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Effects of estradiol, progestogens, and of tibolone on breast proliferation and apoptosis

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ABSTRACT

Aim To study the effects of estrogen therapy, alone or combined with progestogens, and of tibolone on the expression of proliferation and apoptosis markers in normal breast tissue.

Methods Thirty 250-day-old Wistar rats were castrated and 3 weeks later received one of the following treatments by gavage for 5 weeks: (1) estradiol benzoate; (2) estradiol benzoate + medroxyprogesterone acetate; (3) estradiol benzoate + norethisterone acetate; (4) estradiol benzoate + dydrogesterone; (5) tibolone; (6) placebo. Following treatment, the expression of proliferating cell nuclear antigen (PCNA) and caspase-3 was analyzed by quantitative immunohistochemistry in the breast tissue, and proliferation and apoptosis were analyzed semiquantitatively by microscopic imaging.

Results There was a statistically significant difference among the groups for PCNA, caspase-3 and the caspase-3 : PCNA ratio. Tibolone was associated with the lowest proliferative activity, followed by estradiol benzoate + dydrogesterone; however, estradiol benzoate + dydrogesterone showed the greatest rate of apoptosis.

Conclusions The various progestogens can have more or less proliferative and pro-apoptotic effects than estradiol alone. Among the treatment schemes analyzed, the estradiol + dydrogesterone combination resulted in a higher apoptosis rate in relation to the proliferation rate and tibolone was associated with the lowest proliferation.

INTRODUCTION

Observational and randomized studies have shown that estrogen and progestogen therapies following menopause increase the risk of developing breast cancer to a greater extent than using estrogen alone[1,2]. However, it is possible that the various progestogens can have different impacts on the risk of developing breast cancer[3].

Breast cancer cell culture studies have shown that some synthetic progestogens induce a proliferative response, whilst others do not have this effect or it is less intense[4]. The interaction between estrogens and the various progestogens is certainly complex as is its involvement in the appearance of cancer; however, the effects on epithelial proliferation and cell apoptosis can be important mechanisms that are involved. The diverse effects that hormonal treatments have on these parameters have already been reported for breast cancer cell cultures[5].

Normal, non-neoplastic breast tissue has not been the focus of many studies, and no studies to our knowledge to date have compared the effects of various progestogens on proliferation and apoptosis in breast tissue, and this has raised the interest in studying such aspects. The aim of this study was to evaluate the effects of estrogen and progestogens, including tibolone, on the expression of epithelial proliferation and apoptosis markers in normal breast tissue of castrated rats.

METHODS

All of the procedures used in the study described below were approved by the animal research ethics committee of the Faculdade de Medicina do ABC.

Thirty 250-day-old Wistar rats were randomly selected and subjected to bilateral oophorectomy, under anesthesia with

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ketamine and xylazine injected intraperitoneally. The ventral longitudinal abdominal approach was used for identifying and ligating the ovarian pedicles and then removing the gonads.

Three weeks after the surgical procedure, microscopic analysis of vaginal smears of all of the rats was carried out to confirm hypoestrogenism. Subsequently, the animals were randomly divided into six groups of five animals each, and the groups received one of the following treatments administered daily by gavage for 5 consecutive weeks: Group EB: estradiol benzoate (EB) administered orally in a dose of 1 mg/kg; Group EB/MPA: EB 1 mg/kg combined with medroxyprogesterone acetate (MPA) 0.2 mg/kg; Group EB/NETA: EB 1 mg/kg combined with norethisterone acetate (NETA) 0.2 mg/kg; Group EB/DI: EB 1 mg/kg combined with dydrogesterone (DI) 2 mg/kg; Group TIB: tibolone (TIB) 1 mg/kg; Group CTR: oral placebo.

The animals were kept in a calm environment with a constant temperature of 23°C, a 12-h light period per day, and water and food *ad libitum*.

At 250 days of life, the fertility of female rats is declining; this is roughly comparable to the menopausal transition for women. A 5-week treatment corresponds to about eight estrous cycles of this animal, roughly comparable to eight menstrual cycles in women.

