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ORIGINAL STUDY

Evaluation of endometrial progesterone receptor expression after 12 weeks of exposure to a low-dose vaginal estradiol insert

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Abstract

Objective: To evaluate endometrial progesterone receptor (PGR) expression in menopausal women who used vaginal 4-μg and 10-μg estradiol (E2) inserts or placebo.

Methods: REJOICE was a randomized, placebo-controlled trial investigating vaginal E2 inserts in women with moderate to severe dyspareunia due to menopause. In this post hoc analysis, 25 eligible women with endometrial biopsies were randomly selected from each treatment group (4-μg and 10-μg E2 vaginal inserts and placebo). Endometrial biopsy sections were immunostained using an anti-PR (A and B) monoclonal antibody. Cell staining was quantified using an artificial intelligence feature-recognition algorithm. Mean PGR expression levels were analyzed between baseline and week 12.

Results: PGR expression results were available for 22 women in the 4-μg E2 group, and 25 women each for the 10-μg E2 and placebo groups. Similar PGR expression levels were observed at baseline (0.301-0.470 pmol/mg) and after 12 weeks of treatment (0.312-0.432 pmol/mg) for all treatment groups, with no significant differences between baseline and week 12.

Conclusions: No meaningful differences in endometrial PGR expression were observed with the vaginal E2 (4- and 10-μg) inserts at week 12 from baseline, supporting the hypothesis that local exposure to E2 from a low-dose, vaginal insert placed near the vaginal introitus will not be sufficient to upregulate endometrial PGR expression. Coupled with the lack of histologic changes and systemic absorption, our data suggest that these softgel vaginal E2 inserts would not be expected to stimulate endometrial hyperplasia leading to a potential endometrial safety issue in postmenopausal women with moderate to severe dyspareunia, a symptom of vulvar and vaginal atrophy. Further study on the endometrial safety of softgel vaginal E2 inserts is under way.

Key Words: 17β-estradiol – Dyspareunia – Endometrial biopsy – Progesterone receptor – Vaginal estrogen.

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Dyspareunia and vaginal dryness, symptoms of vulvar and vaginal atrophy (VVA), occur in postmenopausal women as a result of the thinning, drying, and loss of elasticity of the vaginal epithelium due to reduced estrogen levels,[1] and are components of the genitourinary syndrome of menopause (GSM).[2] VVA, without treatment, can become progressively worse[3] and can negatively impact quality of life.[4] Vaginal estrogens have been shown to be an effective and safe treatment for women with moderate to severe symptoms of menopausal VVA.[5,6]

The randomized, placebo-controlled REJOICE trial (NCT02253173), a 12-week, phase 3 study, found that the softgel, 17β-estradiol (E2) vaginal insert (TX-004HR), significantly improved the maturation index of the vaginal epithelium, dyspareunia, and vaginal dryness in menopausal women with VVA and moderate to severe dyspareunia. Endometrial biopsies after 12 weeks of treatment showed no histological changes to the endometrium,[7] and a pharmacokinetics study demonstrated negligible systemic E2 absorption after treatment.[8] The 4-μg and 10-μg E2 doses were approved by the FDA as Imvexxy (estradiol vaginal insert; TherapeuticsMD, Boca Raton, FL) for the treatment of moderate to severe dyspareunia, a symptom of VVA, due to menopause.[9]

Vaginal versus oral administration of estrogens has been shown to provide lowered systemic exposure when used for the treatment of local VVA symptoms.[10] While studies have shown no significant increase in endometrial or breast cancer and cardiovascular risk with vaginal estrogens,[11,12] the direct effects of vaginal estrogens on the endometrium have not been well studied. Because of this lack of direct, definitive evidence to support endometrial safety with low-dose vaginal estrogen, the Food and Drug Administration (FDA) has required the addition of the boxed warning of systemically administered estrogens, which includes risks of endometrial cancer, for all vaginal estrogen products.[9,13-16]

To assess whether vaginal E2 may have a direct effect on the endometrium, the progesterone receptor (PGR) was used as a biomarker of E2 exposure. Endometrial PGR expression is one of, if not, the most sensitive gene response to estradiol.[17] We hypothesized that there may be insufficient E2 exposure from the 4-μg and 10-μg E2 vaginal inserts placed in the lower part of the vagina to increase endometrial PGR expression. If bioavailable estradiol is insufficient to stimulate PGR expression, we would not expect inserts to cause endometrial proliferation or hyperplasia/cancer. The objective of this analysis was to quantify the expression of PGR in endometrial tissues of women who used the softgel vaginal E2 or placebo insert in the REJOICE study.

