Study protocol and statistical analysis plan

Study title Effect of Oral Estradiol and Progesterone Therapy on Vaginal Cytokines in Postmenopausal Women

NCT number NCT02224313

Date 08/04/2014

Participants

Twenty healthy women (10 premenopausal and 10 postmenopausal women) were asked to participate in this study. Premenopausal women were between the ages of 20-40 years, had regular menstrual cycles (regular menses during prior 3 months and had a luteal phase serum P4 >3ng/mL), and were not using any hormonal contraception. Postmenopausal women were between the ages of 45-65 years, amenorrhea for at least 12 months, had an intact uterus, were not on HT and had no contraindications to HT. All women were screened for genitourinary tract infections including bacterial vaginosis, chlamydia, gonorrhea and trichomoniasis and underwent a screening PAP smear. Women who did not have evidence of inflammation or infection based on vaginal wet preparations and PAP smears were included in the study. All participants provided a written Informed consent. The protocol was approved by the Eastern Virginia Medical School Institutional Review Board and registered on Clinicaltrial.gov NCT02224313 (August 25, 2014).

Study Design

Each woman was followed at three visits during one menstrual cycle in premenopausal women or during 28 days in postmenopausal women. For premenopausal women, visit 1 was during the follicular phase (days 7-10 of menstrual cycle), visit 2 was during ovulatory phase (days 12-16 of menstrual cycle), and visit 3 during luteal phase (days 19-23 of menstrual cycle). For postmenopausal women, visit 1 was at baseline (no hormones taken), visit 2 was after taking oral E2 1.0 mg daily for 14 days, and visit 3 was after taking oral E2 1.0 mg plus oral P4 100 mg daily for another 14 days. (Table 1)

At each visit, peripheral venous blood sample was obtained for estimation of serum E2 and P4 levels. A vaginal speculum examination was performed with a lateral vaginal wall swab for pH and vaginal epithelial cells for maturation index. A cervico-vaginal lavage using 5.0 mL of sterile saline was then carried out. The participants were instructed not to have intercourse for 48 hours prior to each examination and AbaCard p30 test confirmed the absence of semen in the vaginal canal at each visit.

Serum E2 and P4 analysis

Peripheral blood samples were allowed to clot at room temperature for 15 minutes, then centrifuged for 15 minutes at 4000 G. The resultant supernatant was aspirated into individual storage tubes and frozen until assayed for serum E2 and P4 level with a chemoluminescence technique.

Cervico-vaginal cytokine analysis

Cervico-vaginal lavage was performed by repetitive flushing of the lateral, anterior and posterior walls and fornices of the vagina with 5mL sterile saline using a 6-inch dropper. The resulting fluid sample was centrifuged for 10 minutes at 500 g to remove the cellular component

and debris, and the supernatant aspirated and stored in aliquots at -20°C for later testing. Cytokine levels of IL-8 and IL-1β were measured in duplicate using cytokine specific sandwich ELISA kits (Millipore Sigma, Darmstadt, Germany). The individual wells were read at optical density 450 nm per manufacturer instructions and cytokine values in pg/mL were obtained by plotting along a standard curve of pure cytokine. As the IL-1β concentrations fell below the lower limit of the kit detection, the samples were subsequently concentrated 4-fold with a centrifugal filter (Amicon Ultra-4 10K Centrifugal Filter Device from Millipore Sigma) and the ELISA assay for IL-1β was repeated. The coefficients of variation for both IL-8 and IL-1β are <10% for intra-assay and <12% for inter-assay, as determined by the manufacturer. Total protein concentration in the sample was determined with the bicinchoninic acid assay (Millipore Sigma). The levels of cervico-vaginal IL-8 and IL-1β were normalized for the total protein concentration and presented as picogram per microgram of total protein (pg/ug protein).

Statistical analysis

Baseline descriptive statistics were reported as means±standard deviation for continuous variables and were compared between premenopausal and postmenopausal women with a student t-test. Mixed effects model for repeated measures compared serum E2, serum P4, cervico-vaginal IL-8 and IL-1β levels between premenopausal and postmenopausal women for each visit. Changes of these levels across 3 visits were also explored within premenopausal and postmenopausal group. Pairwise comparison of measures between visits was predicted according to the mixed effects model presented as mean difference±standard error. Correlation between serum E2, serum P4 with cervico-vaginal IL-8 and IL-1β levels was evaluated with Pearson's pairwise correlation stratified by premenopausal and postmenopausal group. The

correlation of changes in serum E2, serum P4 with changes in cervico-vaginal IL-8 and IL-1β levels was also evaluated. All statistical tests were 2-sided and a p-value of 0.05 or less was considered statistically significant. The statistical analyses were performed in STATA software version 14.1 (StataCorp I.P. College station, Texas).