At the end of the treatment period, the rats were euthanized in a CO_2 chamber. The second left thoracic mammary gland of each animal was immediately isolated, resected and fixed in 10% buffered formalin.

The detailed procedures for the immunohistochemical analysis were the same as those previously published by our group[6]. Briefly, immunohistochemistry was carried out to analyze protein immunolabeling for proliferating cell nuclear antigen (PCNA) and caspase-3 in 3-µm-thick histological slices. The primary antibodies used were anti-PCNA and anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, USA) diluted in bovine serum albumin (BSA) in a ratio of 1 : 300. The slides were analyzed using a Nikon Eclipse TS100 optic microscope (Nikon Instruments, Melville, USA) using the same light intensity and condenser height for all slides. The areas that best represented the immunolabeling of the slide were always chosen by the same pathologist (A.M.M.), in a blind manner for treatment groups, and analyzed by $400\times$ magnification. Photomicrographs (640×480 pixels) of consecutive, non-overlapping fields were obtained using a Nikon Coolpix 4300 (Nikon Corporation, Japan) digital camera with maximum zoom. Immunohistochemical labeling was quantified using the Scion ImageLab® for Windows software (Scion Corporation, Frederick, USA).

A histological analysis of five highly magnified fields was also carried out by the same pathologist (A.M.M.), in a blind manner for treatment groups, with ductal epithelial proliferation and apoptosis being classified in a semiquantitative manner as grade 0 (absent or minimal) to 3 (maximum).

Statistical analysis

The data obtained were organized in electronic spreadsheets using the Microsoft Excel®2007 software (Microsoft Corporation®, San Diego, USA). Statistical analysis was carried out using the software WinSTAT®, version 2007.1 (R. Fitch Software, Germany). Continuous numerical data are presented in the mean $\pm$ standard deviation format. Normal distribution was tested using the Kolmogorov–Smirnov test. Group comparisons were carried out by analysis of variance (ANOVA) and multiple comparisons were corrected using the least significant difference method. The Kruskal–Wallis test was carried out for ordinal numerical data (semiquantitative histological analysis) or data without a normal distribution, or when homogeneity of variance had not been proven. Spearman rank correlation analysis was carried out for quantitative and semiquantitative parameters. A significance level of 5% was adopted.

RESULTS

A rat from the tibolone group was found dead one morning with evidence of having been attacked by the other rats in the group. Therefore information from 29 animals was analyzed. The average weight of the rats were 310.6 ± 25.9 g at the beginning of the study.

Table 1 shows the immunohistochemistry results for the markers PCNA, caspase-3 and the caspase-3 : PCNA ratio for each group. It can be noted that there was a statistically significant difference for all parameters. In multiple comparisons, statistically significant differences were found amongst all the groups for PCNA, with the exception of the comparison for EB versus EB/NETA. In the case of caspase-3 expression, the majority of comparisons amongst the groups was statistically significant, with the exception of CTR vs. EB, CTR vs. EB/MPA, CTR vs. EB/NETA, EB vs. EB/MPA and EB/NETA vs. EB/DI.

Table 1 Results of the immunohistochemistry quantification of proliferating cell nuclear antigen (PCNA), caspase-3 and the caspase-3 : PCNA ratio, according to treatment group

	Estradiol benzoate	*Estradiol benzoate + MPA*	*Estradiol benzoate + NETA*	*Estradiol benzoate + dydrogesterone*	*Tibolone*	*Control*	*p Value*
PCNA	62.8 ± 4.8	87.8 ± 7.1	64.0 ± 8.1	40.8 ± 3.3	28.1 ± 4.2	118.4 ± 3.5	<0.001
Caspase-3	78.1 ± 10.6	81.5 ± 10.1	104.7 ± 22.9	111.4 ± 24.3	25.5 ± 6.2	84.4 ± 10.6	<0.001
Caspase-3 : PCNA ratio	1.24 ± 0.07	0.93 ± 0.11	1.69 ± 0.54	2.73 ± 0.57	0.93 ± 0.27	0.71 ± 0.09	<0.001

MPA, medroxyprogesterone acetate; NETA, norethisterone acetate

In the case of the caspase-3 : PCNA ratio, multiple comparisons showed statistically significant differences for EB/DI when compared to all the other groups and for EB/NETA compared to almost all the other groups, except EB. The other groups were not statistically different among themselves. Figure 1 is a graphical representation of the results.