METHODS

The REJOICE trial (NCT02253173) was a 12-week, phase 3, prospective, randomized, double-blind, placebo-controlled, multicenter study conducted at 89 sites in United States and Canada. Enrollment occurred from October 2014 to October 2015. The trial was conducted in accordance with Good Clinical Practice guidelines of the FDA. The protocol and its amendments, subject consent form, and recruitment materials were approved by central or local institutional review boards (IRBs).

Inclusion and exclusion criteria have been described elsewhere.[7] Healthy menopausal women (40-75 y; body mass index $\leq$38 kg/m^2) could participate if they had $\leq$5% superficial cells on vaginal cytological smear; vaginal pH $>$ 5.0; a most bothersome symptom (MBS) of moderate to severe vaginal pain associated with sexual activity (dyspareunia); onset of moderate to severe dyspareunia after menopause; and were sexually active (with vaginal penetration) and anticipated having sexual activity during the study. Participants with a uterus had to have a screening endometrial biopsy sample sufficient for analysis. Written informed consent was provided by all participants.

Key exclusion criteria included endometrial hyperplasia or cancer; undiagnosed vaginal bleeding; liver or kidney disorder; history of thromboembolic disorder, coronary or cerebrovascular disease; malignancy; endocrine disease; any clinically important abnormalities on screening physical examination, assessments, mammogram, electrocardiogram or laboratory tests; heavy smoking ($\geq$15 cigarettes/d); users of e-cigarettes or marijuana; or a history of alcohol or drug abuse. The following products could not be used prior to vaginal pH assessment: oral products (within 8 wk) containing estrogens, progestins, androgens, or selective estrogen receptor modulators (SERMs); transdermal or vaginal (rings, creams, gels) hormone products (within 4 wk); intrauterine progestins (within 8 wk); progestin implants/injectables or estrogen pellets/injectables (within 6 mo); and vaginal lubricants or moisturizers (within 7 d). Other products that could not be used were investigational drugs (within 60 d) or an intrauterine device (within 12 wk).

Randomization was performed using a reproducible, computer-generated method by the study statistician. Enrolled participants were randomized 1:1:1:1 to either TX-004HR containing 4 μg, 10 μg, or 25 μg E2, or matching placebo in softgel capsules. Women self-administered one softgel capsule into the vagina at approximately the same time of day, daily for 2 weeks, and then twice weekly for 10 weeks.

The primary efficacy endpoints were changes from baseline to week 12 with the E2 vaginal insert compared with placebo for percentages of vaginal superficial cells and parabasal cells, vaginal pH and severity of the MBS of dyspareunia associated with VVA. The primary safety endpoints included adverse events and endometrial hyperplasia. Endometrial biopsies were collected at baseline and at week 12, or end of treatment.

In this post hoc analysis, at least 20 women from each of the two E2 (4-μg and 10-μg E2 vaginal insert) and placebo groups were randomly selected from all of the US sites. Women had to have a normal baseline endometrial biopsy, an on-therapy biopsy after study day 70, and tissue readings from all pathologists. Blinded, paraffin-embedded, fixed endometrial samples were sent to the Pathology & Histology Core at Baylor College of Medicine (Houston, TX). Each

endometrial cell block was sectioned at 5 μm and immunostained to visualize PGR expression using an anti-PGR (A and B) monoclonal antibody (PgR1294; Agilent, Santa Clara, CA). This is a well-characterized monoclonal antibody used to detect PGR in breast cancer.[18] Cell staining was quantified using a trainable feature-recognition algorithm using Vectra3 imaging and inForm software (Akoya Biosciences, Marlborough, MA). Mean expression levels between baseline and week 12 were analyzed by 2-sided *t* tests. A nonparametric Kruskal-Wallis ANOVA was used to compare the difference between baseline and week 12 across all groups, and values from baseline and week 12 across all groups. All statistical analyses were performed using the SigmaStat 3.5 software (Systat Software, Inc., Point Richmond, CA).

RESULTS

Of the 75 endometrial biopsies analyzed, 72 had sufficient tissue for analysis. PGR expression results were available for 22 women in the 4-μg E2 group, and 25 women each for the 10-μg E2 and placebo groups. Women included in this post hoc analysis had a mean age of 59 years, and body mass index of 26 kg/m^2 (Table 1). All endometrial biopsies at baseline were read by at least one pathologist and found to have normal atrophic endometrium.

