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Estrogen increases collagen I and III mRNA expression in the pelvic support tissues of the rhesus macaque

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KEY WORDS
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Objective: Our aim was to study the effect of estradiol and raloxifene on collagen synthesis, by measuring the expression collagen I and III mRNA.

Study design: Nineteen nulliparous young adult rhesus macaques underwent oophorectomy and were treated for 5 months with estradiol alone, raloxifene, or no hormone. Tissue samples were acquired from the lateral vaginal wall, and included the paravaginal attachment and levator ani muscle. Expression of mRNA for collagen I and III was measured by in situ hybridization.

Results: Estradiol increased mRNA for collagen I and III compared with no hormone and raloxifene treatment (ANOVA, $P < .05$). Collagen mRNA was localized to fibroblasts in the vaginal connective tissue and the connective tissue investments of striated muscle. Collagen mRNA was not expressed in epithelial, smooth, and striated muscle cells.

Conclusion: Estrogen, but not raloxifene, increases collagen gene transcription and indicates stimulation of collagen synthesis in pelvic floor connective tissues.

Pelvic organ prolapse (POP) is a prevalent disorder in postmenopausal women.[1,2] Disordered collagen metabolism has been associated with pelvic floor disorders in several reports.[3-6] Surgeons have a strong clinical bias that estrogen improves the mechanical integrity of the pelvic floor tissues, but few data exist to support estrogen as a therapy for POP. Reay Jones described decreased thickness and decreased resilience of the uterosacral ligaments removed at hysterectomy from postmenopausal women not on hormone therapy compared with premenopausal women.[7] In a rhesus macaque model, we have shown that estradiol therapy increases expression of cystatin C, an inhibitor of collagen proteases.[8] Reports on women differ on whether estrogen increases or decreases vaginal collagen content by altering the balance between collagen synthesis and degradation.[9-12]

Raloxifene, a selective estrogen receptor modulator (SERM) often used to treat osteoporosis, has been

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implicated in the development of pelvic organ prolapse. In a small blinded, randomized controlled trial, Vardy reported progression of vaginal prolapse measured by POPQ exam in women taking raloxifene compared with women taking estrogen or placebo.[13] In contrast, Goldstein reported no increase in surgery for POP for women taking raloxifene when compared with placebo after 3 years. Raloxifene has been shown to influence collagen metabolism in bone and breast cancer cell lines, but little is known regarding the effect of raloxifene in the vagina.[14,15]

Nonhuman primates share many features with women that make them excellent experimental models for analyzing hormone action on the pelvic floor.[16] The effects of estradiol (E2) and progesterone on the reproductive tract are nearly identical in women and macaques.[17] Other similarities include a semi-upright posture, similar pelvic floor anatomy, similar parturition,[18] and occurrence of vaginal prolapse in multiparous animals.[16]

We hypothesized that estrogen, but not raloxifene, would induce increases in collagen gene transcription in the vagina, and that such increases underlie new collagen synthesis. To explore this hypothesis, we evaluated the total collagen content and the expression and localization of collagen I and III mRNA in the pelvic support tissues after long-term treatment with estradiol and raloxifene. Collagen types I and III are the most abundant collagen subtypes in tissues that confer tensile strength.

Material and methods

Experimental animals

Animal care and husbandry was provided by the Division of Animal Resources of the Oregon National Primate Research Center (ONPRC) at the Oregon Health and Science University (OHSU) approved by the ONPRC/OHSU Institutional Animal Care and Use Committee. Nineteen nulliparous, young adult females underwent oophorectomy, and immediately were given 5-month treatment regimens consistent with postmenopausal hormone therapy. Eight animals were treated with an estradiol implant, 4 received oral raloxifene (1 mg/kg) daily, and 7 were estrogen deprived for 5 months. Estradiol 17β was administered via 5 cm Silastic capsules (0.34 cm i.d.; 0.46 cm o.d.; Dow Corning, Midland, Mich) filled with crystalline E2 (Sigma Chemical Co., St Louis, Mo). Progesterone was administered via similar (6 cm) Silastic capsules filled with P (Sigma Chemical Co.). The Silastic implants used in these experiments produced serum levels of 80.7 + 43 pg E2 and 4.9 + 1.1 ng P/mL, which are within the normal range for cycling rhesus macaques. After 5 months, the pelvic floor was collected en bloc at necropsy and dissected grossly as described previously.[16] The urinary bladder and urethra were dissected sharply from the anterior vagina, and the colon was separated posteriorly. Full-thickness samples of anterior wall from mid-vagina were frozen in liquid nitrogen for isolation of total RNA. Slices transecting the lateral wall from the vaginal lumen to the levator ani muscle were frozen in Tissue-tek OCT (Miles, Inc, Elkhart, Ind) and cryosectioned for in situ hybridization. In addition, the dense connective tissue layer of the lateral paravaginal attachment was isolated from the other tissue layers for analysis of collagen content. All samples were taken from the mid-vagina because this region may be accurately and rapidly dissected in order to maximize RNA integrity.

Measurement of collagen content

Collagen was measured in samples of the densely ligamentous paravaginal attachment by amino acid analysis. Hydroxyproline is unique to collagen and comprises 14% of collagen types I and III dry weight.[19] Multiplying the hydroxyproline content by 7.14 provides a reliable estimate of collagen content, expressed as a ratio (mg collagen/mg total protein).

Isolation of RNA

RNA was analyzed in a subset of animals, 4 with estradiol treatment, 3 with no hormone, and 4 with raloxifene. Samples were thawed in 10 volumes TRIzol reagent (Invitrogen, Carlsbad, Calif), immediately homogenized with a Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY), and total RNA was isolated following the standard TRIzol protocol. The TRIzol-extracted sample was precipitated with ethanol and then combined with RNeasy lysis buffer (Qiagen, Valencia, Calif) for further purification with the RNeasy Midi Kit. RNA bound to the RNeasy filters was treated with RNAse-free DNAse (Qiagen cat #79254; Qiagen) on the filter following the manufacturer's instructions. Concentrations of the total RNA in the final extract were quantified by UV absorbance on a Beckman 640B Spectrophotometer (Beckman Instruments, Inc, Fullerton, Calif). RNA integrity was determined by fractionation on denaturing agarose gels stained with ethidium bromide.

Production of macaque-specific riboprobes

Two micrograms of total RNA was reverse transcribed with the Omniscript Reverse Transcription Kit (Qiagen), oligo-dt primer (Invitrogen), and RNAsin (Promega Corp, Madison, Wis). The resulting cDNAs were PCR amplified using HotStarTaq Master Kit (Qiagen) and human collagen primers (type I, alpha 1 chain: