EPIDEMIOLOGY

Breast epithelial cell proliferation is markedly increased with short-term high levels of endogenous estrogen secondary to controlled ovarian hyperstimulation

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Abstract Oocyte donors have high serum estradiol (E2) levels similar to the serum levels seen in the first trimester of pregnancy. We report in this article our studies comparing cell proliferation, Ki67 (MIB1), and estrogen and progesterone receptor levels (ERα, PRA, and PRB) in the breast terminal duct lobular units of oocyte donors, women in early pregnancy, and in normally cycling women. Breast tissue and blood samples were obtained from 10 oocyte donors, and 30 pregnant women at 5–18 weeks of gestation. Breast tissue samples were also obtained from 26 normally cycling women. In the oocyte donors: peak E2 (mean $\sim$15,300 pmol/l) was reached on the day before oocyte (and tissue) donation; peak progesterone (P4; mean 36.3 nmol/l) was reached on the day of donation; Ki67 was positively associated with level of E2, and the mean Ki67 was 7.0% significantly greater than the mean 1.8% of cycling women. In the pregnant women: mean E2 rose from $\sim$2,000 pmol/l at 5 weeks of gestation to $\sim$27,000 pmol/l at 18 weeks; mean P4 did not change from $\sim$40 nmol/l until around gestational week 11 when it increased to $\sim$80 nmol/l; mean Ki67 was 15.4% and did not vary with gestational age or E2. Oocyte donors have greatly increased levels of E2 and of breast-cell proliferation, both comparable in the majority of donors to the levels seen in the first trimester of pregnancy. Whether their short durations of greatly increased E2 levels are associated with any long-term beneficial effects on the breast, as occurring in rodent models, is not known.

Keywords Oocyte donors · Breast epithelium · Estradiol · Pregnancy

Introduction

Estradiol (E2) and progesterone (P4) are critically important in the pathogenesis of breast cancer [1, 2]. Infertility

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treatments involving controlled ovarian hyperstimulation are known to cause a transient large increase in serum E2 comparable to the levels seen in the first trimester of pregnancy, whereas P4 levels are no greater than those that are seen in the luteal phase of the menstrual cycle [3, 4]. The effects of this short-term high endogenous E2 exposure on normal breast tissue are unknown.

As part of studies of the changes in human breast associated with pregnancy, we have studied breast tissue from naturally cycling nulliparous and parous women and from women immediately after a pregnancy termination [5]. We report here our studies of the epithelium of the breast terminal duct lobular unit (TDLU) at the end of the ovarian stimulation phase in 10 oocyte donors (women having ovarian hyperstimulation to donate oocytes for use by other women). We report on proliferation (Ki67), estrogen receptor α (ER α), and progesterone receptors A and B (PRA and PRB) in these oocyte donors, and compare these results to those obtained from 30 women sampled between 5 and 22 weeks of gestation (weeks since last menstrual period, LMP) [5] and to the results obtained from 26 cycling women.

Materials and methods

All study protocols described here were approved by the Institutional Review Boards (IRBs) of the USC Keck School of Medicine and where appropriate of the Department of Defense Congressionally Directed Breast Cancer Research Program. The prospectively collected samples were obtained after the women had signed an informed consent. The samples obtained retrospectively from the cycling women were used after the women had been contacted and given consent for their samples to be used.

Oocyte donors

Women attending the In vitro Fertilization Clinic at the University of Southern California (USC) to donate oocytes for the use of other women were invited to volunteer for this study. Women who expressed a desire to participate underwent a routine clinical breast examination; no abnormalities were found. The research-related procedures included a menstrual and reproductive history questionnaire, a blood sample on the day of oocyte retrieval and a breast biopsy immediately after the oocyte retrieval or on the day before if necessary.

Subjects underwent standard clinical protocols for ovarian stimulation. Daily subcutaneous injections of follicle stimulating hormone for approximately 10–14 days with regular monitoring of serum E2 and ultrasound measurement of ovarian follicles. When the follicles were

determined to be mature, human chorionic gonadotropin (10,000 IU) was administered by subcutaneous injection and oocytes were retrieved under intravenous sedation by trans-vaginal ultrasound guided needle aspiration approximately 36 h later.

