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Levormeloxifene inhibits vaginal tropoelastin and transforming growth factor beta 1 production

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Abstract

Purpose: To measure the effects of levormeloxifene on vaginal smooth muscle cell (SMC) proliferation, tropoelastin and transforming growth factor (TGF)-β1 production.

Methods: Primary SMC cultures were performed from vaginal wall biopsies. SMC were incubated with levormeloxifene (0.1 μM, 1 μM), in 96-well plates and cell proliferation was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay at 24 hours. Tropoelastin production was measured by the Fastin Assay kit and TGF-β1 levels were assessed by ELISA.

Results: SMC proliferation was significantly increased by levormeloxifene [relative cell number, mean ± SE, levormeloxifene 0.1 μM 130 ± 13% of control (P=NS), 1 μM 151 ± 19% of control (P<0.05)]. Tropoelastin production was significantly decreased by levormeloxifene [mean ± SE, levormeloxifene 0.1 μM 75 ± 4% of control (P=NS), 1 μM 64 ± 2% of control (P<0.05)]. In addition, TGF-β1 production was significantly decreased [mean ± SE, levormeloxifene 0.1 μM 79 ± 11% of control (P=NS), 1 μM 72 ± 14% of control (P<0.05)].

Conclusions: Levormeloxifene increases vaginal SMC proliferation, inhibits tropoelastin and TGF-β1 production.

Key words: smooth muscle, levormeloxifene, elastin, transforming growth factor

Introduction

Pelvic organ prolapse (POP) and urinary incontinence (UI) are disabling conditions that have a major impact on the quality of life (Nygård et al., 2008; Olsen et al., 1997). In spite of the high prevalence, the pathophysiology of these disorders is incompletely understood. Parity, aging, and a chronic increase in abdominal pressure have been proven to be predisposing factors in the development of pelvic floor disorders (Word et al., 2009). There has also been growing evidence to support the role of genetics and disturbances in the extracellular matrix in the development of POP and UI (Word et al., 2009; Kerkhof et al., 2009; Reisenauer et al., 2008).

The vaginal wall consists of an epithelial cell layer, smooth muscle cells, fibromyoblasts and the intervening extracellular matrix that is largely composed of collagen and elastin. Elastin
provides resilience to the vaginal tissues and in part is responsible for the vagina’s ability to regain its normal structure after delivery (Word et al., 2009). Recent experiments on different strains of knock out mice have demonstrated the importance of elastin fibers for maintaining structural and functional integrity of the pelvic floor (Drewes et al., 2007; Liu et al., 2006; Rhan et al., 2009). Human studies have revealed a marked decrease and fragmentation of elastin fibers in patients with POP (Kerkhof et al., 2009; Reisenauer et al., 2008). Abnormal elastin homeostasis was also noted in women with stress UI (Soderberg et al., 2009; Goepel and Thomssen, 2006; Wen et al., 2007; Chen et al., 2007). These findings support the role of connective tissue disturbances, especially elastins’, in the evolution of POP and UI.

Levormeloxifene is a selective estrogen receptor modulator that was developed for the prevention and treatment of postmenopausal osteoporosis (Skrumsager et al., 2002; Alexandersen et al., 2001). A phase III multicenter randomized double blind placebo controlled trial revealed a significant increase in the rates of POP (7% vs. 2%) and UI (17% vs. 4%) in women treated with levormeloxifene versus the control arm (Goldstein and Nanavati, 2002). In addition to this finding in humans, post partum ovariectomized female rats treated with levormeloxifene had a higher incidence of urethral relaxation, with a striking decrease in intercellular matrix staining, compared to controls (Breyer et al., 2010).

Understanding the mechanism by which levormeloxifene causes these side effects may provide an insight into the pathogenesis of POP and UI. We hypothesize that levormeloxifene inhibits elastin secretion by inhibiting TGF-β1 production which in turn may contribute to the development of prolapse and incontinence. The aim of this study was to measure the effects of levormeloxifene on vaginal SMC proliferation and tropoelastin and transforming growth factor (TGF)-β1 production.

