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Lowering oral contraceptive norethindrone dose increases estrogen and progesterone receptor levels with no reduction in proliferation of breast epithelium: a randomized trial

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Abstract

Background—This study was conducted to compare breast epithelial-cell proliferation and estrogen and progesterone receptor levels in women taking one of two oral contraceptives (OCs) containing the same dose of estrogen but different doses of the progestin norethindrone (NET).

Study Design—Thirty-three women were randomly assigned 1:1 to one of two OCs with 35-mcg ethinylestradiol (EE2) but different doses of NET - 1 mg or 0.4 mg. At the end of the active pill phase of the third OC cycle, a breast biopsy was performed and the percentages of epithelial

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Authorship

DT, DVS, MCP, AHW and CLP participated in the design of the study. DH supervised the preparation of the slides and analyzed the slides with the ACIS II system. DH, DVS, and MFP consulted on the interpretation of issues arising in the IHC analysis. MFP performed the review of the biopsy material to ensure that there was no evidence of any abnormalities requiring further clinical evaluation. LHL performed all the biopsies that provided the tissues used in this study. CLP coordinated the study. MCP supervised the statistical analysis. DVS, MCP, AHW, and CLP conceived of the study. MCP, AHW, and CLP drafted the manuscript. All authors critically read the manuscript and approved the final draft.

Competing interests

The authors declare that they have no competing interests.

cells of the terminal duct lobular units were measured for Ki67 (MIB1), progesterone receptors A and B (PRA and PRB), and estrogen receptor α (ER α).

Results—The biopsies from 27 women had sufficient epithelium for analysis. The percentage of cells positive for PRA, PRB, and ER α were approximately double with the lower progestin dose (PRA: $p = 0.041$; PRB: $p = 0.030$; ER α : $p = 0.056$). The Ki67 percentage was not reduced with the lower progestin dose (0.4-mg NET - 12.5% vs 1.0-mg NET - 7.8%).

Conclusions—The increase in PRA, PRB, and ER α positive cells with the 60% lower progestin dose OC appears likely to account for its failure to decrease breast-cell proliferation. This breast-cell proliferation result is contrary to that predicted from the results of lowering the medroxyprogesterone acetate dose in menopausal hormone therapy.

Keywords

Breast epithelial cells; Combined oral contraceptives; Estrogen receptor; Progesterone receptor; Progestin dose; Proliferation markers

1. Introduction

Breast epithelial-cell proliferation is higher in women on menopausal estrogen-progestin therapy (MEPT) than in women on menopausal estrogen therapy (MET) [1], and epidemiological studies have shown that MEPT significantly increases a postmenopausal woman's risk of breast cancer and that the effect is greater than with MET [2, 3]. Analysis of the studies of the effects of MEPT on breast cancer risk showed that a lowering of the dose of the progestin, medroxyprogesterone acetate (MPA), from 10 mg to 2.5 mg significantly reduced the increased breast cancer risk from the MEPT despite extending the number of days the MPA was given from ~10 per 28-day cycle (sequential MEPT) to every day (continuous-combined MEPT) [2].

The large comprehensive meta-analysis conducted by the Collaborative Group on Hormonal Factors in Breast Cancer found that breast cancer risk was slightly increased in current and recent users of oral contraceptives (OCs) [4], but how much of this increase is due to earlier diagnosis because of more frequent contact with the medical system is not completely clear. OCs effectively block ovulation and are thus associated with profoundly reduced levels of serum progesterone. Since the progestin exposure of the breast of a woman on an OC is thus almost solely from the OC progestin, the MEPT results discussed above would predict that the level of progestin in an OC would be positively associated with its effect on breast cancer risk. This was not seen in the Collaborative Groups' analysis and the results of more recent studies have also not been able to provide definitive results on the effect of progestin type or dose [5–9]. In order to gain an understanding of the biology behind this, we decided that it would likely be informative to study the effect of progestin dose on the breast directly by studying the effects on cell proliferation and steroid receptor levels in breast epithelium of two commonly prescribed Food and Drug Administration (FDA) approved OCs containing the same dose of estrogen but different doses of the progestin norethindrone (NET): Ortho Novum 1/35[®] contains 35-mcg ethinyl-estradiol (EE2) and 1-mg NET, and Ovcon 35[®] contains the same dose of EE2 (35 mcg) but only 0.4-mg NET, a 60% reduced dose of progestin. A 10-mg dose of MPA is generally considered as approximately equivalent to a 1-mg dose of NET [10], so the doses being considered in these OCs are roughly comparable to the doses of MPA in sequential and continuous-combined EPT.

The results of lowering the progestin dose in MEPT predicted that the 0.4-mg NET OC would be associated with a marked reduction in breast-cell proliferation compared to the 1-mg NET OC.

2. Materials and methods

The study protocol was approved by the Institutional Review Board (IRB) of the University of Southern California Keck School of Medicine and by the IRB of the Department of Defense Congressionally Directed Breast Cancer Research Program prior to the enrollment of any participants. All participants gave written informed consent, and, in particular, were fully informed in detail concerning the potential risks associated with the ultrasound-guided core breast biopsy sample collection (see below).

2.1. Study participants

Women attending clinics at Los Angeles County/University of Southern California Women's and Children's Hospital who were being prescribed an OC solely for contraception were invited to volunteer for this study.

To be eligible for the study a subject had to be: premenopausal aged 18–34 years; currently taking and willing to switch type of OC or wishing to start taking an OC for contraception; a non-smoker; and willing to refrain from consumption of grapefruit or grapefruit juice during the study (grapefruit interferes with metabolism of exogenously administered OCs) [11]. Subjects with any of the following were ineligible: abnormal breast examination; history or current therapeutic or prophylactic use of anticoagulants; known bleeding disorder or history of unexplained bleeding or bruising; history of breast cancer or previous diagnostic breast biopsy; known allergy to local anesthetic; currently pregnant or pregnant within the previous 6 months; or having any standard contraindication to being prescribed an OC [12].

Women, who expressed a possible desire to participate, were fully informed in detail concerning the potential risks and, in particular, with the risks associated with the ultrasound-guided core breast biopsy procedure. If they continued to express a desire to participate, they were requested to sign an informed consent. After providing written informed consent, the participants underwent a routine clinical breast examination. If there was an abnormal finding on the breast examination, the subject would have been excluded from the study, but no such abnormalities were found. Eligible subjects were administered a menstrual and reproductive history questionnaire, and height and weight were measured. Subjects were provided with three 28-day cycle packs of Ortho Novum 1/35[®] or Ovcon 35[®] with instructions to take the pills each evening. The sequence for the 1:1 treatment allocation was determined using a random-number table constrained by the use of randomly permuted blocks. The brand name was provided to the attending physician in a sealed envelope only to be opened after the subject had been enrolled and had completed the above procedures.

A breast biopsy was performed during the third consecutive OC cycle at the end of the third week of active OC pill use. The radiologist performing the biopsy was blinded to OC type.

2.2. Tissue procurement

An ultrasound-guided 14-gauge core-needle breast biopsy was performed in a region of ultrasonographically normal-dense breast tissue in the upper-outer quadrant of the breast. After anesthetizing the breast with 1% lidocaine, a 4-mm incision was made to facilitate entry of the biopsy needle. Multiple core biopsy samples were obtained through the same single incision. The biopsy specimens were formalin-fixed and paraffin-embedded (FFPE) in a routine manner at the University of Southern California Department of Pathology.

2.3. Immunohistochemistry

Immunohistochemical (IHC) analysis of the FFPE samples was performed for Ki67 (MIB1; a proliferation marker), progesterone receptor A (PRA), progesterone receptor B (PRB), and estrogen receptor α (ER α). Multiple adjacent FFPE sections were cut at 5 μ m, deparaffinized and hydrated. All slides were subjected to antigen retrieval which was performed by heating the slides in 10 mmol/l sodium citrate buffer (pH 6) at 110°C for 30 min in a pressure cooker in a microwave oven [13]. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ in phosphate-buffered saline for 10 min, followed by blocking of nonspecific sites with SuperBlock blocking buffer (Pierce, Rockford, IL, USA) for 1 h both at room temperature [14].

The sections were incubated for analysis with the following antibodies: MIB1, the mouse monoclonal antihuman Ki67 antibody (Dako Cytomation, Carpinteria, CA, USA) at a concentration of 1:500; PRA, the mouse monoclonal antibody NCL-PGR-312 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) at a concentration of 1:5,000; PRB, the mouse monoclonal antibody NCL-PGR-B (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) at a concentration of 1:100; and ER α , the mouse monoclonal antibody ER Ab-12 (Clone 6FH) (Neomarkers, Kalamazoo, MI, USA) at a concentration of 1:100. After incubation with the primary antibodies, antibody binding was localized with the ABC staining kit from Vector Laboratories (Burlingame, CA, USA) according to the manufacturer's instructions and peroxidase activity was detected using 3,3'-diaminobenzidine substrate solution (DAB; Biocare, Concord, CA, USA). A wash step with phosphate-buffer solution (PBS) for 10 min was carried out between each step of the immunostaining. Slides were counterstained with hematoxylin and mounted in mounting medium for examination. A clear distinction between luminal-epithelial cells and myoepithelial cells in terminal-duct-lobular units (TDLUs) is frequently difficult to make on conventionally stained slides. In these IHC studies, we counted the total numbers of luminal-epithelial + myoepithelial cells (together referred to as epithelial cells) and the percentage of them positive for the relevant marker in the TDLUs.

