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Mammary gland and endometrial effects of testosterone in combination with oral estradiol and progesterone

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Abstract

Objective: The goal of this pilot study was to evaluate the effects of testosterone (T) cotherapy on mammary gland and endometrial measures in a postmenopausal primate model.

Methods: Twenty-five surgically postmenopausal cynomolgus monkeys were randomized by social group to receive daily treatment with (1) placebo, (2) oral micronized 17β-estradiol (1 mg/d equivalent in women) + progesterone (200 mg/d equivalent in women) (E + P), or (3) E + P with T administered via subcutaneous pellets for 8 weeks at a high dose (15 mg) followed by 8 weeks at a low dose (1.5 mg) (E + P + T). The main outcome measures were breast and endometrial epithelial proliferation, as measured by Ki67/MIB1 immunolabeling.

Results: Intralobular breast proliferation did not differ significantly among groups after 8 weeks of treatment but was marginally higher ($P = 0.03$) in the E + P + T group after 16 weeks of treatment. No significant increase in proliferation was seen for E + P alone. Comparable changes in mammary gland markers of estrogen-receptor activity were seen for the E + P and E + P + T groups. In the endometrium, the addition of T did not increase endometrial glandular proliferation or estrogen-receptor activity or result in any distinct histologic changes.

Conclusions: The findings of this study do not support the idea that T antagonizes the effects of combined hormone therapy on breast proliferation or markers of estrogen-receptor activity. Overall, the short-term effects of T cotherapy on the mammary gland and endometrium were minimal.

Key Words: Testosterone – Hormone therapy – Estradiol – Progesterone – Breast – Endometrium – Proliferation.

Androgens have emerged in recent years as an important issue in postmenopausal women's health.[1-3] The postmenopausal period is associated with decreased levels of not only ovarian estrogen and progesterone (P) but also endogenous androgens,[4] and age-related changes in circulating androgens have been associated with several chronic diseases.[5-8] Limited evidence also suggests that exogenous androgen therapy may have potential benefits on various health parameters, including sexual function, bone density, and lean body mass.[1,9,10] Nevertheless, androgens have not been a part of traditional postmenopausal hormone therapy (HT) regimens due, in large part, to concerns over safety and lack of efficacy data for specific menopausal indications.[1,2]

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Androgen effects on breast cancer risk are particularly controversial.[1,3,9,11-13] With some exceptions, in vitro and rodent studies have generally found that androgens attenuate estrogen-induced proliferation in breast cancer cells or induced mammary tumors.[9,13,14] These findings are supported by two small studies in postmenopausal macaques showing moderate antagonism of combined estrogen + progestogen therapy on breast proliferation by testosterone (T).[15,16] Similar results were also reported in a recent trial of postmenopausal women, which found increased breast cell proliferation after combined HT alone but not after the addition of a T patch.[17]

In contrast to these findings, epidemiologic studies of postmenopausal women point to potential adverse effects of androgens on the breast. Several prospective observational studies have shown a positive association between endogenous serum androgens and breast cancer risk,[3,11,12] with approximately two to three times greater risk for postmenopausal women in the upper quartile of serum T concentrations compared with those in the lower quartile.[18-23] A recent study also found higher risk of relapse and lower event-free survival in postmenopausal women with breast cancer with high endogenous T compared with those with low T.[24] Other studies have found no significant relationship between serum androgens and breast cancer risk, particularly after adjusting for serum estrogen and other risk factors.[25-27] In one of the few epidemiologic reports to examine the effects of exogenous androgen therapy on breast cancer risk, a relative risk of 2.48 for breast cancer was found in women using esterified estrogens (EE) + methyltestosterone (mT) compared with never users of HT.[28] This increase in risk with EE + mT was significantly greater compared with that of estrogen–only therapy and marginally greater compared with estrogen + progestogen therapy (but was only significant during the first 5 years of therapy). Such data have contributed to concerns that androgens may actually augment estrogen effects on the breast epithelium.[29]

Recent evidence indicates that certain types of long-term combined HT may increase the risk of breast cancer in postmenopausal women.[30-32] These findings have led to uncertainty regarding the safest types of HT and increased interest in alternative types of HT that may provide a better safety profile. Such alternatives include androgen cotherapies such as T. The goal of the current study was to investigate the effects of adding T to combination estrogen + progestogen therapy on the breast and endometrium in a postmenopausal primate model. We hypothesized that a combination of estradol (E) + progesterone (P) + T would result in less proliferation and markers of estrogen receptor activity compared with E + P alone.

METHODS

Animal subjects

For this study, 25 adult female surgically menopausal cynomolgus monkeys (*Macaca fascicularis*) with a mean (SE) age of 8.8 (0.3) years were used. All animals had been ovariectomized for 2.3 years and housed since that time in stable social groups of three to five animals each. Previous studies in these animals were described elsewhere.[33-36] Macaques are Old World primates with greater than 90% overall genetic coding sequence identity to humans,[37] including important genes related to breast cancer risk.[38] Female macaques have a 28-day menstrual cycle and ovarian hormone profile highly similar to that of women,[39] and previous work from our laboratory and others have demonstrated similarities between macaque and human mammary gland biology, including cytokeratin expression,[40] sex steroid receptor expression,[41] responses to endogenous and exogenous sex steroids,[33,42-44] and the presence of hyperplastic and neoplastic mammary gland lesions.[45,46] All procedures in this study were conducted in compliance with State and Federal laws, standards of the US Department of Health and Human Services, and guidelines established by the Wake Forest University Animal Care and Use Committee. The facilities and laboratory animal program of Wake Forest University are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

Study design and treatments

The animals received no hormone treatment for 6 weeks before the start of the current experiment. Animals were randomized by social group to the following treatments: (1) placebo control (Con; n = 7); (2) oral micronized 17β-estradiol (E; 1 mg/d) (Estrace; Bristol-Myers Squibb, New York, NY) + oral micronized P (200 mg/d) (Prometrium; Solvay Pharmaceuticals, Marietta, GA) (n = 8); or (3) E + P + T, delivered via 90-day release of subcutaneous pellets containing either 15 or 1.5 mg of T (Innovative Research of America, Sarasota, FL) (n = 10). Total treatment time was 16 weeks, divided into 8-week phases for high- and low-dose T. After 8 weeks, the animals were anesthetized with ketamine and buprenorphine; high-dose pellets were removed (from the interscapular subcutis) and low-dose pellets were implanted (into the caudal aspect of the left brachium). Doses of T were designed to represent high-dose supraphysiologic (15 mg) and low-dose physiologic (1.5 mg) concentrations. Daily doses are expressed in human equivalents; absolute daily doses (in milligram per kilogram of body weight) were 0.05 for E, 11.1 for P, and an estimated 0.06 (high dose) and 0.006 (low dose) for T. Throughout the experiment, the animals were fed a standard control diet with casein/lactalbumin as the protein source. The diet contained 17.8% of calories from protein, 34.5% from fat, 47.7% from carbohydrates, and 0.20 mg cholesterol/kcal.

The animals were dosed with E and P each morning between 9:00 and 11:00 A.M. Estradiol was administered within a fruit punch (Crystal Light) vehicle, whereas P was injected into a small marshmallow or piece of banana or tangerine (to minimize parenteral absorption). Control animals received a placebo fruit punch and fruit piece. For dosing, all animals were previously trained to enter a catch cage, drink the fruit punch from a syringe, and then eat the marshmallow or fruit. Individual oral drug doses were calculated based on body weight.

Tissue collection and processing

Mammary gland samples were collected after 8 weeks (high-dose T) and 16 weeks (low-dose T) of treatment. For interim biopsies, the animals were anesthetized with ketamine and buprenorphine, and a small (~0.4 g) sample of mammary gland was removed from a preselected breast quadrant. Biopsies were performed by an experienced veterinary surgeon (C.J.L.), and the animals were monitored and given analgesia during recovery following approved clinical procedures. After 16 weeks of treatment, animals were sedated with ketamine and euthanized using sodium pentobarbital (100 mg/kg IV), as recommended by the Panel on Euthanasia of the American Veterinary Medical Association. After collection, half of each mammary gland sample was fixed at 4°C in 4% paraformaldehyde for 24 hours, transferred to 70% ethanol, trimmed, embedded in paraffin, and sectioned to 5 μm in thickness for hematoxylin and eosin (H&E) and immunohistochemical staining. The other half was snap-frozen in liquid nitrogen for later use in gene expression assays. At necropsy, uteri were also collected, weighed, and sectioned transversely at the point of greatest diameter. Half was fixed for histologic, morphometric, and immunohistochemical evaluations, whereas the endometrium from the other half was trimmed and snap-frozen. Those who conducted the histopathologic assessments were blinded to treatment group.

Immunohistochemistry

Immunostaining procedures were performed on fixed mammary gland and uterine tissues using commercially available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67/MIB1; Dako, Carpinteria, CA; 1:50 dilution), progesterone receptor (PGR) (NCL-PGR; Novocastra, Newcastle-upon-Tyne, UK; 1:100 dilution), estrogen receptor α (ESR1) (NCL-ER-6F11; Novocastra; 1:100 dilution), and androgen receptor (AR) (AR-2F12 and AR-318; Novocastra; 1:10 dilution). Staining methods included antigen retrieval with citrate buffer (pH 6.0), biotinylated rabbit antimouse F_c antibody as a linking reagent, alkaline phosphatase-conjugated streptavidin as the label, and Vector Red as the chromogen (Vector Laboratories, Burlingame, CA). Cell labeling was quantified by a computer-assisted manual counting technique using a digital grid filter to select cells for counting across at least three microscopic fields[47] and our modified procedure of cell selection, described previously.[48] This technique provides more systematic and less biased cell counts and covers a larger area of tissue than counting a greater number of cells sequentially. Each immunostain batch included negative control slides, which used the same protocol as for study slides except that nonimmune serum (from the same species as primary antibody) was used in place of the primary antibody. Numbers of positively stained cells were measured as a percentage of the total number examined (100 cells) for each anatomic compartment (lobular epithelium and extralobular ducts for mammary gland and superficial/deep glands and stroma for endometrium). Those who carried out all measurements were blinded to treatment group.

Quantitative gene expression

Expression levels of mRNA transcripts for genes associated with cellular proliferation (Ki67 antigen [MKI67]), estrogen/progesterone action (ESR1), PGR, and signal transducer and activator of transcription 5A [STAT5A]), and androgen action (AR and kallikrein 3 [KLK3]) were determined using quantitative real-time reverse transcriptase–polymerase chain reaction. Mammary gland and endometrial total RNA was extracted, purified, quantitated, qualitatively evaluated for intactness, and reverse transcribed using techniques described previously.[49] Cynomolgus macaque-specific probe-probe sets for internal control genes (glyceraldehyde-3-phosphate dehydrogenase and β-actin), ESR1, AR, and KLK3 were generated through the Applied Biosystems (ABI) TaqMan Assay-by-Design service (Foster City, CA), whereas commercially available rhesus (STAT5A) or human (PGR and MKI67) TaqMan assays were used for remaining assays (Table 1). All probes were designed to span an exon-exon junction to eliminate genomic DNA amplification. Normal premenopausal breast tissue and hormone receptor–negative tumor tissue were run as positive and/or negative controls on each plate. Reactions were performed on an ABI Prism 7000 system using standard reagents and thermocycling protocol.[49] Relative expression levels were determined using the ΔΔCt method described in ABI User Bulletin 2 (available online at http://www3.appliedbiosystems.com/cms/groups/mcb-support/documents/generaldocuments/cms_040980.pdf). The Ct values for glyceraldehyde-3-phosphate dehydrogenase and β-actin were averaged for use in internal calibration, whereas reference breast tissue RNA was run in parallel as an external calibrator.

Uterine ultrasound and histomorphometry

Uterine area was determined by transabdominal ultrasound using a Sonosite 180 portable ultrasound machine with a 5.0-MHz linear transducer (Sonosite, Bothell, WA). Maximal transverse cross-sectional area was measured on static representative digital images using public domain software (National Institutes of Health ImageJ 1.33j, available online at http://rsb.info.nih.gov/nih-image/). Endometrial thickness and glandular area were quantified by histomorphometry, as described previously.[50] Briefly, H&E-stained slides were digitized using an Infinity 3 digital camera (Lumenera, North Andover, MA), and measurements were taken with Image-Pro Plus software version 5.1 (Media Cybernetics, Silver Spring, MD). For endometrial thickness and glandular area, microscopic fields (3 for thickness, 6 for area) were randomly selected at an objective magnification of $\times 20$, and measurements were taken at the point of greatest perpendicular depth. Area was determined by manual tracing of glandular endometrial units and expressed as a percentage of the total area examined. Those who carried out all measurements were blinded to treatment group.

