Effect of cigarette smoking on human oviductal ciliation and ciliogenesis

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Objective: To investigate the effect of cigarette smoke exposure on ciliation and ciliogenesis in human oviductal epithelium.
Design: Molecular analysis using human tubal segments.
Setting: Academic medical center.
Patient(s): Twenty women undergoing elective tubal sterilization procedure.
Intervention(s): Expression of ciliated cell-specific markers was compared in tubal segments from smokers and nonsmokers using quantitative immunohistochemistry and Western blot analysis. The expression of transcription factors in the motile ciliogenesis program was compared using quantitative polymerase chain reaction and quantitative immunohistochemistry.
Main Outcome Measure(s): Oviductal ciliation and expression of transcription factors involved in ciliogenesis.
Result(s): No significant differences were detected in density of ciliation between groups. Neither number of years of smoking nor pack-year history correlated with density of ciliation. Expression of ciliogenic transcription factors FOXJ1, RFX2, and RFX3 was consistent between groups.
Conclusion(s): Few studies have evaluated the relationship between smoking and ciliated epithelium in human oviducts. Cigarette smoking does not seem to result in quantitative differences in the density of ciliation nor expression of ciliogenesis factors. Our findings suggest that pathophysiologic mechanisms other than ciliation account for the increased risk of ectopic pregnancy in women who smoke. (Fertil Steril® 2013;99:199–205. ©2013 by American Society for Reproductive Medicine.)
Key Words: Fallopian tube, oviduct, cigarette smoke, cilia, ciliogenesis

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Cigarette smoking in women of reproductive age has remained alarmingly prevalent over the past 20 years, and currently, nearly one in four women smoke cigarettes (1). Women who smoke sustain nearly a fourfold increased risk of ectopic pregnancy compared with women who never smoked, and this association is independent of other known risk factors (2, 3). A dose–response relationship between smoking and tubal pregnancy has been reported (4). Despite substantial epidemiologic and clinical evidence for the association, the mechanisms by which cigarette smoke contributes to ectopic pregnancy are poorly understood.

The human oviduct, or fallopian tube, is lined by tufts of motile cilia—cellular protrusions with an axonemal microtubular skeleton composed of α- and β-tubulin heterodimers (5). Dynamic variation in oviductal ciliation supports the existence of regulatory mechanisms governing ciliogenesis. The transcriptional control of ciliary gene expression has become clearer in recent years, with FOXJ1 and RFX transcription factors emerging as key regulators of a set of genes involved in cilia biogenesis and physiology. Interestingly, preclinical studies revealed RFX3 and FOXJ1 to share common direct target genes, implying synergy in the regulation of the motile ciliogenic program (6). Although FOXJ1 expression has been demonstrated in the fallopian tube, RFX expression has not been previously reported.

The coordinated action of the ciliated columnar epithelium in the fallopian tube is integral to the dynamic and directional transport of the immotile cumulus–oocyte complex from the distal ampullary segment toward the endometrial cavity (7). Any insult to

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the epithelium that decreases either the density or function of ciliated columnar cells has the potential to change this microenvironment from a fluid to a static one, thereby increasing the risk of delayed transit of the embryo and subsequent implantation in an intratubal ectopic location. Normal ciliary activity is related to ciliary cell quantity (8), and deciliation of the fallopian tube has been reported in tubal pregnancy specimens (9).

Studies have previously demonstrated a decrease in ciliated columnar epithelium in the bronchial tract of smokers (10). Yet, in this location, it is difficult to delineate whether the degradation of ciliated cells is thermal or biochemical. Cigarette smoke is composed of nearly 4,000 chemical compounds, such as nicotine, polycyclic aromatic hydrocarbons, cadmium, and cyanide, any of which may be toxic to oviductal ciliogenesis and ciliation. In the hamster oviduct, a lower percentage of ciliated cells in the ampullary oviductal epithelium of animals exposed to cigarette smoke was reported (11). Few studies investigating the association between tobacco use and ectopic pregnancy in human oviductal epithelium have been conducted. Herein we present data from a study specifically designed to assess differences in oviductal ciliation and ciliogenesis in women who smoke.

MATERIALS AND METHODS

Study Population

This study was performed on 20 prospectively collected tubal specimens from women undergoing elective tubal sterilization. The study was approved by the institutional review board of the Madigan Healthcare System, and all women provided written, informed consent before study inclusion. Subjects were parous premenopausal women (age range, 25–40 years) with regular menstrual cycles, scheduled for an interval laparoscopic tubal sterilization procedure. Subjects using any form of hormonal treatment within 3 months of surgery were excluded. Additional exclusion criteria included current pregnancy or postpartum within 6 months of delivery, current breastfeeding, reported history of *Chlamydia trachomatis* infection, pelvic inflammatory disease, or pelvic malignancy. Before surgery, subjects completed a detailed questionnaire regarding gynecologic, medical, and smoking history. A 10-mL serum sample was obtained on the day of surgery for assessment of the serum cotinine level. Serum cotinine, a metabolite of nicotine, was measured via commercial gas chromatography/mass spectrometry with a lower limit of detection of 20 ng/mL (Quest Diagnostics).

Tissue Specimens

Fallopian tube specimens were collected from patients at the time of laparoscopic tubal ligation by the Pomeroy method (12). At surgery, the fallopian tube was atraumatically grasped using the laparoscopic Babcock clamp through an endoloop at a point 5 cm from the cornua, and the endoloop was cinched down (Fig. 1). The 2–3-cm segment of isthmic–ampullary tube was then excised with endoshears and removed through a laparoscopic port. Cautery was not used during the surgical removal of oviductal specimens. A portion of the tubal segment was fixed immediately in 10% buffered formalin for 24 hours at room temperature before paraffin embedding. The remaining segment was immediately rinsed in 1% phosphate buffered saline (PBS) at 4°C, dissected free of adventitia, equilibrated in RNAlater (Ambion), snap-frozen, and stored at −70°C.

Immunohistochemistry of Fallopian Tube for Ciliated Cell Markers

Slide-mounted, paraffin-embedded oviduct sections of 5-μm thickness were dewaxed with Hemo-De (Scientific Safety Solvents) and rehydrated through graded alcohols into PBST buffer (PBS + 0.05% Tween-20). All specimens used in the molecular analyses were histologically confirmed to be from the ampullary segment of the fallopian tube. For the detection of cilia and ciliated cells, α-tubulin and LhS28 immunohistochemical staining was performed using the EnVision G/2 double stain system (Dako, product code K5361). A monoclonal antibody directed against acetylated α-tubulin (dilution 1:1,000; Abcam) was applied to rehydrated paraffin sections and allowed to incubate for 10 minutes at room temperature. Endogenous peroxidase was blocked. In separate immunostaining, sections were incubated for 10 minutes with the monoclonal antibody LhS28. Considered a marker for ciliated epithelial cells, LhS28 specifically immunostains ciliary basal bodies at the apical surface (13) (dilution 1:1,000; Abcam). A secondary antibody (Richard-Allen Scientific) linked to peroxidase and diaminobenzidine (DAB) was used to visualize the binding of the primary antibody for each specimen. For detection of RFX2 and RFX3, primary antibodies (RFX2 dilution 1:100 and RFX3 dilution 1:50; Lifespan Biosciences) were incubated with tissues at 4°C in a humidified chamber overnight, and DAB-based detection was performed. The slides were counterstained with hematoxylin (Richard-Allen Scientific), dried, and overlaid with coverslip using nonaqueous media. Positive and negative specificity controls were used throughout.

Quantification of Immunostaining

The slides were visualized using an inverted microscope (Olympus IX71) and examined for α-tubulin and LhS28, RFX2, and RFX3 reactivity by an investigator blinded as to cohort assignment. White balanced images of representative tubal lumen per specimen were selected at random and captured at ×60 or ×20 using the Olympus DP manager. Images were processed using ImageJ software (http://rsbweb.nih.gov/ij/) for quantitative analysis via the color deconvolution method. Images were "defined" to erase background and artifacts but retain approximately one nucleated cell depth bordering the lumen and interiors using the ImageJ freehand tool. Vectors for hematoxylin (blue) and DAB (brown) were used in the analysis. The lower limit threshold was set at "0" for all colors, and the upper limit threshold for color brown was set at "100" for α-tubulin and LhS28 and "135" for RFX2 and RFX3 to allow automated measurements. Measurements were taken for integrated density (calculation of area times mean gray value) for brown. Results are an average of integration density of target over nucleated cells (brown over blue) of 10 slide preparations per specimen on average.