At baseline, mean PGR expression levels in the endometrium ranged from 0.301 pmol/mg to 0.470 pmol/mg for all treatment groups (Fig. 1). Similar PGR expression levels for each group were observed after 12 weeks of treatment, with levels ranging from 0.312 pmol/mg to 0.432 pmol/mg (Fig. 1). No significant differences in mean PGR expression levels from baseline to week 12 were observed in any group. No significant differences were observed for the comparison of the difference (week 12 to baseline) between the three treatment groups (Kruskal-Wallis, $P = 0.311$) and comparison of baseline and week 12 values between the three treatment groups (Kruskal-Wallis, $P = 0.071$). Figure 2 shows representative images of PGR staining.

TABLE 1. *Participant demographics and baseline characteristics*

Characteristic	TX-004HR 4-μg E2 ($n = 22$)	TX-004HR 10-μg E2 ($n = 25$)	Placebo ($n = 25$)
Age, y	59.2 ± 5.5	59.4 ± 5.6	57.7 ± 5.3
Age category, *n* (%)			
<65 y	17 (77.3)	21 (84.0)	22 (88.0)
≥65 y	3 (13.6)	4 (16.0)	3 (12.0)
Ethnicity, *n* (%)			
Not Hispanic or Latino	19 (86.4)	21 (84.0)	19 (76.0)
Hispanic or Latino	3 (13.6)	4 (16.0)	6 (24.0)
BMI, kg/m^2	25.6 ± 4.2	26.3 ± 4.9	26.7 ± 5.2
Smoking, *n* (%)			
Former smoker	13 (59.1)	7 (28.0)	10 (40.0)
Never smoked	9 (40.9)	18 (72.0)	13 (52.0)
Current smoker	0	0	2 (8.0)
Alcohol use, *n* (%)			
Current	16 (72.7)	16 (64.0)	16 (64.0)
Never	4 (18.2)	8 (32.0)	8 (32.0)
Former	2 (9.1)	1 (4.0)	1 (4.0)

Data expressed as mean ± SD.
BMI, body mass index; E2, 17β-estradiol.

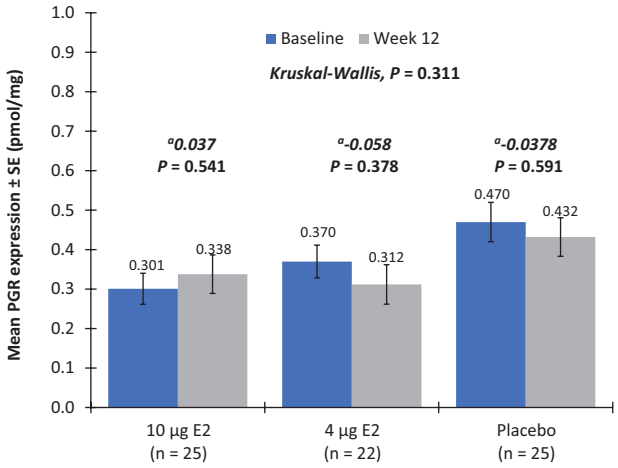

FIG. 1. Mean expression of the progesterone receptor (PGR) in endometrial biopsies at baseline and week 12 from postmenopausal women receiving E2 vaginal inserts or placebo inserts in the REJOICE study. One sample per woman at each timepoint was assessed with the PGR monoclonal antibody (PgR1294; Agilent, Santa Clara, CA) and analyzed with the Vectra3 imaging and inform software (Akoya Biosciences, Marlborough, MA). [a]Mean difference between week 12 and baseline values and 2-sided *t* test *P* values. Kruskal-Wallis *P* value also shown for the difference between baseline and week 12 values across groups. E2, 17β-estradiol; SE, standard error.