**Materials and Methods**

Tissue samples of the anterior vaginal wall were obtained from four women without POP undergoing abdominal hysterectomy for benign gynecologic reasons at the University of Miami, Miller School of Medicine, Jackson Memorial Hospital, Miami, FL between 12/1/2006 and 12/31/2008. All patients underwent an assessment of POP stages on the basis of the Pelvic Organ Prolapse Quantification System (Bump et al., 1996). Women with endometriosis, immunological and connective tissue diseases, recent use of vaginal hormones, and women with prior pessary use were excluded. Institutional Review Board approval was obtained prior to the start of the study and all patients signed an informed consent form prior to surgery. The site of tissue collection was standardized due to the fact that the vaginal wall composition may vary throughout. After removal of the uterus, full-thickness samples of the anterior vaginal wall were obtained from the vaginal cuff at the anterior midline portion of the vaginal wall, with Metzenbaum scissors. Care was taken to avoid crush injury to the site of the vaginal wall biopsy. Demographic and pertinent clinical information was recorded prospectively and stored in a dedicated database.

**Isolation and characterization of vaginal primary smooth muscle cells**

After removal of the uterus, full-thickness samples of the anterior vaginal wall were obtained
from the vaginal cuff at the anterior midline portion under sterile condition from four women without POP. Specimens were preserved in cold DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1 mM glutamine, 0.075% Na₂HCO₃ and 100 μg/mL penicillin-streptomycin. After a maximum time of 4 hours from tissue harvesting the tissue was mechanically minced in cold cultured media and the fragments were washed 3 times in PBS and were plated onto fibronectin coated plastic dishes (Thermo Fischer Scientific, Rochester, NY). After the initial outgrowth, clones with a morphology resembling the smooth muscle phenotype were patch cloned and propagated in culture. For cell characterization a 5000 cell/well fibronectin coated 8-well chamber slide was plated. Once cell confluence reached 80% cells were washed twice with PBS and fixed in 4% PFA for 30 min at 37°C. To verify the intracytoplasmic distribution of F-actin fibers 4% PFA fixed cells were exposed to a permeabilizing solution of Tryton X100 0.1% and Rhodamine Phalloidin for 45 min at 37°C. Slides were then treated with mounting media (Vectashield, Vector Laboratories, Burlingame, CA) containing DAPI for nucleic acid staining and image acquisition was performed in glycerol immersion by confocal microscopy. In addition, the 8-well chamber slides were used to carry out the immunocytochemical staining. After blockage of endogenous peroxidase activity with a solution of hydrogen peroxide and methanol, slides were sequentially treated with the primary mouse antibody, biotinylated anti-mouse immunoglobulin, and Streptavidin-biotin-peroxidase complex (LSAB™+/HRP kit, Dako, Carpinteria, CA). Diaminobenzidine was used as a chromogen in the presence of hydrogen peroxide. Slides were then counterstained with hematoxylin. All reactions were carried out at room temperature (22°C). To identify the SMC, anti-smooth muscle actin antibodies were used (monoclonal mouse, 1:250, 30 min incubation, clone 1A4, catalog # 0851 Dako, Carpinteria, CA). Caldesmon expression was studied with a monoclonal mouse antibody, 1:100, 30 min incubation, (clone h-CD catalog # M3557 Dako, Carpinteria, CA). An antigen retrieval step was used for caldesmon using citrate buffer and a steamer for 30 min. As a negative control normal mouse serum was substituted for the antibody.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay

To perform the MTT proliferation assay, SMC from the fourth to sixth passages (5,000 cells per well) were cultured in fibronectin (50 μg/mL) coated 96-well culture plates (Costar, Cambridge, MA), in a total volume of 200 μL DMEM/F-12 with 10% FBS. Cells were incubated with levormeloxifene (0.1 μM or 1 μM) (Novo Nordisk, Bagsvaerd, Denmark) in 96-well plates and cell proliferation was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay at 24 hours utilizing a commercially available MTT assay kit (American Type Culture Collection, Catalog #30-1010K, Manassas, VA). The two doses of levormeloxifene represented the highest and lowest serum levels in women treated with this drug to prevent osteoporosis (Novo-Nordisk, Novo Nordisk Investigator’s Brochure, 1998). Treatment and control groups were performed in 6 replicate wells. The relative number of viable cell was determined at 24 hours by incubating the cells with 1 mg/mL of MTT for 4 hours. The live cells utilized MTT resulting in the accumulation of formazan crystals which were then solubilized with acid isopropanol (90% isopropyl alcohol, 2.5% SDS, 0.004 N HCl) for 1 hour. The optical density of the solution was measured at 570 nm.
**Fastin assay for elastin**