The markers MIB1, PRA, PRB, and ER α are all nuclear. For each marker, we used the Automated Cellular Imaging System II (ACIS II; Clariant, Aliso Viejo, CA, USA) to assess all TDLUs on a single slide or the first 100 target areas containing TDLUs selected systematically from left to right and top to bottom on the slide if there were a large number of epithelial cells present. The ACIS II is a cellular imaging system that digitizes the images and permits the user to identify and quantitate relevant areas on a high-resolution computer screen based on color differentiation. The pathologist conducting the IHC was blinded to OC type.

2.4. Statistical analysis

We analyzed these data using the statistical package program Stata 11 (Stata Corporation, College Station, TX, USA). Differences in expression and tests for trend in expression were tested for significance by standard t-tests and regression tests after adjustment for age and ethnicity (African American, Hispanic Whites, non-Hispanic Whites) and after transformation of the variables to achieve more normal distributions of values (logarithmic transformation of MIB1 and square root transformations of PRA, PRB, and ER α). All statistical significance levels (p-values) quoted are two-sided. In this pilot study, we planned on obtaining tissue samples from 30 women, 15 in each of the two OC groups. This sample size afforded us 80% power with a 1-sided alpha of 5% to detect a 50% reduction in breast-cell proliferation in the 0.4-mg OC group based on an estimated standard deviation as observed by Anderson et al. [15]. The results in women who had not taken a hormonal

contraceptive for at least 10 weeks before starting the study were checked to see if their results differed from the overall results.

3. Results

Thirty-three women were enrolled in the study with 17 and 16 randomly assigned to Ortho Novum 1/35[®] and Ovcon 35[®], respectively. Fig. 1 shows the flow of participants through the study. All 33 completed the study including contributing a breast-biopsy specimen. Five of the breast-biopsy specimens contained insufficient TDLU epithelial tissue for analysis and one of the remaining women was diagnosed with a follicular cyst on the day of biopsy, leaving 27 evaluable patients – 14 on Ortho Novum 1/35[®] and 13 on Ovcon 35[®]. The means (and 95% confidence intervals) of the proportion of epithelial cells with positive nuclear staining for Ki67, PRA, PRB and ER α for the 27 women are given in Table 1.

The Ki67 mean value increased 60% from 7.8% to 12.5% with the reduction in NET dose from 1.0 mg to 0.4 mg, although this increase in breast-cell proliferation was not statistically significant ($p=0.27$). PRA, PRB and ER α also increased with the reduction in NET dose. PRA and PRB were statistically significantly higher in the lower-dose progestin OC group. The PRA mean value increased 120% from 7.6% in the 1.0-mg NET group to 16.7% in the 0.4-mg NET group ($p = 0.041$). The PRB mean value increased 98% from 12.0% in the 1.0-mg NET group to 23.7% in the 0.4-mg NET group ($p = 0.030$). The ER α mean value increased 102% from 9.0% in the 1.0-mg NET group to 18.2% in the 0.4-mg OC group, although this difference was only of borderline statistical significance ($p = 0.056$).

The results in the 21 women who had not taken a hormonal contraceptive for at least 10 weeks before starting the study were very similar to the overall results shown in Table 1.

4. Discussion

The predicted reduction in TDLU breast epithelial-cell proliferation with the 60% reduction in progestin dose was not observed. Although the observed mean value of Ki67 was higher in the lower progestin dose group, it was not statistically significant. This failure to observe a simple dose-effect of this progestin on breast epithelial-cell proliferation is likely to be at least part of the explanation of why epidemiological studies have in general failed to identify differences in risk of breast cancer by dose of progestin in the OC [4–9].