Vaginal cytology and epithelial thickness

To evaluate treatment effects on vaginal maturation, vaginal keratinocytes were collected with a cotton swab, rolled onto a glass slide, fixed, and stained using a modified Papanicolaou method. Maturation value was calculated as follows: (0.2 × % parabasal cells) + (0.6 × % intermediate cells) + (% superficial cells). Vaginal epithelial thickness was quantified by histomorphometry at the end of the study on H&E-stained slides, using techniques as for endometrial thickness.

TABLE 1. *Primer/probe sets for target genes evaluated by qRT-PCR*

Gene ID	GenBank number	Species	ABI assay ID
ACTB	DQ464112	Mf	(custom)
AR	NM000044	Mf	(custom)
ESR1	DQ469336	Mf	(custom)
GAPDH	DQ464111	Mf	(custom)
KLK3	NM001648.2	Mf	(custom)
MKI67	NM002417.3	Hs	Hs00606991_m1
PGR	NM000926	Hs	Hs00172183_m1
STAT5A	NM003152	Mm	Rh02844604_m1

ABI indicates Applied Biosystems; Hs, *Homo sapiens;* Mf, *Macaca fascicularis* (cynomolgus macaque); Mm, *Macaca mullata* (rhesus macaque); qRT-PCR, quantitative real-time polymerase chain reaction.

Serum hormones

Serum concentrations of E, P, and T were measured from samples collected 2 to 4 hours (acute) and 20 to 28 hours (lag) after oral E + P dosing. Blood was collected by femoral venipuncture after sedation with ketamine, and serum concentrations were quantitated by radioimmunoassay using commercially available kits from Diagnostic Systems Laboratories (Webster, TX) (E, DSL-4800 ultrasensitive; P, Coat-A-Count) and Diagnostic Products Corp (Los Angeles, CA) (total T, Coat-A-Count). For E and P assays, serum was extracted with ethyl ether using standard procedures.

Serum lipids

Blood was collected at baseline and after 8 weeks (high-dose T) and 16 weeks (low-dose T) of treatment for measurement of serum lipid/lipoprotein concentrations. Samples were collected after food had been withheld for approximately 18 hours. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride concentrations were measured using enzymatic methods on the COBAS FARA II analyzer (Roche Diagnostics Inc, Montclair, NJ), with protocols and reagents supplied by Boehringer Mannheim. Serum samples from all time points were run at the same time. Lipid assays were run in a clinical chemistry laboratory at Wake Forest University School of Medicine, which is fully standardized with this method and is in the continuing surveillance phase of the Centers for Disease Control and Prevention (Atlanta, GA) Lipid Standardization Program. HDL concentrations were measured using the heparin-manganese precipitation procedure.[51] Low-density lipoprotein (LDL) cholesterol plus very-low-density lipoprotein (VLDL) cholesterol was calculated as the difference between total plasma cholesterol and HDL.

Statistics

This study was designed as a pilot investigation. Statistical power was calculated based on previous mammary lobular epithelial proliferation data, determined by Ki67 immunolabeling, using conjugated equine estrogens (CEE) + medroxyprogesterone acetate (MPA) as the hormone treatment.[43] For this measure, we estimated the minimum difference between control and HT means to be 14% positive cells, with an overall SD of 12.0%. The sample size in each group providing a greater than 70% chance at a 0.05 significance level to detect a statistically significant increase in proliferation was eight per group. Data were analyzed using analysis of variance (ANOVA) for breast and uterine endpoints, body weight, and serum hormones. A mixed general linear model with baseline covariance was used for serum lipid data. All variables were evaluated for their distribution and equality of variances between diets. Owing to non-normal distribution, immunohistochemistry and serum hormone data were evaluated using a nonparametric Kruskal-Wallis test followed by two-sided Wilcoxon rank sum pairwise analysis. All data obtained from the quantitative real-time reverse transcription–polymerase chain reaction were log transformed to improve distribution and then retransformed to original scale and reported as percent control with 90% CI. Data are otherwise reported as mean ± SE. A two-tailed Fisher's exact test was used to evaluate treatment group differences in lesion prevalence on histology. Missing datapoints included a subset of mammary gland biopsies (8 wk) lacking lobuloalveolar (n = 5; one Con and four E + P) or ductal (n = 9; two Con, six E + P, and one E + P + T) epithelium on sectioning and unmeasurable uterine ultrasound images (n = 5; one Con and one E + P + T at 0 wk, one Con at 8 wk, and two Con at 16 wk). All pairwise *P* values were adjusted for the number

TABLE 2. *Treatment effects on body weight and reproductive tract measures*

	Con	E + P	E + P + T	*P* (ANOVA)
Body weight, kg				
Baseline (0 wk)	4.57 ± 0.35	4.54 ± 0.32	4.46 ± 0.29	0.97
High-dose T (8 wk)	4.69 ± 0.39	4.59 ± 0.37	4.59 ± 0.33	0.98
Low-dose T (16 wk)	4.68 ± 0.37	4.67 ± 0.35	4.54 ± 0.31	0.94
Uterine area, cm^2				
Baseline (0 wk)	0.79 ± 0.12	0.88 ± 0.10	0.72 ± 0.09	0.53
High-dose T (8 wk)	0.63 ± 0.21	1.57 ± 0.18^a	1.38 ± 0.16^b	0.008
Low-dose T (16 wk)	0.68 ± 0.19	1.88 ± 0.15^c	1.72 ± 0.14^c	<0.001
Endometrial thickness, mm				
Low-dose T (16 wk)	0.95 ± 0.16	2.34 ± 0.15^c	2.02 ± 0.14^c	<0.001
Endometrial glandular area, %				
Low-dose T (16 wk)	9.6 ± 1.5	14.8 ± 1.4	12.7 ± 1.2	0.051
Vaginal maturation index				
Baseline (0 wk)	54.3 ± 5.6	51.4 ± 5.3	55.4 ± 4.7	0.85
High-dose T (8 wk)	47.5 ± 5.5	78.5 ± 5.1^c	73.7 ± 4.6^a	<0.001
Low-dose T (16 wk)	48.3 ± 3.2	80.9 ± 3.0^c	83.0 ± 2.7^c	<0.001
Vaginal epithelial thickness, μm				
Low-dose T (16 wk)	190 ± 73	615 ± 68^c	560 ± 61^c	<0.001

Values represent mean ± SE. Letters indicate significant differences with the control group ($^aP < 0.01$, $^bP < 0.05$, $^cP < 0.001$). No significant differences were noted between the E + P and E + P + T groups. n = 7, 8, and 10 for the Con, E + P, and E + P + T groups, respectively, for all measures except uterine area; for this latter measure, n = 6, 8, and 9 at baseline; n = 6, 8, and 10 at 8 weeks; and n = 5, 8, and 10 at 16 weeks for the Con, E + P, and E + P + T groups, respectively. Con, control (placebo); E, oral 17β-estradiol; P, oral micronized progesterone; T, testosterone administered via subcutaneous pellets at high (8 wk) and low (16 wk) doses; ANOVA, analysis of variance.

TABLE 3. *Treatment effects on serum hormone concentrations*

		Con	E + P	E + P + T	*P* (ANOVA)
Estradiol, pg/mL					
2 wk	2-4 h PD	<5	428.1 ± 64.6[a]	303.0 ± 57.8[a]	<0.001
8 wk	20-28 h PD	<5	25.3 ± 4.9[b]	16.9 ± 4.4[b]	0.01
10 wk	2-4 h PD	<5	366.4 ± 78.8[a]	239.0 ± 70.5[a]	<0.001
10 wk	20-28 h PD	<5	23.3 ± 4.9[a]	18.6 ± 4.4[a]	<0.001
16 wk	20-28 h PD	<5	24.9 ± 5.8[a]	36.3 ± 5.2[a]	<0.001
Progesterone, ng/mL					
2 wk	2-4 h PD	<1	13.2 ± 3.6[a]	33.7 ± 3.2[a,c]	<0.001
8 wk	20-28 h PD	<1	2.8 ± 0.2[a]	2.6 ± 0.2[a]	<0.001
10 wk	2-4 h PD	<1	22.4 ± 2.7[a]	24.8 ± 2.4[a]	<0.001
10 wk	20-28 h PD	<1	3.4 ± 0.4[a]	3.9 ± 0.3[a]	<0.001
16 wk	20-28 h PD	<1	6.6 ± 2.0[a]	8.9 ± 1.7[a]	<0.001
Testosterone, ng/dL					
0 wk	NA	12.8 ± 2.7	11.2 ± 2.5	12.8 ± 2.3	0.90
2 wk	2-4 h PD	6.5 ± 56.0	2.5 ± 52.4	669.1 ± 46.8[a,c]	<0.001
8 wk	20-28 h PD	11.4 ± 17.8	8.8 ± 16.6	196.4 ± 14.9[a,c]	<0.001
10 wk	2-4 h PD	10.8 ± 19.6	12.8 ± 18.3	128.3 ± 16.4[a,c]	<0.001
10 wk	20-28 h PD	7.4 ± 14.8	1.7 ± 13.9	98.1 ± 12.4[a,c]	<0.001
16 wk	20-28 h PD	14.5 ± 3.4	11.5 ± 3.2	17.3 ± 2.8	0.47

Serum was collected 2 to 4 hours and/or 20 to 28 hours PD after 2 and 8 weeks of the low-dose T (wk 1-8) and high-dose T (wk 9-16) treatment phases. For conversion to SI units, multiply by the following conversion factors: 3.67 for estradiol (pmol/L), 3.18 for progesterone (nmol/L), and 0.035 for testosterone (nmol/L). Values represent means ± SE. Letters indicate significant differences with the control group ($^aP < 0.01$, $^bP < 0.05$) or with the E + P group ($^cP < 0.01$). n = 7, 8, and 10 for the Con, E + P, and E + P + T groups, respectively, for all measures. Con, control (placebo); E, oral 17β-estradiol; P, oral micronized progesterone; T, testosterone administered via subcutaneous pellets; NA, not applicable; ANOVA, analysis of variance; PD, postdosing.

of pairwise tests (n = 3) using the Bonferroni correction. Data were analyzed using the SAS statistical package (version 8; SAS Institute, Cary, NC). A two-tailed significance level of 0.05 was chosen for all comparisons.

RESULTS

Body weight and reproductive tract measures

No baseline differences in body weight, uterine area, or vaginal maturation index were noted among treatment groups ($P > 0.1$ for all; Table 2). Increases in uterine area, vaginal maturation index, and vaginal epithelial thickness were seen for E + P and E + P + T (both high and low T doses) compared with the control ($P < 0.05$ for all), whereas no significant effects on body weight were observed (Table 2). Endometrial thickness was also greater in the E + P and E + P + T groups after the low-dose T phase ($P < 0.001$), whereas endometrial glandular area was only marginally higher (ANOVA, $P = 0.051$) compared with control. The E + P and E + P + T groups did not differ significantly for any of these measures.

Serum E, P, and T

The oral E dose resulted in acute and lag serum E concentrations of ~200 to 500 pg/mL (734-1,835 pmol/L) and ~15 to 30 pg/mL (55-110 pmol/L), respectively. The oral P dose provided acute and lag serum P concentrations of ~10 to 40 ng/mL (42-127 nmol/L) and ~2 to 10 ng/mL (6-32 nmol/L), respectively (Table 3). These concentration ranges are within the daily range for postmenopausal women taking oral micronized E and P.[52,53] Serum E and P were significantly higher at acute and lag time points for both the E + P and E + P + T groups compared with the control group ($P < 0.05$ for all); the only difference between these groups was acute serum P, which was higher for E + P + T (high-dose T) compared with E + P at 2 weeks but not thereafter. The high-dose T pellet resulted in serum T concentrations of ~500 to 750 ng/dL (17-26 nmol/L) at 2 weeks and ~150 to 250 ng/dL (5-9 nmol/L) at 8 weeks, whereas the low-dose T pellet resulted in serum T concentrations of ~75 to 150 ng/dL (3-5 nmol/L) at 10 weeks and ~10 to 25 ng/dL (0.4-0.9 nmol/L) at 16 weeks.

Mammary gland Ki67, PGR, and ESR1 expression

The proliferation marker Ki67 is widely used as a prognostic indicator in human breast cancer[54] and a risk marker of hormone exposure in preclinical studies.[33,42,43,46] At 8 weeks, the E + P and E + P + T (high-dose T) groups had similar levels of proliferation (lobular and ductal epithelium). Neither treatment group differed significantly from the control group, although a trend was noted for increased proliferation in the E + P group (ANOVA, $P = 0.05$; Fig. 1A). Comparable changes were also seen between the E + P and E + P + T groups in PGR expression (Fig. 1B). At 16 weeks, significantly greater lobular proliferation was present for E + P + T (low-dose T; $P = 0.03$) but not E + P treatment compared with control (Fig. 1A), whereas lobular and ductal PGR expression was higher in both hormone treatment groups ($P < 0.05$ for all; Fig. 1B). No significant group differences were noted for ESR1 expression at either time point (Fig. 1C). Immunolabeling for AR was not quantified because of a lack of staining in mammary gland lobular and ductal epithelium in all treatment groups, in contrast to distinct nuclear staining in positive control human and macaque prostate gland epithelium (Fig. 2).