Ultrasound guided 14-gauge core-needle breast biopsy tissue was collected from a region of ultrasonographically normal dense breast tissue in the upper outer quadrant of the breast. Samples of these tissues were formalin fixed paraffin embedded (FFPE) in a routine manner at the USC Department of Pathology.

Pregnant women

The recruitment of pregnant women has been described previously [5]. Briefly, the pregnant samples were collected from women who had undergone a pregnancy termination within the preceding 10 min; and the blood and tissue samples were collected and processed in like manner to that described above for oocyte donors.

Normally cycling women

The recruitment of normally cycling women has also been described previously [5]. Briefly, the samples from cycling women were obtained from women undergoing a reduction mammoplasty; some of these samples were collected prospectively and others from FFPE tissue blocks, that had been routinely processed at the USC Department of Pathology. These were much larger tissue samples than are obtained at core-needle biopsy but were processed in like manner. No blood samples were obtained at the time of surgery from the retrospectively identified women and hormone values are not reported on here for the cycling women.

Immunohistochemistry

Immunohistochemical (IHC) analysis of the FFPE samples was performed as follows: Multiple adjacent FFPE sections were cut at 5 μm, deparaffinized and hydrated. All slides were subject 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. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 20 min, followed by blocking of nonspecific sites with SuperBlock blocking buffer (Pierce, Rockford, IL, USA) for 1 h both at room temperature (see [5]).

The sections were incubated with the following antibodies: MIB1, a proliferation marker, the mouse monoclonal antihuman Ki67 antibody (Dako Cytomation, Carpenteria, 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 ERα, the mouse monoclonal antibody ER Ab-12 (Clone 6F11) (Neomarkers, Kalamazoo, MI, USA) at a concentration of 1:100. After incubation with the primary antibodies, antibody binding was localized with the secondary antibody for 45 min and then 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.

We generally assessed all TDLUs on a single slide. A clear distinction between luminal-epithelial cells and myoepithelial cells in TDLUs is frequently difficult to make on conventionally stained slides. For this reason we counted the total numbers of luminal-epithelial + myoepithelial cells (epithelial cells) and the percentage of them positive for the relevant marker using the Automated Cellular Imaging System II (ACIS II, Clarient, Aliso Viejo, CA, USA), which digitizes the images and permits the user to identify and quantitate relevant areas on a high-resolution computer screen based on color differentiation. The ACIS II software program does not function optimally when both nuclear and cytoplasmic staining is present. Due to some background cytoplasmic staining in addition to nuclear positivity found in the ERα slides from the pregnant subjects, we used conventional light microscopy and manual counting for assessing the TDLUs in these cases; we counted 500 epithelial cells except in a few cases with scant epithelial tissue. Only nuclear staining was regarded as positive staining.

Blood specimens

The blood specimens obtained during oocyte stimulation and at breast biopsy were processed in a standard manner and the serum frozen at −20°C. E2 and P4 were quantified by specific radioimmunoassay as described previously [6, 7]. SHBG was measured by a chemiluminescent immunoassay on the Immulite Analyzer (Siemens Medical Solutions Diagnostics, Malvern, PA, USA). The coefficients of variation for E2, P4 and SHBG were 14.7, 7.8, and 3.7%, respectively. No serum results were given in our previous publication on pregnant and naturally cycling women [5]. Non-SHBG-bound E2 was calculated by the method of Södergård et al. [8] using the parameters given by Dunn et al. [9].

Statistical analysis

We analyzed these data using the standard 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 transformation of the variables to achieve more normal distributions of values (square root transformations of ERα, PRA, and PRB; and logarithmic transformation of MIB1) [5]. E2, P4, and SHBG values were logarithmically transformed. Testing for the effects of prior births, age and ethnicity on the results were carried out by inclusion of terms for these in regression analyses. All statistical significance levels (P values) quoted are two sided.

Results

We recruited 13 oocyte donors who provided informed consent. One decided to withdraw from the study prior to undergoing the breast biopsy. The remaining 12 completed the study protocol. The biopsies from two of the women had no epithelium in the specimen—these women were excluded from further study. Of the remaining 10, eight had their biopsy on the day of oocyte donation and two on the day before due to their unavailability on the donation day.