Studies of breast-cell proliferation in OC users (Supplementary Table 1) [15–27] have contained a wide range of estrogen and progestin doses: no study reported an effect of progestin dose (although these were ‘combined’ doses across various progestins) and only the Anderson et al. [15] study reported lower proliferation in OCs with lower EE2. It is of interest to note that early studies reported by Anderson et al. [15] and Williams et al. [16] found little difference between OC users and normally cycling women in TDLU breast-cell proliferation as measured by thymidine labeling index (TLI). The more recent studies of Olsson et al. [22] and Isaksson et al. [24] using Ki67 as the marker of cell proliferation have each found some evidence that average proliferation on OCs is greater than over the menstrual cycle -- 10.6% vs 9.0%, and 4.8% vs 2.2%, respectively. The Ki67 figure of 7.8% we observed with the much more commonly used 1-mg NET OC should not be taken as an average figure for the OC cycle since there is clear evidence that proliferation is lower in the placebo week and there is some evidence that proliferation may be at its maximum towards the end of the active pill phase when we took our breast-biopsy samples [15].

Concomitantly with this failure to observe a decrease in Ki67 with the reduction in NET dose, the levels of each of PRA, PRB, and ER α approximately doubled. This is the first report of an effect of NET dose on PRA, PRB, and ER α expression levels in the breast.

Whether this increase in the proportion of cells expressing steroid receptor with decreasing progestin dose explains the failure to see a decrease in Ki67 is unclear. We did not measure the pharmacokinetics of EE2 and NET in these women and were thus not able to see whether these values together with the proportion of cells expressing receptor were associated with the Ki67 values. The proportion of cells expressing receptors themselves (within an OC type) were not associated with the Ki67 values. It is not known whether this effect of NET dose on receptor levels holds true for other progestins. Increasing progesterone levels after ovulation in the normal menstrual cycle are associated with markedly lower ER expression in almost all studies (Supplementary Table 2) [16, 28–33], but the changes in PR expression over the menstrual cycle in the same studies are inconsistent (Supplementary Table 3) [16, 28–33].

Studies of ER expression in the breast during an OC cycle have found lower levels in the three weeks on active estrogen-progestin than in the week on placebo, and the levels during OC use are lower than the levels seen during the menstrual cycle (Supplementary Table 2). However, studies of PR expression have found higher levels in the three weeks on active estrogen-progestin than in the week on placebo, and the results from the three studies that have investigated how the PR expression levels during OC use compared to the levels seen during the menstrual cycle have produced inconclusive results (Supplementary Table 3). There have been no reports on the effects of dose of EE2 or on the effects of the dose and type of progestin in the OC on these findings.

The results presented here provide clear evidence that decreasing the dose of the progestin NET in an OC from 1 mg to 0.4 mg increases ER α , PRA and PRB in the breast epithelium. There is indirect evidence suggesting that decreasing the dose of EE2 in an OC will decrease PR, but it may increase ER [1]. It is possible that an OC with the same NET dose but lower EE2 dose may be associated with a decreased proliferation of breast epithelium. We are currently investigating this possibility in a trial similar to the one reported here. Whether it is possible to adjust the doses in OCs to achieve an average breast-cell proliferation rate which is the same as or less than that occurring in a normal menstrual cycle is unknown.

5. Conclusions

Lowering the NET dose by 60% from 1 mg to 0.4 mg in a 35-mcg EE2 OC did not decrease breast-cell proliferation and approximately doubled the proportion of breast-epithelial cells expressing PRA, PRB, and ER α . These latter results demonstrate that a simple dose-effect relationship between progestin dose and breast-cell proliferation is not to be expected. The breast-cell proliferation result is contrary to that predicted from the results of lowering the MPA dose from 10 mg to 2.5 mg in MEPT. This MEPT result strongly suggests that further investigation of the dose-effect relationship between other progestins in OCs is warranted. Investigating the dose-effect relationship with EE2 may also be informative. The long-term aim of such studies is to find an estrogen-progestin combination that will decrease the breast cancer risk in OCs -- it is clear that simply reducing the progestin dose, at least of some progestins, is insufficient to achieve this aim.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Flowchart of participants.

Table 1Ki67, PRA, PRB and ER α percentage associated with two 35-mcg EE2 OCs with different NET doses

Measure	NET dose		p ^a
	1 mg	0.4 mg	
Ki67	7.8 (4.4 – 13.9)	12.5 (7.0 – 22.3)	0.27
PRA	7.6 (5.3 – 10.4)	16.7 (8.6 – 27.3)	0.041
PRB	12.0 (8.8 – 15.8)	23.7 (14.1 – 35.7)	0.030
ER α	9.0 (5.5 – 13.3)	18.2 (10.0 – 28.8)	0.056

Values shown are means with 95% confidence limits

^aTwo-sided significance level for difference between NET doses.