DISCUSSION

Our data are an important step toward determining the effects of locally administered, low-dose, vaginal E2 on endometrial PGR expression. We observed no meaningful differences in endometrial PGR expression with the 4-μg and 10-μg E2 softgel vaginal inserts at week 12 from baseline. These findings support the hypothesis that local exposure to a low-dose, vaginal E2 insert, placed near the vaginal introitus (lower/outer part of the vagina), does not significantly increase the expression of PGR in the endometrium. These results are consistent with the lack of effect on endometrial histology reported with the primary safety results,[7] and negligible systemic E2 absorption,[8] with the E2 vaginal insert in women from the REJOICE trial. As previously published, stimulation of the endometrium with estrogen (delivered orally) has been associated with endometrial hyperplasia leading to endometrial cancer.[19,20] Collectively, our data suggest that the amount of E2 reaching the endometrium (no uterine first pass effect) with the vaginal insert is insufficient or below the minimal amount necessary to stimulate the endometrium and would therefore not be expected to lead to endometrial hyperplasia. These data also support the activities of professional women's health organizations, including The North American Menopause Society (NAMS) and the American College of Obstetricians and Gynecologists (ACOG), who have advocated for an update of the product labeling for low-dose vaginal estrogens by removing their boxed warning that pertains to endometrial safety with systemic estrogen products.

The effect of vaginal E2 on the endometrium is difficult to measure and while changes in blood levels of E2 are not

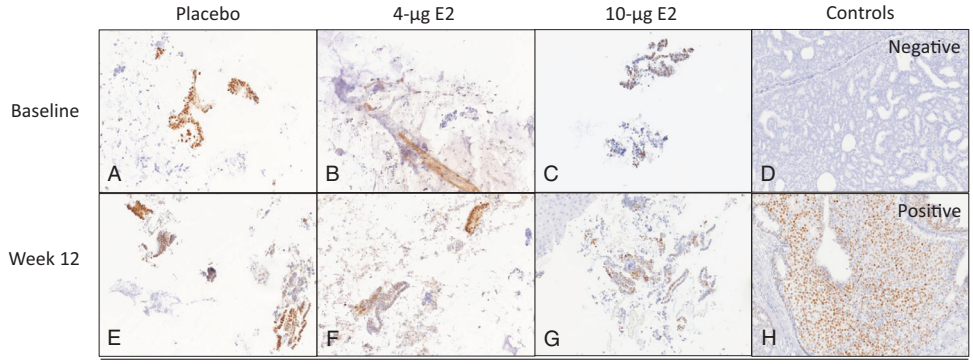

Figure	Total no. of cells	No. of positive cells	PGR expression ratio
A	336	150	0.446
B	355	108	0.304
C	262	99	0.378
D	2629	5	0.002
E	358	189	0.528
F	305	92	0.302
G	476	140	0.294
H	2157	1155	0.535

FIG. 2. Representative images of progesterone receptor staining. Cell staining was quantified using a trainable feature-recognition algorithm (Pathology Core and Lab, Baylor College of Medicine, Houston, TX).

detectable, it does not necessarily mean there are no direct effects on the endometrium. We used PGR expression as a marker of a direct endometrial effect from estradiol since endometrial tissue is very sensitive to estrogen. Progesterone plays a major role in female reproduction by preparing the uterus for implantation and establishing and maintaining pregnancy.[21] Its actions on the endometrium, myometrium, and cervix are mediated via two PGR isoforms (PR-A and PR-B), which are ligand-activated transcription factors.[21] In uterine cells, PGR expression is stimulated by estrogens through the estrogen receptor-α; therefore, progesterone responsiveness is dependent on the presence of estrogens.[17] In fact, very low levels of estrogen are required for progesterone responsiveness as reported in studies of PGR expression in the luteal phase.[22,23] Thus, PGR expression was used as a rapidly responding and sensitive response marker for E2 in this study.

A uterine first pass effect has been described postulating that steroids placed in the upper part of the vagina would be preferentially delivered to the uterus and subsequently passed into the systemic circulation; however, we have discovered that this may not be true for steroids placed in the lower part of the vagina.[24,25] Pharmacodynamic differences due to drug placement within the vagina may be explained by the embryologic origins of the female reproductive system. While the uterus, cervix, and approximately the upper half of the vagina are derived from the Müllerian ducts (paramesonephric ducts), the lower third to half of the vagina is derived from the urogenital sinus.[26] These embryological differences may also explain the distinct lymphatic and circulatory drainage of both parts of the vagina. These regional characteristics for differential lymphatic drainage are highlighted in studies of vaginal cancer showing that tumors arising in the upper two-thirds of the vagina metastasize to the pelvic lymph nodes, whereas tumors in the lower third of the vagina metastasize to the inguinal and femoral nodes.[27] As a result, the vascular and lymphatic circulatory patterns in the vagina follow these embryonic developmental templates, and in theory medications administered into the upper vagina are likely to be distributed preferentially into the pelvic organs.

