The effects of estrogen, progesterone and polypropylene mesh on vaginal smooth muscle cell proliferation

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Abstract

Objective: To measure the effects of estrogen, progesterone and polypropylene mesh on vaginal smooth muscle cell proliferation. Methods: Primary smooth muscle cell (SMC) cultures were performed from vaginal biopsies which were then incubated with estradiol (0.1 µM, 1 µM, 10 µM), progesterone (5 nM, 50 nM, 0.5 µM), or polypropylene mesh to assess cell proliferation. Results: In vitro vaginal SMC proliferation was significantly increased by estradiol but not by progesterone or by polypropylene mesh (relative cell number, mean ± SD, control vs. estrogen 0.24 ± 0.02 vs. 0.28 ± 0.02, P=0.01; control vs. progesterone 0.24 ± 0.02 vs. 0.23 ± 0.02, P=NS and control vs. mesh, 0.24 ± 0.02 vs. 0.24 ± 0.01, P=NS). In addition, estradiol increased cell proliferation in a dose responsive fashion (estradiol dose: 0.1 µM, 1 µM, 10 µM) compared to control (P=0.01). Conclusion: Vaginal SMC proliferation is significantly increased by estrogen.

Key words: cystocele, smooth muscle, cell proliferation, mesh

Introduction

Cellular elements (smooth muscle cells, fibroblasts and epithelial cells) of the pelvic floor connective tissue play a key role in the organization of the extracellular matrix which, with its components like collagen and elastin fibers, helps to establish resilience to stretching and expansive forces (Kerkhof et al., 2009). For proper maintenance of tissue homeostasis cells undergo division in cycles and may enter a resting phase or end stage leading to cell death or apoptosis. Previously, we have published a study demonstrating an increase in the rate of apoptosis both in the USL and vaginal wall biopsies of patients with uterine or anterior vaginal wall prolapse (Takacs et al., 2008; Takacs et al., 2009). It was concluded that an imbalance towards apoptosis, without compensatory cell proliferation, may lead to a reduced number of cellular elements and weakened extracellular matrix in the pelvic floor connective tissues resulting in pelvic organ prolapse (POP) (Takacs et al., 2008; Takacs et al., 2009). Additionally,
it appears that estrogen and progesterone may have an effect on the rate of cell proliferation (Liu et al., 2006, Ewies et al., 2008). Blakemann et al. (2001) have demonstrated that estrogen increases the rate of squamous epithelial proliferation in the lower urinary tract but did not appear to affect cell proliferation in the deeper subepithelial tissues. These findings suggest a mechanism by which estrogen exerts its effect on the lower urinary tract and may provide an explanation for the success of estrogen in the treatment of some conditions causing lower urinary tract dysfunction (Blakeman et al., 2001).

Utilization of grafts in the surgical management of POP has become popular even though the associated risks are still significant (Iglesia et al., 1997). Very little is known regarding the effects of mesh on the subsequent reorganization of the vaginal connective tissues (Elmer et al., 2009). One of the most frequently used synthetic meshes is a Type I polypropylene macroporous knitted monofilament mesh (Dwyer, 2006). This mesh type has been very popular in the management of abdominal wall hernias and numerous publications have evaluated its effects on fibroblast cultures (Weyhe et al., 2007; Weyhe et al., 2008). However, in vitro data on the effects of these meshes on the vaginal connective tissues, vaginal fibroblast and smooth muscle cells has been limited. To study the effects of hormones and meshes in the vaginal area quite difficult secondary to ethical consideration, we have opted to conduct our investigation under in vitro conditions.

Our aim was to understand the effects of hormones and mesh on cell proliferation. To test our hypothesis that estrogen and polypropylene mesh increase vaginal smooth muscle cell proliferation, we collected vaginal wall biopsies from women without POP to perform primary cell cultures and measure the effects of hormones and mesh on cell proliferation.

**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, USA between 12/1/2006 and 12/31/2008 (Table 1). 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, USA) supplemented with 10% FBS, 1 mM glutamine, 0.075% Na₂HCO₃, 100 µg/ml penicillin-
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After a maximum time of 4 hours from tissue harvesting, the tissue was mechanically minced in cold cultured media, fragments of the tissue were washed 3 times in PBS and plated into fibronectin coated plastic dishes (Thermo Fischer Scientific, Rochester, NY, USA). After the initial outgrowth, clones with morphology resembling smooth muscle phenotype were patch cloned and propagated in culture. For cell characterization, 5,000 cell/well of a 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, USA) 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, USA). Diaminobenzidine was used as 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 smooth muscle cells, anti-smooth muscle actin antibody was used (monoclonal mouse, 1:250, 30 min incubation, clone 1A4, catalog #0851 Dako, Carpinteria, CA, USA). Caldesmon expression was studied with a monoclonal mouse antibody, 1:100, 30 min incubation, (clone h-CD catalog #M3557 Dako, Carpinteria, CA, USA). An antigen retrieval step was used for caldesmon using citrate buffer and a steamer for 30 min. For 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 estradiol (0.1 µM, 1 µM, 10 µM) (Sigma-Aldrich, St. Louis, MO), progesterone (5 nM, 50 nM, 0.5 µM) (Sigma-Aldrich, St. Louis, MO) and polypropylene mesh (Gynemesh, Ethicon, Somerville, NJ, USA) in 96-well plates and cell proliferation was assessed by 3-[4,5-
dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide (MTT) assay at 24 hours utilizing a commercially available the MTT assay kit (American Type Culture Collection, Catalog #30-1010K, Manassas, VA, USA). 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 are then solubilized with acid isopropanol (90% isopropyl alcohol, 2.5% SDS, 0.004 N HCl) for 1 hour. The optical density of the solution is measured at 570 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, USA).