Figure 1 shows the E2 and P4 values of the individual subjects in the 7 days before oocyte retrieval. E2 increased steadily in each subject until the day before oocyte retrieval—on the day of retrieval, E2 had fallen from a mean of ∼15,300 to ∼6,000 pmol/l. P4 also increased steadily in each subject; the mean value increased from 1.1 nmol/l at 7 days before oocyte retrieval to 4.1 nmol/l at 2 days before retrieval, and then, after hCG treatment, to 18.0 nmol/l on the day before oocyte retrieval, and to 36.3 nmol/l on the day of retrieval. For comparison, in naturally cycling women, the follicular phase maximum E2 is ∼1,100 pmol/l, the luteal phase maximum E2 is ∼510 pmol/l, and the luteal phase maximum P4 is ∼40 nmol/l [10].

Figure 2 shows the relationships of serum E2 and P4 to gestational age in the pregnant women [5]. E2 increased steadily with gestational age, from ∼2,000 pmol/l at 5 weeks to ∼27,000 pmol/l at 18 weeks of gestation. P4 did not change from the mid-luteal peak of ∼40 nmol/l until around week 11 of gestation, after which it increased to ∼80 nmol/l.

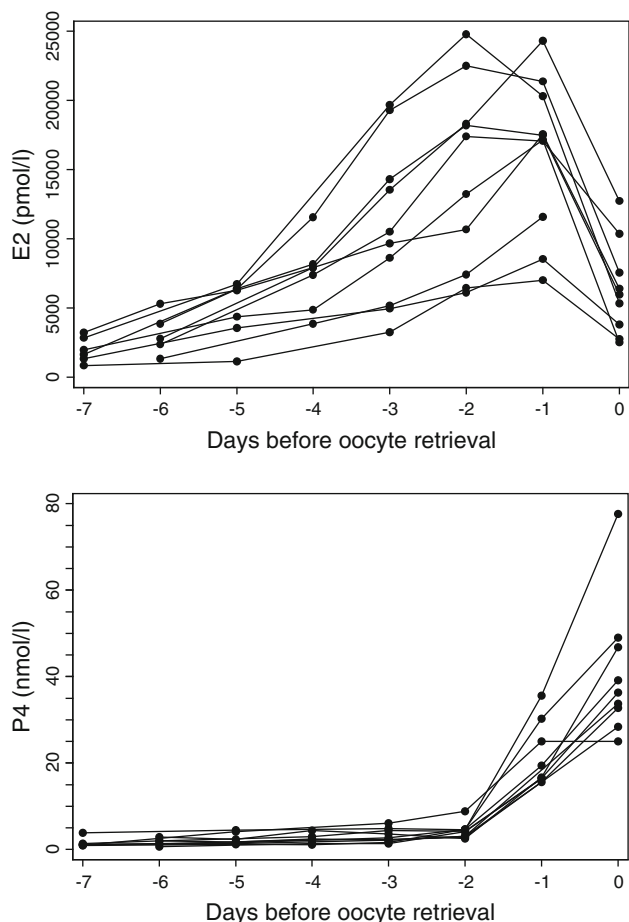Figure 3 shows the relation between Ki67 (MIB1) for the oocyte donors plotted against their E2 on the day before

Fig. 1 Individual oocyte donor estradiol (E2) and progesterone (P4) values in the week before oocyte retrieval

Fig. 2 Individual pregnant woman estradiol (E2) and progesterone (P4) values versus gestational age

biopsy, and for the 30 pregnant women plotted against their E2 on the day of biopsy, while Fig. 4 shows the relation between Ki67 for the 10 oocyte donors and the 30 pregnant women plotted against their P4 on the day of biopsy.

For the oocyte donors, there was a strong positive relationship between Ki67 and E2 on the day before biopsy (see Fig. 3)—correlation, $r = 0.76$ ($P = 0.010$); while the correlation between Ki67 and E2 on the day of biopsy was much weaker—$r = 0.17$ ($P = 0.65$) (data not shown). For the oocyte donors, there was no significant relationship between Ki67 and P4 on the day of biopsy or P4 on the day before biopsy. For the pregnant women, there was no relationship between Ki67 and E2 or P4.

The means (and 95% confidence intervals) of the proportion of epithelial cells with positive nuclear staining for Ki67, ERα, PRA, and PRB are given in Table 1.