The described regional characteristics of the vagina may explain the difference in systemic E2 absorption observed between two different E2 inserts, which have different vaginal placement.[28] The 10-μg vaginal E2 tablet (Vagifem, estradiol vaginal inserts, Novo Nordisk, Plainsboro, NJ), which was inserted higher (upper third) in the vagina with an applicator, had significantly higher E2 systemic absorption than the 10-μg TX-004HR insert, which was inserted digitally in the lower (outer) part of the vagina ($AUC_{0\text{-}24}$ [133 vs 50 pg∗h/mL] and C_{max} [20.4 vs 14.4 pg/mL], respectively for Vagifem vs TX-004HR).[28] The pharmacokinetic substudy of the REJOICE trial, using the 4-μg and 10-μg E2 vaginal inserts, did not find significant changes in E2, estrone, or estrone conjugates parameters ($AUC_{0\text{-}24}$, t_{max}, C_{min}, C_{avg}, C_{max}) compared with placebo (except for E2 C_{max}, which was higher for the 10-μg E2 dose than placebo on day 1 but not on day 14).[8] In contrast, other estrogen products placed higher in the vagina may stimulate the endometrium by reaching the systemic circulation or by uterine proximity (uterine first pass).[14,29]

Based on these data, several professional organizations, including NAMS and ACOG, have stated that there is no need for women to use an additional progestogen for endometrial protection and that no endometrial surveillance is necessary with the use of vaginal estrogen unless a woman presents with unexpected vaginal bleeding.[5,30,31] While the FDA requires a

boxed warning for all estrogen therapies, the majority of the data found in the literature do not show an association between low-dose vaginal estrogens and endometrial cancer. No association was found in the Women's Health Initiative Observational Study (1993-2005) and Extension Study (2005-2010), a large cohort trial that evaluated 45,663 women.[11] Similarly, the Nurses' Health Study (1992-2002) found a nonsignificant association of vaginal estrogen use with endometrial cancer (HR = 1.52, 95% CI 0.78-2.98), which was lower after removing women who had previously used systemic estrogen and adjusting for those who had used systemic estrogen plus progestin or progestin alone (HR = 1.24, 95% CI 0.64-2.41).[32] A systematic review of 38 studies (20 randomized controlled trials, 8 interventional studies, and 10 observational studies) also did not support an increased risk of endometrial hyperplasia or cancer with low-dose vaginal estrogens.[12]

Some limitations of our study include the relatively low number of biopsies analyzed, no systemic estradiol measurements at baseline and at 12 weeks, and the fact that this was a post hoc analysis. While endometrial PGR expression is very sensitive to estrogens, the systemic level of estradiol/estrogens needed to have an endometrial response is still unknown. Because of the low number of samples, these results should be cautiously interpreted as the difference in the mean baseline and week 12 values may not have been great enough to reject the possibility that change was due to random sampling variability. However, no increase in PGR expression was observed in the endometrium, such that hyperplasia would not be expected to be an issue given the understanding of the biology of what receptors are activated by estrogens and the fact that vaginal therapy should be given at the lowest dose and for the shortest amount of time. Furthermore, no cases of hyperplasia were observed in the REJOICE trial with the softgel vaginal E2 inserts.[7] Additional studies are ongoing to further elucidate the effects of the vaginal E2 inserts on the endometrium.

When placed near the vaginal introitus (lower third of the vagina), the softgel 4-μg and 10-μg E2 inserts do not seem to have an impact on the endometrium. Our data also lend support to published pharmacokinetic data[8] that show negligible systemic absorption with the E2 vaginal insert, by suggesting that little, if any, E2 from this low-dose vaginal E2 insert is available at the endometrium, based on the absence of change in endometrial PGR expression with 12 weeks of treatment; and thus, likely would not pose a risk for endometrial hyperplasia leading to endometrial safety issues. While our data are not definitive, our results are an important step toward determining whether any local endometrial exposure to E2 from the vaginal insert has an effect on the endometrium.

CONCLUSION

Endometrial expression of the E2-sensitive PGR did not increase with low-dose 4-μg and 10-μg E2 vaginal inserts placed in the lower (outer) part of the vagina, suggesting that negligible E2 reaches the endometrium with vaginal E2 insert use. Coupled with the lack of histologic changes and systemic absorption, our data suggest that these softgel vaginal E2 inserts would not be expected to stimulate endometrial hyperplasia leading to a potential endometrial safety issue in postmenopausal women with moderate to severe dyspareunia, a symptom of VVA.

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