliferation in the TDLU; raloxifen does not induce proliferation and may be a safe estrogen receptor modulator regarding its effects on mammary glands during menopause.

Key words: Hormone replacement; Rat; Mammary gland; Ki-67 antigen; Proliferation.

Introduction

Menopause marks the end of the reproductive period and the beginning of senescence in women. Morphological and functional changes in the ovaries during climacterium perturb hormonal status with loss of ovarian estrogen and progesterone production. Specifically, estrogen insufficiency transforms a physiologic event into a pathologic cycle disturbing life quality and renders menopause the most critical point in a woman's life. Hormone replacement therapy (HRT) applied during this difficult period aims to supplement the quality of life rather than increase life expectancy.

Mammary glands are complementary reproductive organs and their pathological transformations are within the scope of gynecological practice. Subsequently, mammary development defects in ovarian dysfunction, lactation and related pathologies as well as the possible effects of oral contraceptives and HRT on mammary glands are frequently encountered problems. Recently there has been substantial debate and controversy on the use of HRT in clinical practice. Besides the generally accepted positive effects of HRT on cognitive abilities, favorable changes in the cardiovascular and urogenital systems, elimination of vasomotor symptoms and more importantly a reduction in osteoporotic fractures, some desirable effects related to the mammary gland and endometrium are frequently encountered. In vitro studies revealed a biphasic effect of estrogen on mammary gland tissue: cellular proliferation at physiological but inhibition at pharmacological doses. On the other hand, the effect of progesterone is still controversial; while some authors propose that progesterone has an anti-mitogenic effect and inhibits the proliferative effect of estrogen, others say it aggrevates the proliferative effects of estrogen [1].

Raloxifene is a tamoxifen analog belonging to the benzothiophen group of selective estrogen receptor modulator families [2]. The alkyl-amino-etoxic side chain is responsible for the antiestrogenic effect of raloxifene and nitrogen in this side chain specifically reacts with aspartate in the estrogen receptor to induce an antiestrogenic effect. Under in vivo conditions, raloxifen inhibits the proliferation of breast cancer cells more effectively than tamoxifen [3].

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Mammary glands are modified apocrine sweat glands specialized for milk production which consist of glandular and ductal structures in fibrous stroma and adipose tissue. Each breast contains 15-20 conically shaped lobes, each covered by a group of lobules made up of many lactiferous ducts. Lactiferous ducts join to form intralobular teminal ducts, each of which opens to an extralobular terminal ducts which in turn opens to collecting ducts. The basic component of the mammary lobule is the alveolus (acinus), covered by a milk secreting epithelium. The structures starting from the alveolus and leading to the ductus (epithelial cells, lactiferrous ducti, intralobular and extralobular terminal ducti) collectively form the 'terminal ductal lobular unit (TDLU). Russo *et al.* [4] underscored the importance of TDLU as the point of origin of breast cancer and since then this terminology has been accepted by investigators in the field.

Immunohistochemical methods to evaluate cellular proliferation have become available recently and supplement cytological information. Basically, there are two methods to demonstrate cellular proliferation in mammary tissue: antiproliferating cell nuclear antigen (anti-PCNA) and Ki-67. The latter is an antigen used as a cellular proliferation marker; it is coded by a single gene located on chromosome 10 which defines Mr 345 and 395 kDa antigens. The expression of Ki-67 is closely related to the cell cycle thereby making it a suitable marker to assess the growth fraction of tumors [5]. Ki-67 expression starts at G1 phase and continues to increase during the cell cycle; its expression decreases just after mitosis. Ki-67 protein contains large amounts of proline-glutamic acid-serine-threonin (PEST) motives; therefore it can be catabolized quickly. Considering these properties, resulting in a very short half-life after mitosis, Bruno and Darzynkiewicz [6] suggested using Ki-67 as a specific marker of proliferation.

There are different therapeutic approaches regarding HRT in menopausal women. While some protocols employ only estrogen, others combine progesterone with estrogen either continuously or intermittently. In the present rat model of HRT application in ovariectomized (OVX) rats, all three protocols were simulated.

The aim of the present study was to investigate the effects of different HRT regimens and estrogen receptor modulator raloxifene on epithelial cell proliferation in the mammary gland. In a rat model of surgical menopause, various estrogen/progresterone combinations and raloxifene were administered and resulting changes in cell proliferation were assesed by Ki-67 immunocytochemistry and histological evaluation.