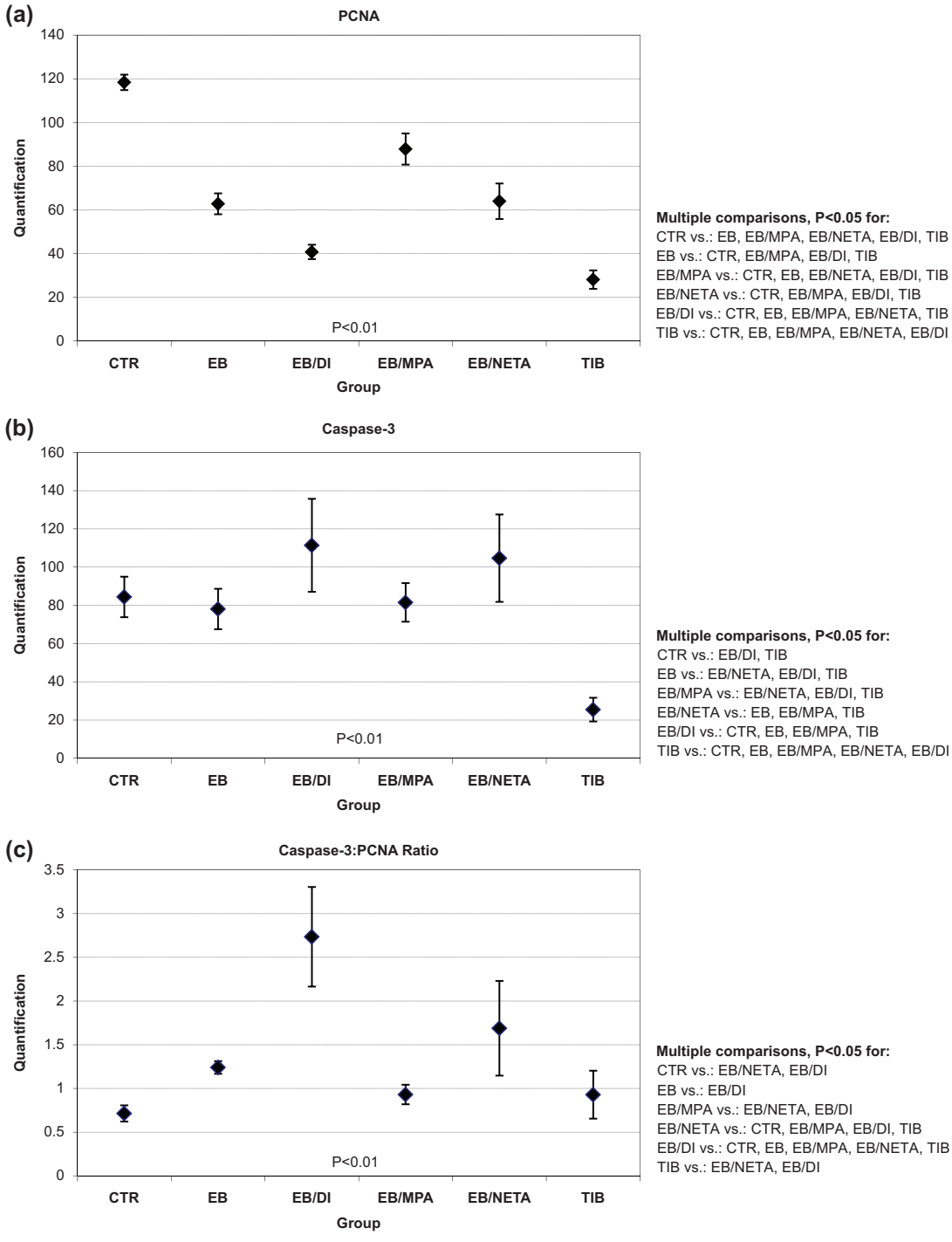

Figure 1 Graphical representation of the means and standard deviations obtained for the immunohistochemical quantification of proliferating cell nuclear antigen (PCNA), caspase-3 and the caspase-3 : PCNA ratio, per group (differences between groups, $p < 0.001$). Multiple comparisons: (a) PCNA: $p < 0.05$ for all the pairs compared, *with the exception of* EB vs. EB/NETA (n.s.); (b) Caspase-3: $p < 0.05$ for all, *except for* CTR vs. EB, CTR vs. EB/NETA, CTR vs. EB/MPA; EB vs. EB/MPA and EB/NETA vs. EB/DI (n.s.); (c) Caspase-3 : PCNA ratio: $p < 0.05$ for EB/DI vs. CTR, EB/DI vs. EB, EB/DI vs. EB/MPA, EB/DI vs. EB/NETA, EB/DI vs. TIB, EB/NETA vs. CTR, EB/NETA vs. EB/MPA, EB/NETA vs. TIB, for all other comparisons, not significant. EB, estradiol benzoate; NETA, norethisterone acetate; MPA, medroxyprogesterone acetate; DI, dydrogesterone; TIB, tibolone; CTR, control

Although the semiquantitative analyses using a regular optical microscope did not reveal any statistically significant differences, there was a statistically significant correlation between caspase-3 and the degree of apoptosis ($r = 0.42$, $p = 0.016$).

DISCUSSION

We have found no other studies that compare the effects of hormonal treatments with so many progestogens on proliferation and apoptosis in normal breast tissue as much as ours does. In summary, the least epithelial proliferation was observed in the tibolone group, followed by the estradiol + dydrogesterone group, and the most apoptosis was found in the estradiol + dydrogesterone group, followed by the control group, in such a way that the highest apoptosis/proliferation ratio was found in the estradiol + dydrogesterone group.

From the graphical analysis and based on multiple comparisons, a clear difference can be noticed in the effects of the various progestogens on epithelial proliferation expressed by PCNA. Whilst the estradiol + MPA combination had the highest proliferative activity, dydrogesterone had less and the least proliferation was seen with tibolone.

In a previous publication, we had already observed greater proliferation with estradiol + MPA treatment than with estradiol alone[7]. In the same manner, however, in monkeys, greater breast proliferative activity has recently been shown with estrogen + MPA as opposed to only estrogen or tibolone[8]. The low breast proliferative activity of tibolone has also been demonstrated in another research model[9,10].