Mammary gland gene expression

We next measured the mRNA expression of several intramammary markers related to proliferation (MKI67) and

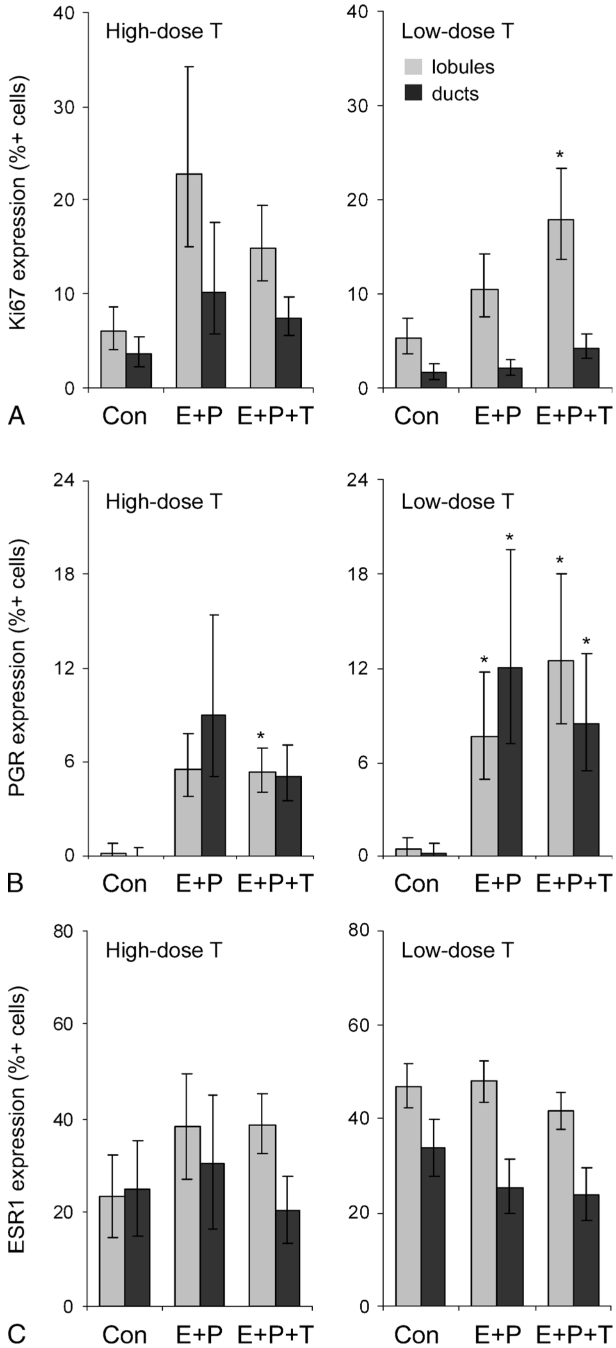

FIG. 1. Mammary gland effects of high-dose and low-dose testosterone cotherapy (T) given with oral estradiol and progesterone (E + P) on expression of markers of proliferation (Ki67) (**A**), estrogen receptor activation (progesterone receptor [PGR]) (**B**), and estrogen receptor α (ESR1) (**C**) as determined by immunolabeling. At 8 weeks, n = 6, 4, and 10 for lobular epithelium and n = 5, 2, and 9 for ductal epithelium for the control (Con), E + P, and E + P + T groups, respectively. At 16 weeks, n = 7, 8, and 10 for the Con, E + P, and E + P + T groups, respectively, for both lobular and ductal epithelium. *$P < 0.05$ compared with the control group (Con).

ESR1, PGR, and AR signaling. In the mammary gland, expression of the estrogen-sensitive PGR was significantly higher with E + P ($P = 0.02$) but not E + P + T treatment after the low-dose T phase (Table 4). Expression of progestogen-sensitive STAT5A was higher in the E + P and E + P + T groups after the low-dose T phase and in the E + P + T (but not E + P) group after the high-dose phase ($P < 0.05$ for all). No statistically significant treatment effects were seen on the mammary expression of ESR1, MKI67, AR, or the androgen-sensitive KLK3 (Table 4).

Mammary gland histology

Mammary gland tissues were evaluated on routine histology for evidence of proliferative changes. Lobules were qualitatively more apparent in the E + P and E + P + T groups (Fig. 3A-C). At 8 weeks, findings included columnar cell change (a benign proliferative lesion[46]) in three biopsies (one Con, one E + P, and one E + P + T) and atypical ductal hyperplasia in one biopsy (E + P + T) (Fig. 3D). At 16 weeks, findings included columnar cell change in two cases (both E + P) and columnar cell hyperplasia in one case (Con).

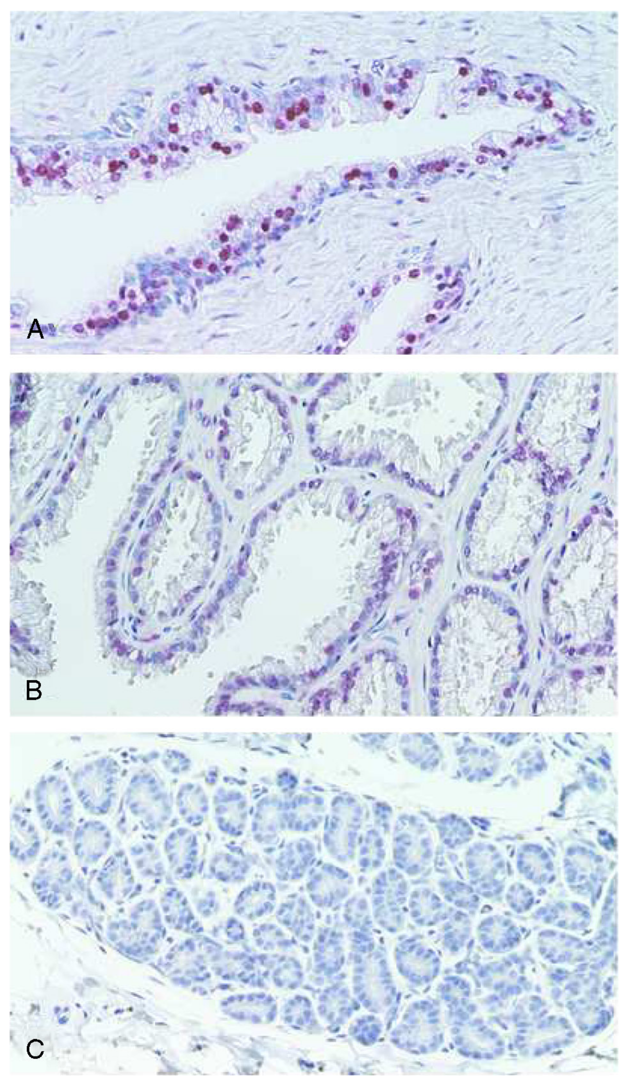

FIG. 2. Lack of androgen receptor (AR) expression within mammary gland epithelium. Representative images of positive control human (**A**) and macaque (**B**) prostate glands showing positive AR immunolabeling (red nuclei), in contrast to mammary gland lobular epithelium (**C**).