Materials and Methods

Experimental animals: Sexually mature female Sprague-Dawley rats (190-250 g) were used in the experiments (n = 78). Subjects were ovariectomized and drug treatments started three weeks after surgery. Following recovery from surgery, rats were kept under standard colony conditions (four/cage, 20-22°C, 12-hour light/dark cycle) with *ad-lib* food and water.

The animals were handled at all times under the guidelines for animal care and experimentation of the pertinent European Communities Council Directive (86/609/EEC), and all procedures were approved by the Institutional Animal Ethics Committee of Ege University.

Surgery: Rats were anesthetized by pentobarbitone sodium (40 mg/kg, intraperitoneal, Pentotal). Ovaries were pulled out through small bilateral flank incisions (1 cm each), the junction between the Fallopian tube and the ovary were ligated, the side of the ovary was cut and the ovaries were removed. The horns were returned into the abdominal cavity through the openings and skin incisions were closed with sutures.

Drugs: All hormone/drug treatments were applied for 20 days (5 cycles), intraperitoneally at the same time each day. Pilot studies in the literature revealed no differences between subcutaneous or intraperitoneal routes of injection [7]. 17β-Estradiol (50 μg/kg, 17β-Estradiol, Sigma) and medroxyprogesterone (2.5 μg/kg, medroxyprogesterone, Sigma) were dissolved in peanut oil (1 ml/kg, Sigma). The vehicle was used for control injections. OVX rats received 17β-estradiol or vehicle injections at 16:00h and progesterone injections at 08:00h. Raloxifen (1 mg/kg, Sigma) was dissolved in carboxymethyl cellulose (5% 1 mg/kg, Sigma) and applied at 08:00h. The control group received only the vehicle.

Experimental groups:

1. Estrogen and progesterone control group (peanut oil): PO (n = 8);
2. 17β estradiol group: E (n = 12);
3. 17β estradiol and medroxyprogesterone group: EP (n = 12);
4. 17β estradiol and intermittent medroxyprogesterone applied group: EintP (n = 14);
5. Raloxifene group: R (n = 18);
6. Raloxifene control group (carboxymethyl cellulose): CC (n = 14).

The estrus cycle of Sprague Dawley adult rats is four days on average. Subsequently, while the EP group received an estrogen and progestin combination continuously, in the intermittent protocol (EintP group) progestin was combined with estrogen intermittently at two-day intervals.

Tissue sampling and immunohistochemistry: At the end of drug/vehicle treatments, rats were anesthetized with phenobarbital sodium and perfused intracardially initially with phosphate buffered saline and then with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Mammary glands were dissected, specimens were put into 3.7% buffered formalin for two hours and then immersed into paraffin to be later processed and examined. Tissue sections 3 μm in thickness were kept in an incubator at 55°C overnight for deparafinization, placed into 1 mM ethylene diamine tetraacetic acid (EDTA) at pH:8 for 2-3 min with negative pressure and treated with 3% H_2O_2. Following buffered phosphate solution treatment, DAKO Protein Block X0909 was used to avoid artifacts. Subsequently, Ki-67 Antigen (M7248) was applied at 1:25 dilution and the signal was amplified using biotinylated antimouse IgG (DAKO, 1:25 dilution) and streptavidin peroxydase (DAKO LSAB^R 2 System HRP, K0609). Sections treated with diaminobenzidine (DAB, DAKO, K3467) were processed; terminal ducts and lobules (terminal ductal lobular units, TDLU) were examined under light microscope.

Quantification of immunohistochemical staining: The Ki-67 proliferation index was determined by scoring at least 1,000 cells for each section according to the method of Sadi and Barrack [8]. Specialized mammary gland ducts and TDLU were determined under light microscope and the number of epithelial cells positive for Ki-67 was determined among 1,000 epithelial

cells. Ki-67 proliferation indices in the TDLU and ducts were used for group comparisons.

Statistical analysis: Initially the two control groups (PO and CC) were compared. Since there were no Ki-67 positive cells in either control group and since the histological evaluation was identical, control groups were pooled. Animals with and without Ki-67 positive cells were subjected to Fisher's exact probability test computed by SPSS program for Windows. The level for rejecting the null hypothesis was 0.05.