The Ki67 mean value was increased from 1.8% in the cycling women to 7.0% in the oocyte donors ($P = 0.003$). The Ki67 mean value in the seven oocyte donors whose serum E2 values on the day before biopsy exceeded 10,000 pmol/l was 14.1%, very close to the mean value of 15.4% seen in the pregnant women.
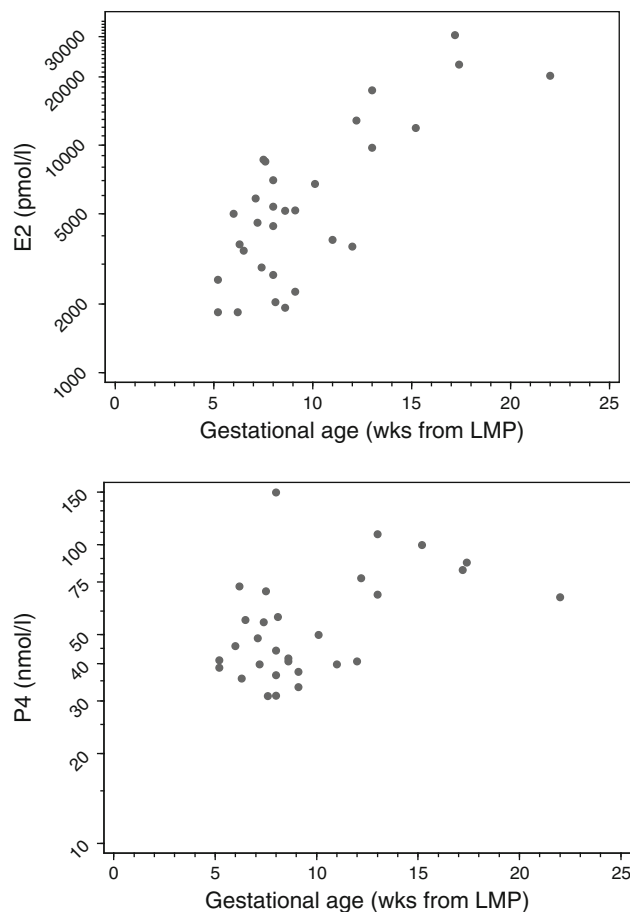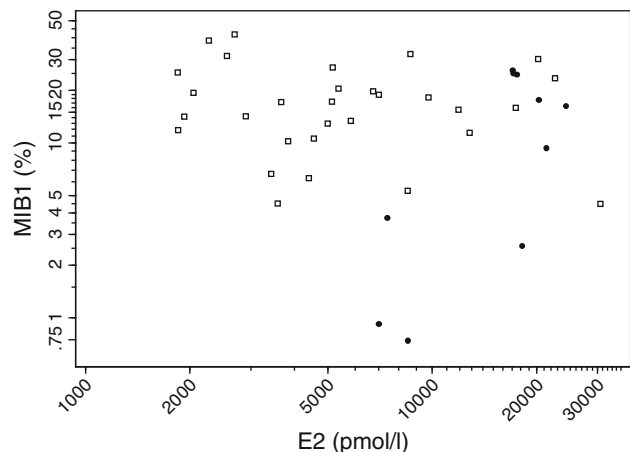

Fig. 3 Individual woman TDLU epithelial cell MIB1 values versus estradiol (E2) values—E2 values for oocyte donors on the day before biopsy (*filled circle*); E2 values for "pregnant" women on the day of biopsy (*open square*)

The ERα mean value was lower in oocyte donors (6.8%) than in cycling women (12.0%). The PRA mean value was slightly lower in the oocyte donors (17.8%) than in the cycling women (23.5%), but was not as low as in the
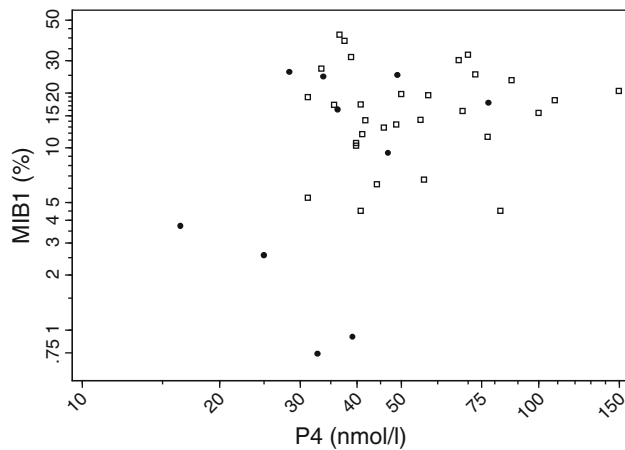
Fig. 4 Individual woman TDLU epithelial cell MIB1 values versus progesterone (P4) values on day of biopsy—oocyte donors (*filled circle*); "pregnant" women (*open square*)