SMC from the fourth to sixth passages (5,000 cells per well) were cultured in 96-well culture plates (Costar, Cambridge, MA), in a total volume of 200 μL DMEM/F-12 with 10% FBS and were grown to near confluence. Cells were incubated with levormeloxifene (0.1 μM or 1 μM) in 96-well plates with serum free DMEM/F-12. Treatment and control groups were performed in 6 replicate wells. Supernatants were collected 24 hours after the initiation of treatment. Supernatants were treated with the Fastin Elastin Assay kit (Biocolor Ltd, UK) as recommended by the manufacturer. The Fastin Elastin Assay is a quantitative dye-binding method for the analysis of elastins extracted from biological materials. The dye label employed is 5,10,15,20-tetraphenyl-21,23-porphine tetra-sulfocated (TPPS). The dye reagent binds to the ‘basic’ and ‘non-polar’ amino acid sequences found in mammalian elastins. Recovered dye-bound elastin from each sample and standard was read in a 96-well plate at 513 nm. All measurements were performed in quadruplicate. The measured elastin protein amounts were normalized to corresponding cell numbers to provide a reliable basis of comparison between samples.

**TGF-β1 levels were assessed by ELISA**

ELISA was performed following the manufacturer’s recommendations (R & D Systems, MN, USA, Catalogue # 240). Initially, 96-well plates were coated with freshly prepared capture antibodies (100 μL/well). After washing, the plates were blocked by adding 300 μL of block buffer (5% Tween-20 in PBS). Samples were activated by adding 0.1 mL of 1N HCl to 0.5 mL samples and were neutralized by adding 0.1 mL of 1.2 N NaOH/0.5 M HEPES, then 100 μL of the detection antibody was added and the plates were incubate for two hours. Following these steps 100 μL of a working dilution of Streptavidin-HRP was added followed by 100 μL of substrate solution. Optical density of each well was determined immediately, using a microplate reader set to 450 nm.

**Statistical methods**

Continuous data were compared using the Student’s t test if the distribution of samples was normal or the Mann-Whitney U test if the sample distribution was asymmetrical. Differences were considered significant when P-value was less than 0.05. All statistical calculations were performed using the SigmaStat software (SPSS Inc, Chicago, IL).

**Results**

The demographic data of the patients are described in Table 1. We have obtained samples from four women with no or minimal pelvic organ prolapse. Two of these women were premenopausal and samples were obtained during the follicular phase of the menstrual cycle while the other two women were in menopause.

Characterization of the cultured cells confirmed the smooth muscle phenotype as previously published (Takacs et al., 2010). The internal F-actin architecture of the cells was consistent with SMC and our cultured cells were staining positively, both with an antibody to smooth muscle actin and h-caldesmon (Takacs et al., 2010).

Under *in vitro* conditions SMC proliferation was significantly increased by levormeloxifene 1
**Table 1.** Clinical characteristics of women undergoing vaginal biopsy and primary smooth muscle culture

<table>
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<th>Women with SMC culture (n=4)</th>
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| Age (mean ± SD)            | 54 ± 4  
| Parity (median, range)     | 2 (1–5)  
| Body Mass Index (mean ± SD) | 28 ± 2  
| Smoking (n, %)              | 0  
| Menopause (n, %)            | 2 (50)  
| Hormone replacement (n, %)  | 0  
| POP stage (median, range)   | 0 (0–1)  

Fig. 1. Primary vaginal smooth muscle cells proliferative response to levormeloxifene. *, *P=0.01.

Fig. 2. Levormeloxifene inhibits primary vaginal smooth muscle cells tropoelastin production. *, *P=0.05.

µM [relative cell number, mean ± SE, levormeloxifene 0.1 µM 130 ± 13% of control (P=NS), 1 µM 151 ± 19% of control (P<0.05)] (Fig. 1) and tropoelastin production was significantly decreased [mean ± SE, levormeloxifene 0.1 µM 75 ± 4% of control (P=NS), 1 µM 64 ± 2% of control (P<0.05), Fig. 1] (Fig. 2). In addition, TGF-β1 production was significantly decreased
Comment

Our study revealed a marked decrease in elastin production and an increase in SMC proliferation by levormeloxifene at a concentration of 1 μM. Furthermore, we also demonstrated a significant decrease in TGF-β1 production by levormeloxifene as compared to controls.