Results

Tables 1 and 2 depict the number of animals in the group with a positive proliferative index and the range of the number of cells positive for Ki-67, respectively. In the TDLU, while no proliferation was observed in the control group (i.e., OVX rats receiving vehicle), in groups receiving estrogen, with or without progesterone, proliferation reached significant levels. Rats treated with raloxifene did not differ from the controls and there was only one rat of 18 where two cells were positive for Ki-67.

Table 1. — *Number of animals with a positive proliferative index in each experimental group; the numbers represent the animals with Ki-67 positive cells in the group (ratio).*

	E (n = 12)	EP (n = 12)	EintP (n = 14)	R (n = 18)	Control (n = 22)
TDLU	8** (66.7%)	3* (25%)	7** (50%)	1 (5.5%)	0
Ducts	1	0	2	0	0

Different from control: **p < 0.001, * p < 0.05 (Fisher's exact test).

Table 2. — *Range of the number of cells positive for Ki-67 (in 1,000 cells counted).*

	E	EP	EintP	R	Control
TDLU	4-100	12-16	2-157	2	–
Ducts	3	–	2-2	–	–

In the ducti, proliferation was negligible with no significant differences between the treatment and control groups, although in one animal in the E group three positive cells and in the EintP group in two animals two positive cells were seen.

In H&E stained sections proliferation in ductal epithelia and the surrounding alveoli was prominent in estrogen-treated rats. Vacuolated epithelial cells, dilatation of the duct cysts and secretion-filled ducts were seen in the sections taken from the EP and EintP groups of rats. The raloxifene group was characterized by atrophic mammary tissue in the stroma rich adipose tissue and this finding matches with postmenopausal findings of human mammary glands. Normal postmenopausal mammary glands characterized by atrophic duct cysts in stroma rich in adipose tissue are represented by the PO control group. Figure 1 shows sections with Ki-67 immunohistochemistry.

Discussion

Breast cancer is the most common cancer among women and the second leading cause of death after lung cancer [9]. Especially with widespread use of HRT during the postmenopausal period, the response of the mammary gland to ovarian hormones has been the subject of many studies. Many researchers draw attention to the potentiating effect of HRT on breast cancer [10-15].

In the WHI study, combined estrogen and progesterone is reported to increase breast cancer risk by 1.26 compared to using estrogen alone. However it is not clear whether this finding is due to the hormonal intervention or reflects an already preexisting and unrecognized tumor [16-18]. Epidemiological evidence supports the role of ovarian hormones on breast carcinogenesis and lifetime exposure to the mitogenic effects of ovarian hormones is a major risk factor in the development of this disease [19-20].

The proliferation index of the mammary gland is higher during the luteal phase of the menstrual cycle when higher progesterone levels dominate, compared to the follicular phase [21-22]. Progesterone, previously defined as antagonizing the proliferating effects of estrogen is now recognized to have a proliferative, even mitogenetic effect on some tissues [23]. Pike and Spicer have shown an increase of mitotic activity in mammary gland epithelium with progesterone during the second half of the menstrual cycle, and identified this effect as an increased risk for breast cancer combined with the mitogenic effect of estrogen [20, 24]. Progesterone has been suggested to increase the risk of breast cancer through increased DNA synthesis, estron production from estron sulphate, and insulin receptor mRNA synthesis in mammary glands [24, 25]. On the contrary, other investigators have suggested that progesterone increases estradiol and estron breakdown by enhancing the activity of estradiol dehydrogenase and estron sulphurtransferase enzymes, respectively [26].

Persson *et al.* [27] found a significant increase in breast cancer by using combined EP-HRT for six years or more, while no similar increased risk was observed with ERT. Although direct comparisons cannot be made, the results from our rat model do not support these reported findings in humans; however, the risk with countinuous estrogen may be lower compared to intermittent progesteron.

Magnusson *et al.* [11] reported increased risk of breast cancer when testosterone-derived progestins were used instead of progesterone-derived progestins in HRT protocols. Different effects of different progestins on the mammary gland were studied by Seeger *et al.* [28] and the results revealed that the type of progestin used may also be important in addition to the type of HRT protocol employed regarding effects on breast cancer.

A large number of studies report similarities between mouse, rat and human mammary glands; therefore, developing animal models provides opportunities to test the mechanism of action and efficacy of different therapeutic strategies in rodents. Hofseth *et al.* [29] compared the mammary gland of postmenopausal women (either surgical or natural menopause) regarding proliferative effects of estrogen replacement therapy (ERT) and found no differences. Benign breast biopsies from 86 postmenopausal women were analyzed with antiproliferating cell nuclear antigen (anti-PCNA) and Ki67 antibodies to measure relative levels of cell proliferation.

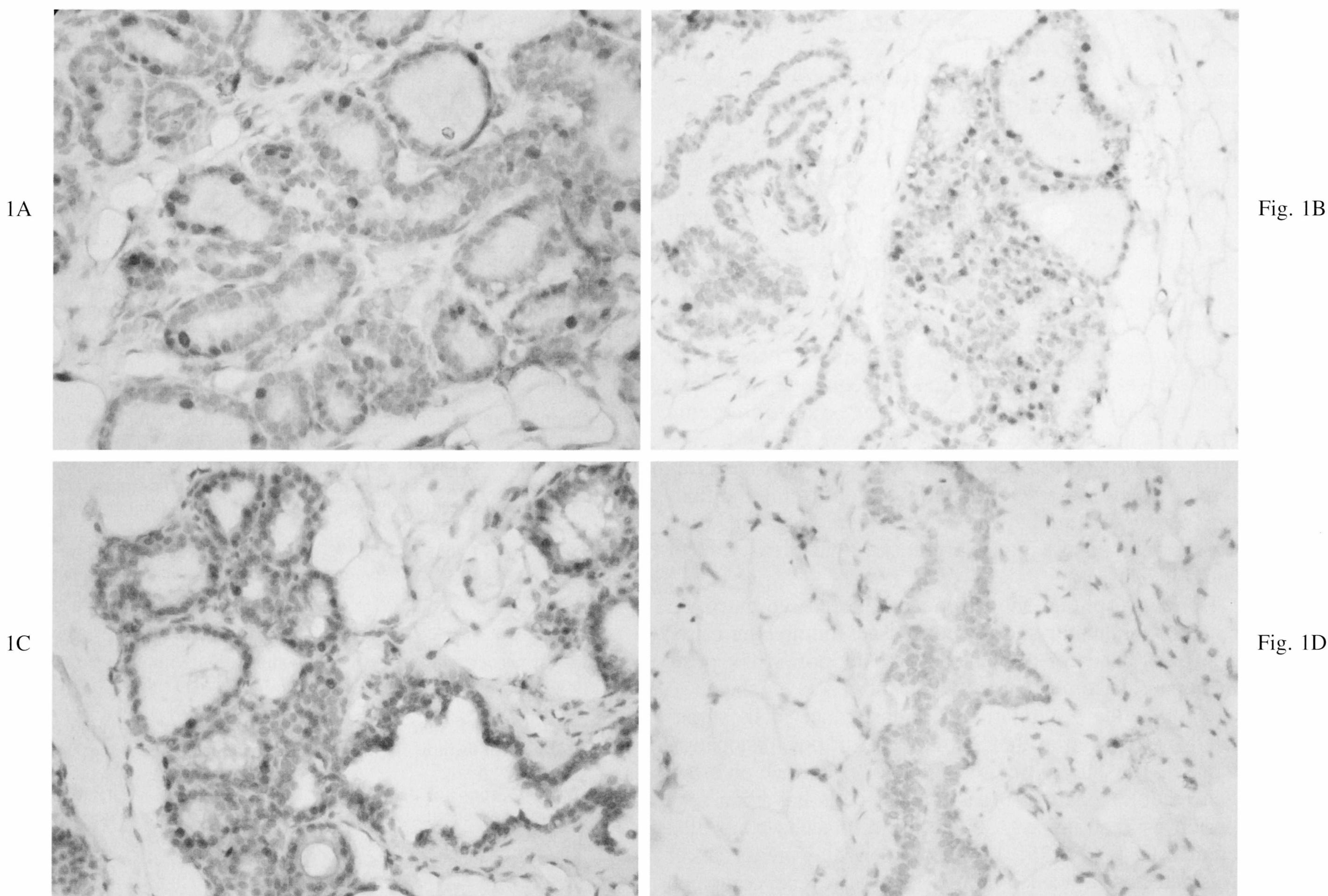

Figure 1. — Ki-67 positive cells in the mammary gland of OVX rats receiving ovarian hormones or raloxifene. A) Estrogen (x 40), B) Estrogen and intermittent progestin (x 20); C) Estrogen and continuous progestin (x 20), D) Raloxifene (duct, x 20).