Table 1 Mean (with 95% confidence limits) percentages of Ki67, PRA, PRB, and ERα in oocyte donors, pregnant women, and naturally cycling women

	Group	Mean[a]	lcl[a]	ucl[a]	P value[b]
Ki67	Oocyte donor (N = 10)	7.0	3.0	16.5	
	Pregnant (N = 30)	15.4	12.4	19.1	0.016
	Cycling (N = 26)	1.8	1.2	2.7	0.003
ERα	Oocyte donor	6.8	4.3	9.9	
	Pregnant	11.0	7.9	14.6	0.15
	Cycling	12.0	9.2	15.2	0.034
PRA	Oocyte donor	17.8	13.4	22.9	
	Pregnant	3.9	2.5	5.5	<0.001
	Cycling	23.5	16.2	32.2	0.37
PRB	Oocyte donor	16.9	11.3	23.6	
	Pregnant	12.8	9.4	16.8	0.31
	Cycling	19.2	13.3	26.0	0.67

[a] Calculations were made with transformed values—logarithmic for MIB1, and square root for ERα, PRA, and PRB values. lcl and ucl are lower and upper 95% confidence interval values, respectively

[b] P values are for comparisons with oocyte donors

pregnant women (3.9%). The difference between the oocyte donors and the cycling women was not statistically significant. The PRB mean values were similar in all three groups of women.

The results shown in Table 1 are the values as measured without adjustment for the potential confounders of parity, age, or ethnicity. Adjustment for ethnicity and age had little effect on any of the values shown. We previously reported that parous naturally cycling women had significantly lower PRA values than nulliparous naturally cycling women [5], and lower values of Ki67, ERα, and PRB, but the differences for these factors were not statistically significant. Parity had no effect on Ki67, ERα, and PRA in

pregnant women, but the PRB mean was marginally statistically significantly greater in the parous group (P = 0.049). Eight of the 10 oocyte donors were nulliparous, so that we had no power to investigate the effects of parity in the oocyte donors. Adjustment for parity made little difference to the comparisons shown in Table 1 and no differences to the statistical significance of the comparisons.

Discussion

This is the first study, to our knowledge, to evaluate the immediate effects of short-term exposure to high levels of endogenous estrogen on the breast epithelium of women. In oocyte donors, the level of breast-cell proliferation was positively associated with their serum E2, and a large increase in breast-cell proliferation, similar to the increase seen in pregnant women, occurred in six of the seven oocyte donors whose serum E2 exceeded 10,000 pmol/l. In contrast, the pregnant women demonstrated the same level of breast-cell proliferation over the whole range of observed serum E2 values from ~1,800 pmol/l to ~30,000 pmol/l with no evidence of a dose–response.

When we re-plotted Fig. 3 using non-SHBG-bound E2 rather than E2, a very similar picture was seen. It is unlikely that the contribution of estriol (E3) and estetrol (E4) to the overall estrogenic milieu in pregnant women explains their higher proliferation because these two hormones are at very low levels through gestational week 8 where most of our subjects with E2 concentrations below 10,000 pmol/l lie (Fig. 3 [11–13]). Higher levels of proliferation in pregnant women may be due to their longer exposure to high levels of E2 and to longer exposure to luteal (or higher) levels of P4, it may also be due to their higher ERα expression (see below).

Prolactin is a breast-cell mitogen and prolactin levels increase starting around week 5 of gestation [14]. The proliferation effect of E2 may be enhanced as prolactin has been reported to induce estrogen receptor expression in the breast [15, 16]. Prolactin levels vary greatly during the day with a maximum during sleep and a rapid fall-off on waking. Time of blood draw was not recorded for our study subjects and therefore does not provide useful information on the comparison of prolactin levels in oocyte donors and pregnant women.