**Results**

The demographic data of our patients are described in Table 1. Characterization of our cultured cells confirmed smooth muscle phenotype (Fig. 1). The internal F-actin architecture of

**Fig. 1.** Vaginal smooth muscle cell culture. A: Initial outgrowth of smooth muscle cells. B: Intracytoplasmic distribution of F-actin fibers by confocal microscopy utilizing Rhodamine Phalloidin immuno-fluorescence. C: Immunostaining with antibody to smooth muscle α-actin. D: Immunostaining with antibody to h-caldesmon.
the cells was consistent with smooth muscle cells (Fig. 1B). In addition, our cultured cells were staining positively both with antibody to smooth muscle actin and h-caldesmon (Figs. 1C and 1D).

Under *in vitro* culture conditions, vaginal smooth muscle cell (Fig. 2) proliferation was significantly increased by estradiol but not by progesterone or by polypropylene mesh after 24 hours incubation (relative cell number, mean ± SD, control vs. estrogen: 0.24 ± 0.02 vs. 0.28 ± 0.02, \( P=0.01 \); control vs. progesterone 0.24 ± 0.02 vs. 0.23 ± 0.02, \( P=\text{NS} \); control vs. mesh, 0.24 ± 0.02 vs. 0.24 ± 0.01, \( P=\text{NS} \); Fig. 2). In addition, estradiol has significantly increased cell proliferation in a dose responsive fashion (estradiol dose: 0.1 \( \mu \text{M} \), 1 \( \mu \text{M} \), 10 \( \mu \text{M} \)) compared to control (Fig. 3). Progesterone (5 nM, 50 nM, 0.5 \( \mu \text{M} \)) did not have a significant effect on the rate of cell proliferation.
Discussion

Primary smooth muscle cell culture was performed to test the effect of estrogen, progesterone and polypropylene mesh on smooth muscle cell proliferation. It was observed that estrogen but not progesterone or polypropylene mesh increased the rate of cell proliferation in smooth muscle cell culture. In addition, estradiol has increased smooth muscle cell proliferation in a dose responsive fashion.

Ewies et al. (2008) demonstrated similar findings by showing that estrogen significantly increased pelvic ligament fibroblast proliferation. This is in sharp contrast with the findings of Liu et al. (2006) who noted that estradiol suppresses fibroblast proliferation derived from cardinal ligament of women with or without prolapse. It appears that estrogen may have a differential effect on cell proliferation based on the location of the cells in the pelvic floor.

Our study is the first to test the effect of polypropylene mesh on vaginal smooth muscle cells in vitro. Experiments with fibroblasts and mesh related to hernia-research revealed that cell proliferation was induced by all types of mesh (Weyhe et al., 2008; Weyhe et al., 2007). Based on these findings, we hypothesized that polypropylene mesh would induce the rate of smooth muscle cell proliferation. However, results from other studies conducted on different organ systems (i.e., inguinal hernia surgery) may not be applicable to the unique vaginal environment and reconstructive pelvic surgery (Schumpelick et al., 2003). Contrary to our hypothesis we found no significant change in the rate of vaginal smooth muscle cell proliferation exposed to polypropylene mesh. This finding is also supported by animal trials from Krambeck et al. (2006) who revealed that polypropylene mesh had the lowest degree of proliferative response compared to five other pubovaginal slings materials. In addition, the only human in vivo study assessing the inflammatory response to polypropylene mesh in pelvic reconstructive surgery indicated that the total cell count and the number of fibroblasts were significantly decreased one year postoperatively after vaginal implantation of polypropylene mesh (Elmer et al., 2009). It should be mentioned, however, that there was a significant postoperative increase in the number of macrophages and mast cells compared to preoperative counts (Elmer et al., 2009). Overall, mesh with little or no effect on cell proliferation appears to provoke the most desired biological response after implantation (Weyhe et al., 2007). Type I polypropylene mesh seems to have these characteristics providing some in vitro data in favor of its use for pelvic reconstructive surgery.

Limitations to this study include the relatively small sample size and the in vitro nature of our experiments may. Furthermore, cultured smooth muscle cells from patients with and without POP would help us to determine if there is a difference in the rate of proliferation and responsiveness to estrogen. Also there are other factors not controlled for in this trial that may have an influence on cell proliferation rates (Connell et al., 2009). Finally, a 3-D culture co-culture model may better represent the complex in vivo pelvic floor supportive structures than a monolayer of smooth muscle cells.

In summary, in vitro vaginal smooth muscle cell proliferation was significantly increased by estradiol but not by progesterone or by polypropylene mesh.
References