In a randomized clinical trial, levormeloxifene was found to increase the risk of POP 3-fold and urinary incontinence 5-fold within 10 months of treatment (Goldstein and Nanavati, 2002). It is interesting to note that an estrogen receptor modulator would have such an effect on the pelvic floor. Though the pathophysiology of this effect is unclear, there has been some scrutiny into the possible mechanism. Increased uterine size, due to tissue edema, and the drugs direct effect on extracellular matrix are some of the prevailing theories but these have not been confirmed (Goldstein and Nanavati, 2002). Ewies et al. (2008) investigated the phenotypic effect of levormeloxifene on cultured fibroblasts and found significant alterations in the actin morphology of static fibroblasts. Levormeloxifene disturbed the fibroblasts ability to maintain the cytoskeleton architecture and the authors speculated that through this mechanism levormeloxifene may disrupt ligamentous integrity and result in POP (Ewies et al., 2008). Animal experiments seem to confirm the ability of levormeloxifene to alter the connective tissues integrity. Post partum ovariectomized female rats that were treated with levormeloxifene had considerably more urethral relaxation, with a striking decrease in intercellular matrix staining, compared to controls (Breyer et al., 2010). Changes in the urethral cellular matrix composition are thought to play a role in the development of UI in rats. Our finding that levormeloxifene inhibits elastin production could be another potential mechanism for levormeloxifene to cause pelvic floor dysfunction.

The role of elastin in the pathogenesis of pelvic floor disorders has been extensively studied. Marked fragmentation and decreases in elastin fibers in patients with POP has been previously documented (el-Kholi and Mina, 1975). Ewies et al. (2003) and Karam et al. (2007) demonstrated
decreased elastin content in the cardinal ligaments and anterior vaginal wall respectively in patients with POP. Fibulin-5, which belongs to the family of fibulin proteins, is believed to play a role in the formation and stabilization of elastin fibers. Previously we demonstrated decreased expression of fibulin-5 in the vaginal wall of women with POP (Takacs et al., 2009). These observations are further supported by mouse models of POP. Severe POP and other elastinopathies were reported in fibulin-5 (Fbln5−/−) knockout mice with evidence of disrupted elastin fibers (Drewes et al., 2007). Lysyl oxidase-like1 (LOX1) is a protein involved in the elastin fiber synthesis and assembly and LOXL1 knockout mice exhibit significant elastin fiber defects and POP in the postpartum period (Lee et al., 2008). POP has also been associated with cutis laxa, a genetic disorder with unclear etiology but associated with markedly decreased elastin (Paladini et al., 2007). These findings clearly indicate the role of elastin in the maintenance of normal pelvic support and its absence or damage in POP. Abnormal elastin homeostasis has also been noted in women with stress UI (Soderberg et al., 2009; Goepel and Thomssen, 2006; Wen et al., 2007; Chen et al., 2007).

Based on previous studies it is recognized that TGF-β1 is a strong stimulator of elastin (Choi et al., 2009; Kahari et al., 1992). TGF-β1 has been widely studied in vascular smooth muscle and skin and has been shown to upregulate elastin in dermal fibroblasts and healthy cultured SMC (Kothapalli et al., 2009; Davidson et al., 1993). TGF-β1 production was significantly decreased by levormeloxifene as compared to controls in our study. We believe that TGF-β1 may play a critical role in elastin homeostasis and down regulation of elastin in POP/UI.

The limitations of our study include the small sample size and the in vitro nature of the experiments. We have tested only two doses of levormeloxifene (the highest and lowest serum levels in women treated with this drug to prevent osteoporosis; Novo-Nordisk, Novo Nordisk Investigator’ Brochure, 1998), because we very extremely limited by the amount of levormeloxifene available for our experiments. This compound is not commercially available any longer and we have received only a very small quantity from company used to produce levormeloxifene. Additionally, we did not compare the effects of levormeloxifene on SMC from women with and without prolapse/incontinence. Levormeloxifene may have an effect on other components of the pelvic architecture not assessed in this study. In addition, we studied only tropoelastin production (an indirect measure of mature insoluble elastin) and did not evaluate the degradation process through the different matrix metalloproteinase expressions. Finally, the methodology for elastin measurement used did not assess the insoluble form or membrane bound forms of elastin. In conclusion, levormeloxifene significantly decreases tropoelastin and TGF β1 production and increases SMC proliferation of vaginal smooth muscle cells.

References


The effect of levormeloxifene on smooth muscle cells