TABLE 4. *Treatment effects on mammary gland and endometrial gene expression*

	Con	E + P	E + P + T
Mammary gland: high-dose T (8 wk)			
ESR1	1.0 (0.8-1.2)	0.7 (0.5-0.8)	1.0 (0.7-1.0)
PGR	1.0 (0.8-1.3)	1.2 (1.0-1.5)	1.5 (1.2-1.8)
AR	1.0 (0.8-1.2)	0.8 (0.7-0.9)	0.6 (0.5-0.7)
MKI67	1.0 (0.6-1.4)	1.4 (1.0-1.8)	0.8 (0.4-1.2)
KLK3	1.0 (0.7-1.4)	2.0 (1.5-2.6)	1.7 (1.3-2.2)
STAT5A	1.0 (0.8-1.3)	1.4 (1.2-1.8)	2.2 (1.8-2.7)[a]
Mammary gland: low-dose T (16 wk)			
ESR1	1.0 (0.8-1.3)	1.6 (1.3-2.0)	1.1 (0.9-1.4)
PGR	1.0 (0.7-1.4)	4.2 (3.0-5.8)[a]	2.2 (1.7-3.0)
AR	1.0 (0.9-1.1)	1.5 (1.4-1.7)	1.4 (1.2-1.5)
MKI67	1.0 (0.0-2.2)	3.1 (1.9-4.2)	1.9 (0.9-2.9)
KLK3	1.0 (0.7-1.4)	1.3 (0.9-1.7)	1.7 (1.3-2.2)
STAT5A	1.0 (0.7-1.3)	3.6 (2.7-4.8)[a]	3.2 (2.5-4.1)[a]
Endometrium: low-dose T (16 wk)			
ESR1	1.0 (0.7-1.4)	0.6 (0.5-0.8)	0.4 (0.3-0.5)
PGR	1.0 (0.7-1.4)	1.8 (1.3-2.4)	1.7 (1.3-2.2)
AR	1.0 (0.8-1.3)	0.6 (0.5-0.7)	0.8 (0.6-0.9)
MKI67	1.0 (0.7-1.4)	1.6 (1.2-2.3)	0.4 (0.3-0.5)[b]
KLK3	1.0 (0.6-1.6)	5.4 (3.5-8.2)[a]	1.6 (1.1-2.3)
STAT5A	1.0 (0.8-1.2)	0.6 (0.5-0.7)	0.6 (0.5-0.7)

Values represent relative mean mRNA fold change (90% CI) from control group, as measured by quantitative reverse transcription–polymerase chain reaction. Letters indicate significant differences with the control group ([a]$P < 0.05$) or with the E + P group ([b]$P < 0.05$). n = 7, 8, and 10 for the Con, E + P, and E + P + T groups, respectively, for all measures. Con, control (placebo); E, oral 17β-estradiol; P, oral micronized progesterone; T, testosterone administered via subcutaneous pellets; ESR1, estrogen receptor α; PGR, progesterone receptor; AR, androgen receptor; MKI67, gene for the proliferation marker Ki67 antigen; KLK3, kallikrein 3; STAT5A, signal transducer and activator of transcription 5A**.**

Endometrial Ki67, PGR, and ESR1 expression

Endometrial gland Ki67 immunolabeling (assessed after low-dose T phase only) tended to be lower in the E + P + T group, although this effect was not significant (ANOVA, $P = 0.10$). Both E + P and E + P + T treatments were associated with greater stromal Ki67 (significant for E + P + T at $P = 0.02$ but not for E + P at $P = 0.12$; Fig. 4A). Progesterone receptor expression did not differ significantly among treatments in glands but was higher in stroma (superficial and deep) in both the E + P and E + P + T groups ($P < 0.05$ for both; Fig. 4B). Glandular ESR1 labeling was higher in both E + P and E + P + T groups ($P < 0.01$ for

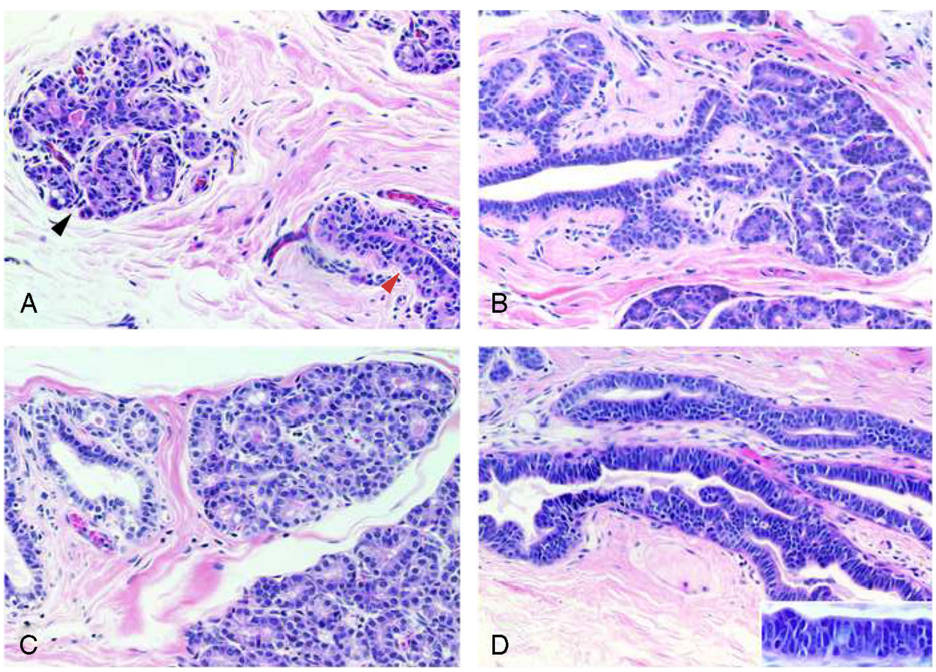

FIG. 3. Treatment effects on mammary gland histology. Representative mammary gland images after treatment with placebo (**A**), oral estradiol and progesterone (**B**), or oral estradiol and progesterone with testosterone cotherapy (**C**). Arrowheads in **A** indicate lobular (black) and extralobular duct (red) compartments. An example of ductal hyperplasia with luminal micropapillary columnar cell change (inset) is shown in **D**. Hematoxylin and eosin stain.

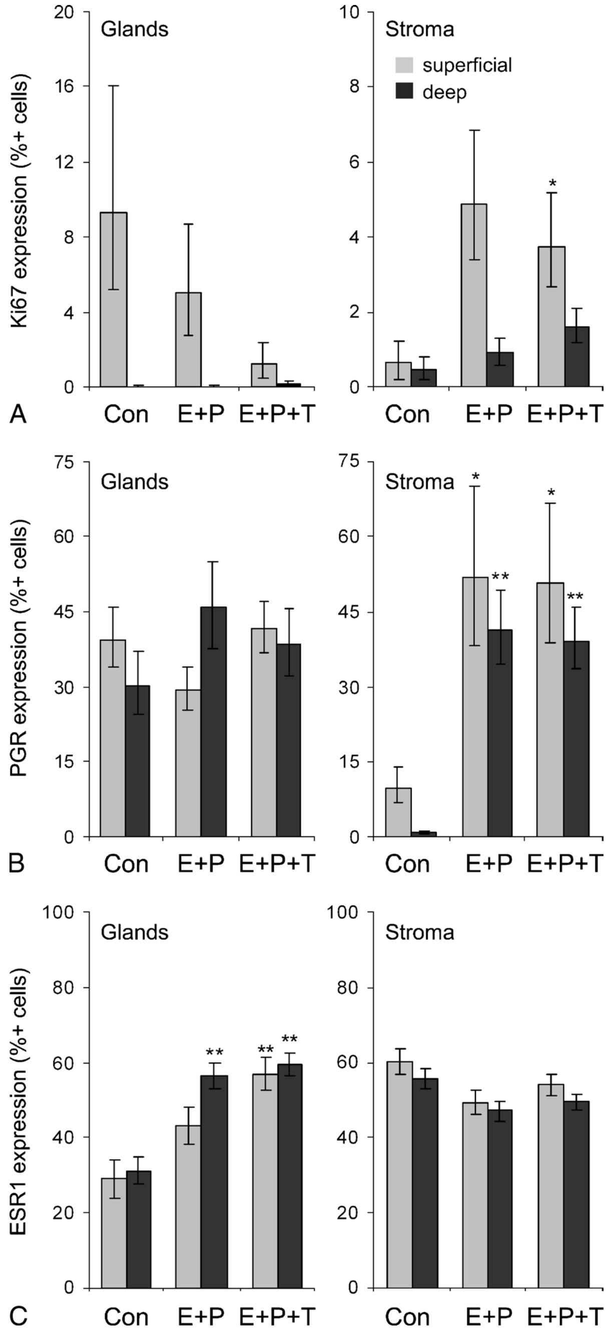

FIG. 4. Endometrial effects of oral estradiol and progesterone given alone (E + P) or with testosterone cotherapy (E + P + T). Data correspond to low-dose T phase only. Figures indicate nuclear expression of Ki67 (**A**), progesterone receptor (PGR) (**B**), and estrogen receptor α (ESR1) (**C**) as determined by immunolabeling. n = 7, 8, and 10 for the control (Con), E + P, and E + P + T groups, respectively, for all measures. *$P < 0.05$ and **$P < 0.01$ compared with the control group (Con).

both), whereas no significant group differences were noted for stromal ESR1 (Fig. 4C). No significant group differences were seen for endometrial PGR, ESR1, AR, and STAT5A gene expression, whereas MKI67 expression was lower in the E + P + T compared with the E + P group ($P = 0.04$), and KLK3 expression was higher in the E + P group ($P = 0.04$) but not the E + P + T group (Table 4).

Endometrial histology

General histologic findings included diffuse glandular and stromal atrophy in all control endometria and varying degrees of stromal edema and expansion, glandular elongation, and spiral arteriolar development in E + P and E + P + T endometria. Lesions included simple glandular hyperplasia in one case (E + P), decidual stromal change in five cases (three E + P and two E + P + T), and superficial hemorrhage in one case (E + P).

Serum lipids

Serum lipid parameters did not differ among groups at baseline (Table 5). Total plasma cholesterol was significantly lower in the E + P group compared with both the control and E + P + T groups ($P < 0.05$ for both) after the high-dose but not the low-dose T phase. A similar pattern was noted for LDL + VLDL. In contrast, serum triglyceride was significantly increased in both the E + P and E + P + T groups ($P < 0.05$ for both) after the high-dose but not the low-dose T phase. Serum HDL concentrations were marginally lower in the E + P and E + P + T groups after the high-dose (ANOVA, $P = 0.13$) and low-dose (ANOVA, $P = 0.05$) T phases, whereas no group differences were observed for the ratio of total cholesterol to HDL (Table 5).

DISCUSSION

The use of T-containing agents as hormone cotherapies has increased in recent years,[28] despite a lack of specific use guidelines and mixed evidence regarding T effects on breast cancer risk. In this pilot study, we found a small increase in intralobular breast proliferation after the addition of low-dose T but no effects on histology or markers of estrogen receptor activity. We found no evidence that T antagonizes the effects of combined HT given in the form of E and P. These findings suggest that the short-term effects of T cotherapy on breast proliferation and hormonally mediated markers are limited, even at higher doses. In addition, we found little evidence that T markedly affects proliferation or estrogen-related responses in the endometrium.