Further studies are needed to assess the possible association between the mitogenic activity of progestins and breast cancer risk in both humans and animal models. Considering related findings in the literature, in our rat model we studied the effect of estrogen alone and in combination with progesterone; since in clinical practice progesterone can be combined with estrogen intermittently to simulate a regular menstrual cycle, we have also included a group with estrogen combined with intermittent progesterone.

We have used Ki-67 immunohistochemistry as an index of the proliferative response of mammary glands to hormonal stimuli because it is used as a marker in breast cancer diagnosis and is accepted as an independent risk factor in determining the recurrence of the tumor [30].

In our study, estrogen-treated rats had a significantly increased ratio of proliferative changes in the TDLU. The effect was more pronounced when estrogen was administered alone or in combination with intermittent progesterone, imitating the estrus cycle. Estrogen paired with progesterone continuously throughout the 20 days of treatment also increased the ratio of animals with proliferative changes in the TDLU compared to controls, but the number of animals affected, and therefore the significance level of the difference, was lower than for the other two groups mentioned.

Considering the available information, it is apparent that the effect of different dosages and administration protocols and of different formulations of estrogens and progestins may be critical regarding impact on breast cancer; therefore future research will be illuminating. Furthermore, since proliferative changes were observed only in some animals following hormone treatments (Table 1), the contribution of other factors on increasing vulnerability, such as genetic predisposition, should not be overlooked.

After Russo *et al.* [4] defined TDLU as the origin of breast cancer, Haslam *et al.* [21] showed higher rates of proliferative activity in TDLU epithelium in mice treated with combined estrogen and progestin compared to mice receiving only estrogen or to controls. In another study by same authors [31] ductal dilatation and side-branching in mammary gland tissue was observed in combined HRT-treated mice which was inhibited by the progesterone

antagonist RU486; therefore the authors blame the progestin component of HRT for this effect. In our study continuous progesterone combined with estrogen gave better results, regarding proliferation in the TDLU, compared to estrogen alone or combined with intermittent progesterone. This finding supports the view that progesterone may be inhibiting the proliferative effect of estrogen.

Haslam *et al.* [21], evaluated the proliferative effects of hormones on mammary glands with Ki-67 and anti-PCNA and found higher indices of Ki-67 especially in TDLU epithelium of HRT users compared to non-users. Hargreaves *et al.* [32] did not find differences in Ki-67 indices among the breast biopsy specimens of estrogen replacement therapy (ERT) users and combined EP users. In our study the proliferative changes and mitogenic effects in the hormone treated groups were significantly different from controls in TDLU while no significant differences were observed in the ducti, confirming the findings of Russo *et al.* [4] and Halsam *et al.* [21]. Therefore our findings also support the notion that breast cancer starts in TDLU.

Benzothiophene raloxifene is a selective estrogen receptor modulator and acts on tissues mimicking estrogen; it is used for prevention of osteoporosis in postmenopausal women [33]. Raloxifene has been shown to antagonize the proliferation of breast cancer cells stimulated by estrogen at the cellular level in postmenopausal women. The antiproliferative effect of raloxifene on estrogen receptor positive breast cancer cells has been supported by randomized, double-blind, placebo-controlled clinical trials [34, 35]. Lower incidence of breast cancer was detected in women treated with raloxifene, compared to placebo in longitidunal studies [35, 36]. In the present study, we showed that raloxifene protects normal mammary gland architecture without any proliferative activity either in TDLU or in the ductal epithelium in OVX rats. To our knowledge, this is the first study in the literature comparing the effects of HRT and raloxifen on mammary gland tissue in an animal model of surgical menopause and showing that no difference exists between the control and raloxifen treated groups regarding Ki-67 proliferation indices and histological findings. The clinical expression of the present results obtained from rats may suggest raloxifen as a good drug of choice in osteoporotic women especially those with coexisting breast disease.

In conclusion, the present study indicates that in OVX rats, while ovarian hormones increase proliferative changes in the TDLU, suggesting increased vulnerability to breast cancer, raloxifene treatment does not have such an effect.

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