Differences in terms of apoptosis are also clear among the various progestogens being studied, the highest rate being observed with dydrogesterone. However, the analysis of apoptosis in relation to proliferation is probably more important than of apoptosis alone, and is expressed in this study through the caspase-3 : PCNA ratio. Once again, the estradiol + dydrogesterone combination revealed the highest apoptosis in relation to proliferation.

Tibolone resulted in the least apoptosis; however, we believe this is due to the fact that epithelial proliferation was also lower, as already mentioned.

Surprisingly, the control group was the one with the highest levels of PCNA, and therefore with the most epithelial proliferation. However, the apoptosis rates of this group were only statistically different to those of tibolone and the estradiol + dydrogesterone combination, with the control group exhibiting more apoptosis than the tibolone group and less than the estradiol + dydrogesterone group. It is hard for us to explain this finding which contradicts our expectations, but it is probably due to multiple other breast proliferation factors that were not evaluated in this experiment.

Overall, our results are in line with observations made by Fournier and colleagues, according to whom, women who received estrogen combined with micronized progesterone or dydrogesterone showed a risk of developing breast cancer similar to those who did not take it and lower than when taking only estrogen. On the other hand, the risk was higher than that of only estrogen, if there was another progestogen other than dydrogesterone or progesterone in the therapeutic hormonal treatment[3].

Furthermore, the low proliferation observed with tibolone supports the findings of a clinical study, in which women who received tibolone had less chance of developing breast cancer[11].

It is important to point out that the findings described here come from normal breast tissue and not from breast cancer cells like the majority of studies on this subject. This is relevant since hormones which could possibly decrease the risk of developing breast cancer[11] could be associated with a greater risk of the cancer recurring in women who have already had the illness, which proves that tumor cells respond in different ways to the various steroids[12].