Previous data regarding androgen effects on the breast are mixed. Some concerns with T relate to the potential for its conversion to E within the mammary gland and other tissues, thereby contributing to postmenopausal estrogen exposure.[4] Mammary gland epithelial cells (at least in tumors) may express ARs,[55] and previous studies in monkeys and women have suggested that activation of ARs by T may antagonize estrogen effects.[15-17] The results of the current study do not support these latter findings. Possible reasons for this discrepancy may relate to the types of hormones and relative doses used in the respective studies. The previous monkey studies[15,16] were short pilot studies (3 d; n = 4-5 groups) that used a much higher sustained dose of E (2.5-mg subcutaneous pellets), resulting in a greater effect on proliferation (+284%) than that seen with lower dose E + P in the current study. The recent trial in postmenopausal women also used a higher oral E dose (2.0 mg/d), a different progestogen

TABLE 5. *Treatment effects on serum lipids*

	Con	E + P	E + P + T	*P* (ANOVA)
TC, mg/dL				
Baseline (0 wk)	253.0 ± 20.6	274.3 ± 19.3	223.3 ± 17.3	0.16
High-dose T (8 wk)	227.1 ± 11.2	175.3 ± 10.9[a]	215.1 ± 9.8[b]	0.009
Low-dose T (16 wk)	171.0 ± 11.9	153.7 ± 11.6	153.2 ± 10.4	0.47
LDL + VLDL, mg/dL				
Baseline (0 wk)	150.9 ± 24.0	188.9 ± 22.5	119.8 ± 20.1	0.10
High-dose T (8 wk)	125.0 ± 8.2	92.9 ± 8.1[c]	128.4 ± 7.2[b]	0.01
Low-dose T (16 wk)	170.3 ± 6.8	186.0 ± 6.8	184.8 ± 6.0	0.20
HDL, mg/dL				
Baseline (0 wk)	102.1 ± 8.6	85.4 ± 8.0	103.5 ± 7.2	0.22
High-dose T (8 wk)	100.9 ± 6.7	88.7 ± 6.5	82.5 ± 5.6	0.13
Low-dose T (16 wk)	79.8 ± 5.9	59.6 ± 5.8	63.4 ± 5.0	0.052
Triglycerides, mg/dL				
Baseline (0 wk)	53.1 ± 8.9	39.6 ± 8.3	37.7 ± 7.4	0.39
High-dose T (8 wk)	24.4 ± 11.2	51.9 ± 7.0[c]	62.2 ± 6.3[a]	0.004
Low-dose T (16 wk)	44.0 ± 8.6	37.9 ± 7.9	51.2 ± 7.1	0.46
TC/HDL				
Baseline (0 wk)	2.64 ± 0.47	3.56 ± 0.44	2.27 ± 0.39	0.11
High-dose T (8 wk)	2.31 ± 0.21	2.15 ± 0.21	2.74 ± 0.18	0.12
Low-dose T (16 wk)	2.25 ± 0.35	2.74 ± 0.35	2.93 ± 0.30	0.34

Values represent mean ± SE. For conversion to SI units (mmol/L), divide by 38.67 for total cholesterol, LDL + VLDL, and HDL and by 88.57 for triglycerides. Letters indicate significant differences with the control group ([a]$P < 0.01$, [c]$P < 0.05$) or with the E + P group ([b]$P < 0.05$). n = 7, 8, and 10 for the Con, E + P, and E + P + T groups, respectively, for all measures. Con, control (placebo); E, oral 17β-estradiol; P, oral micronized progesterone; T, testosterone administered via subcutaneous pellets; ANOVA, analysis of variance; TC, total cholesterol; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; HDL, high-density lipoprotein.

(norethisterone acetate at 1.0 mg/d), and a transdermal T patch rather than a subcutaneous implant. A more stimulatory combined HT formulation may thus be required for any potential antagonistic effects of T to be seen. It is also possible that the current study, as a pilot investigation, was underpowered to detect any such effects if modest. As indicated in "Methods," power was calculated using previous data from CEE + MPA rather than E + P. More recent data indicate that standard doses of E + P may have less stimulatory effects on mammary proliferation than CEE + MPA does.[33] This difference, along with missing mammary proliferation datapoints for the high-dose T phase (related to an unexpected high degree of adiposity of the animals), further limited power to detect group differences in proliferation. An additional factor related to T (as well as E and P) effects is route of administration. Testosterone therapy may be given orally (mT and T undeconate), transdermally (T patches, gels, and creams), intramuscularly (T enanthate), and via micronized T implants,[3] and it is currently unknown how mode of delivery may influence effects on breast, uterus, and other target tissues.

Few previous studies have examined T effects on the endometrium.[3] In the current study, E + P and E + P + T treatments resulted in stromal changes characteristic of combined HT formulations (edema, conspicuous arterioles, and decidual change) without an increase in glandular proliferation, indicating that the P dose adequately antagonized the potential stimulatory effects of E on the epithelium. The addition of T did not increase endometrial glandular proliferation or estrogen receptor activity or result in any detectable histologic changes. For some markers, the changes in the E + P + T group were marginally less than those in the E + P group (eg, MKI67 gene expression), suggesting that certain doses of T cotherapy may exert a mild antagonistic effect on E effects. This pattern is consistent with a recent study in postmenopausal women that found no evidence of histologic endometrial changes or increased endometrial proliferation after 3 months of treatment with oral T undecanoate given alone and modest antagonism of estrogen effects when given alongside E valerate.[56] The lack of stimulatory effect of T on endometrial epithelium is also consistent with previous rodent and cell culture studies,[57,58] although it remains unclear whether T given alone may increase estrogen exposure via local aromatization.

An important issue involving T therapy is the lack of clear guidelines for diagnosing androgen insufficiency.[1] This problem is due, in large part, to the lack of reliable methods for detecting and validating low physiologic concentrations of serum T in postmenopausal women. The variation in seum T values in the control and E + P groups in this study reflects this issue and highlights the inherent clinical challenge of individually calibrating T doses from a low to a high physiologic level. We also observed a marked decrease in serum T over time for high and low T doses, which was probably due to local fibrosis around the pellets. It is unclear how this decline in T during the course of each phase may have affected the results. Also of note is the lack of increased serum E concentrations in the E + P + T group compared to the E + P group, suggesting that aromatization of T was minimal (at the systemic level at least). This latter point is consistent with evidence from a number of T studies in women,[3] which found no evidence of increased serum E after T therapy.

CONCLUSIONS

In this pilot study, we found limited overall effects of T cotherapy on breast and endometrial parameters. In the mammary gland, greater lobular epithelial proliferation was seen after low-dose but not high-dose E + P + T. Although this change may indicate a potential mild stimulatory effect of T, the effect was small and not significantly different from that for E + P alone. The lack of effects on PGR expression in the breast suggests that T does not exert robust effects on estrogen receptor activity. Nuclear AR expression was not detected in mammary gland lobular and ductal epithelium. Treatment with E + P resulted in significantly higher uterine area, vaginal maturation, and endometrial thickness, and neither low- nor high-dose T altered these effects. The addition of T did not increase endometrial glandular proliferation or estrogen receptor activity or result in any distinct histologic changes. Addition of high-dose but not low-dose T resulted in modest attenuation of E + P effects on select serum cholesterol measures.

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