We previously reported that PRA decreased steadily with gestational age in pregnant women [5] and although there was already some decrease early on in pregnancy, PRA only reached very low levels (~1%) after week 12 of gestation. There was a non-statistically significant 24% reduction of PRA in oocyte donors compared with naturally cycling women, but this was small relative to the

reduction seen in pregnant women. We also previously reported that "overall there was little difference in ERα expression between non-pregnant and pregnant subjects," but the data strongly suggested that "ERα expression is increased early on in pregnancy (<8 weeks of gestation) and then declines to lower levels than are seen in non-pregnant subjects." In contrast, ERα expression in oocyte donors was low.

Based on a strictly limited amount of epidemiological data but considerable data from rodent experiments (see below), it is possible that a short-term pregnancy and short-term relatively high levels of estrogen may provide some long-term protection against breast cancer. However, the fetoplacental unit in pregnant women is responsible for major endocrinologic changes which are not present in women undergoing ovarian stimulation. Thus, a number of factors in a pregnant woman may contribute to long-term protection against breast cancer. The effects of the high levels of endogenous E2 and P4 achieved during human full-term pregnancy are twofold: a transient increase in breast cancer risk and this is followed by a significant long-term permanent decrease in risk if the pregnancy occurs before around age 32, with the protective effect being greater the earlier the age at which the pregnancy occurs [16–21]. The mechanism for the protective effect remains unclear, but has been attributed in part to hormonal changes, in particular, a reduction in prolactin levels [1], and may possibly be due to hormone-induced changes in breast function leading to lower breast-cell proliferation and possibly other effects. Breast-tissue mRNA expression differences between parous and nulliparous rodents have been observed [22, 23], but whether such changes occur in humans has not been satisfactorily established. There is some evidence that terminated pregnancies may also provide some degree of protection against breast cancer [24]. There are no data available on the effects of a terminated pregnancy on long-term prolactin levels, breast-cell proliferation, or any other possibly relevant factors.

Full-term pregnancy-induced protection against mammary carcinogenesis is consistently observed in rats [25–27]. There is again some evidence that terminated pregnancies may also provide protection [26], but this has not been found consistently [27]. The protective effect in the rat can also be achieved by administration of exogenous E2 and P4 [25, 28–30], and two studies have found that with E2 + P4 substantial protection could be achieved with as little as 7–10 days of administration [29, 30].

There are large differences in the effects of pregnancy in women and in the rat: the ovary is the sole source of serum estrogen and the major source of serum progesterone in pregnancy in the rat, while in women, the main source of estrogen and progesterone moves from the ovary to the placenta during the latter part of the first trimester [31, 32]. The serum E2 levels in pregnant rats only exceed the values seen in cycling rats during the third week of pregnancy, when it approximately doubles [33–37]. In contrast, the levels of serum E2 are greatly increased in pregnant women—they are increased some fivefold in the first trimester, some 20-fold in the second trimester, and some 40-fold in the third trimester [31]. Whether the results in the rat of short-term E2 exposure at only twice the maximum estrus serum E2 level are of any relevance to the human situation is, thus, not at all clear.

The serum P4 levels in cycling rats vary from 45 to 160 nmol/l [34–36]; the levels steadily increase during pregnancy and reach a maximum of 320 nmol/l in the second week, approximately double the maximum seen in the estrus cycle, and then decline in the third week [38, 39]. The levels of serum P4 in cycling women are lower, at 1.5–40 nmol/l, than the levels in the cycling rat [10]. Serum P4 levels in women increase steadily during pregnancy—they are increased some twofold in the first trimester, some fourfold in the second trimester, and some 10-fold in the third trimester compared to the maximum of around 40 nmol/l at the luteal-phase serum P4 peak [31]. The maximum seen during pregnancy in women is thus not greatly increased over the maximum level seen in the rat estrus cycle, and the results in the rat of short-term P4 exposure at the maximum estrus cycle serum P4 level could possibly be of more relevance to the human situation.

If short-term high levels of serum E2 do provide long-term protection against breast cancer, then we might expect that the breast would change in oocyte donors in a way similar to that seen in pregnant women. The short-term high levels of endogenous E2 did cause a dramatic increase in breast-cell proliferation similar to that associated with pregnancy, but the reduction in PRA was much less than that seen in pregnant women. Studies comparing nulliparous oocyte donors at some time after donation to parous women should be informative.

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