This study has some limitations, such as the fact that it evaluates proliferation and apoptosis objectively with only one marker for each, and also the subjective semiquantitative evaluation; however, the correlation between the subjective evaluation and the immunohistochemistry for apoptosis supports the fact that this objective method was appropriate. Another limitation was the relatively small sample size, but even so, it proved to be statistically suitable for the variables of interest.

On the other hand, it should be pointed out that a comparison of the effects of various progestogens combined with estradiol and of tibolone on normal breast tissue was carried out in one study, which is novel according to our search of the literature.

The appearance of breast cancer is dependent on the complex interaction of numerous factors[13], including the hormones evaluated in this study. Growth factors, oxidative stress factors and other factors are certainly involved, but were out of the scope of this study[13,14].

In conclusion, tibolone was associated with the least breast epithelial proliferation, but also the least apoptosis; estrogen + dydrogesterone combined were associated with less proliferation than estrogen alone and the most apoptosis. Estroprogestative treatment containing medroxyprogesterone acetate was associated with greater epithelial proliferation than estrogen alone.

Conflict of interest Luciano M. Pompei is lecturer for the following pharmaceutical companies: Abbott, Bayer, GSK, MSD, Libbs, and TEVA. Cesar Eduardo Fernandes is lecturer for the following pharmaceutical companies: Bayer, Sanofi and TEVA. The other authors have no conflict of interest to declare.

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References

1. Beral V; Million Women Study Collaborators. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;362:419–27
2. Manson JE, Chlebowski RT, Stefanick ML, *et al*. Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the Women's Health Initiative randomized trials. *JAMA* 2013;310:1353–68
3. Fournier A, Berrino F, Clavel-Chapelon F. Unequal risks for breast cancer associated with different hormone replacement therapies: results from the E3N cohort study. *Breast Cancer Res Treat* 2008;107:103–11
4. Ruan X, Neubauer H, Yang Y, *et al*. Progestogens and membrane-initiated effects on the proliferation of human breast cancer cells. *Climacteric* 2012;15:467–72
5. Chen FP, Chien MH, Chen HY, Ng YT. Effects of different progestogens on human breast tumor cell growth. *Climacteric* 2011;14:345–51
6. Pompei LM, Steiner ML, Theodoro TR, *et al*. Effect of estrogen therapy on vascular perlecan and metalloproteinases 2 and 9 in castrated rats. *Climacteric* 2013;16:147–53
7. Pompei LM, Carvalho FM, Ortiz SC, Motta MC, Cruz RJ, Melo NR. Morphometric evaluation of effects of two sex steroids on mammary gland of female rats. *Maturitas* 2005;51:370–9
8. Wood CE, Branstetter D, Jacob AP, *et al*. Progestin effects on cell proliferation pathways in the postmenopausal mammary gland. *Breast Cancer Res* 2013;15:R62
9. Cline JM, Register TC, Clarkson TB. Effects of tibolone and hormone replacement therapy on the breast of cynomolgus monkeys. *Menopause* 2002;9:422–9
10. Conner P, Register TC, Skoog L, *et al*. Expression of p53 and markers for apoptosis in breast tissue during long-term hormone therapy in cynomolgus monkeys. *Am J Obstet Gynecol* 2005; 193:58–63
11. Cummings SR, Ettinger B, Delmas PD, *et al*. The effects of tibolone in older postmenopausal women. *N Engl J Med* 2008; 359:697–708
12. Kenemans P, Bundred NJ, Foidart JM, *et al*. Safety and efficacy of tibolone in breast-cancer patients with vasomotor symptoms: a double-blind, randomised, non-inferiority trial. *Lancet Oncol* 2009;10:135–46
13. Wren BG. The origin of breast cancer. *Menopause* 2007;14: 1060–8
14. Jezierska-Drutel A, Rosenzweig SA, Neumann CA. Role of oxidative stress and the microenvironment in breast cancer development and progression. *Adv Cancer Res* 2013;119: 107–25