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Prenatal Bisphenol A Exposure Alters Epithelial Cell Composition in the Rhesus Macaque Fetal Oviduct

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ABSTRACT

Bisphenol A (BPA) is an endocrine disrupting compound that is a pervasive environmental contaminant. Although it has been reported to affect the development of a variety of fetal reproductive tissues, data on the effect of fetal BPA exposure on oviducts were extremely limited and were only available in mice. To determine if there are adverse effects of gestational BPA exposure on fetal oviduct, we exposed pregnant rhesus macaques with female fetuses to oral or nonoral BPA during the last trimester of gestation (day 100 to term). After the treatment, fetal oviducts were collected for morphology evaluation. BPA exposure altered the percentages of different cell types (ciliated, nonciliated, and secretory) in the fetal oviduct and resulted in a significant high ciliated cell population in the BPA-exposed fetal oviduct. The distribution of ciliated cells on the epithelium in the BPA-exposed fetal oviduct was also altered. Gestational BPA exposure reduced the expression of mucosubstance and uteroglobin in secretory cells in the fetal oviduct. A comparison of the outcome of the fetal oviduct studies with similar outcomes previously reported in the lung from the same fetuses demonstrates that BPA exhibits opposite effects in these two organs. In conclusion, the BPA-associated alterations in the fetal oviduct could potentially affect the oviduct morphology and function later in life with a negative impact on fertility. The mechanisms of action of the differential response in the oviduct and the lung to BPA exposure require further investigation.

Key words: fallopian tube; nonhuman primate; uteroglobin; mucin; endocrine disruptor.

Bisphenol A (BPA) is a high production volume chemical that is used in a wide variety of consumer products, including polycarbonate and other forms of plastics, resins used to line food and beverage containers, thermal printed papers, and composites used in dentistry. The widespread use exposes humans to BPA on a constant basis (Stahlhut *et al.*, 2009), confirmed by a Centers for Disease Control and Prevention (CDC) report showing that BPA is present in the urine of more than 90% of Americans sampled (Calafat *et al.*, 2008). Although estimates of daily exposure differ markedly (Dekant and Volkel, 2008; Taylor *et al.*, 2011; Vandenberg *et al.*, 2010), the pervasiveness of human exposure to BPA is not disputed (Calafat *et al.*, 2008; Vandenberg *et al.*, 2009).

The endocrine disrupting properties of BPA are extensive and varied, with effects on signaling mechanisms involving estrogen, androgen, aryl hydrocarbon, and thyroid hormone receptors (Peretz *et al.*, 2014; Welshons *et al.*, 2006). Because development of the female reproductive tract is regulated by steroid hormones, many studies have assessed the possible effects of exogenous estrogen exposure such as BPA on female

reproductive health. A recent study summarized published scientific literature from 2007 to 2016 on the potential effects of BPA on female fertility and concluded that BPA may be associated with infertility in women by altering the morphology and function of the female reproductive tract (Ziv-Gal and Flaws, 2016). Interestingly, of the 127 scientific reports analyzed in that study, only 4 focused on the effects of BPA exposure on the oviduct.

The oviduct is the place where fertilization occurs. In women, the morphology of the oviductal epithelium undergoes cyclic changes and is regulated by steroid hormones to support fertilization success (Verhage *et al.*, 1979). The oviductal epithelium interacts with sperm to maintain sperm motility and viability by storing them in the lower region of the oviduct (Suarez, 2008). As the time of ovulation approaches, sperm are gradually released from the oviductal epithelium, and the numbers and the timing of sperm that reach the fertilization site in the upper oviduct are tightly regulated to reduce the incidence of polyspermic fertilization (Hunter *et al.*, 1982; Wilmut and Hunter, 1984). After fertilization, the oviduct supports the embryo before it reaches the uterus. Alterations in oviduct morphology and function would adversely affect fertilization, preimplantation embryo development, and embryo transport.

BPA exposure in adult mice was found to delay embryo transport and implantation, suggesting that the function of oviduct may have been affected (Pan *et al.*, 2015; Xiao *et al.*, 2011). In CD-1 mice, exposure to low-dose BPA in utero induced progressive proliferative lesions in the oviduct when they became adults (Newbold *et al.*, 2007, 2009), indicating that BPA exposure in the oviduct during fetal development could have important consequences for adult reproductive success and offspring health. However, to date, all studies were conducted in mice, and it is unknown whether the same adverse effects would exist in women. The significant species-specific differences in the oviducts of rodents and primates (including women) make it difficult to extrapolate the mouse data for human health implications.

To examine the effects of gestational BPA exposure on oviduct development and to increase the knowledge base in the adverse effects of BPA on reproductive health, here we used the nonhuman primate as a model to better understand the possible mode of action of BPA on the human oviduct. The animals received BPA during the last trimester of gestation, which is the time that human and primate oviductal epithelium differentiate to form the pattern seen in the adult oviduct. The fetal oviducts from animals that received BPA were evaluated, and the findings were placed in context with another epithelial tissue, the lung, that has been evaluated from the same animals.

MATERIALS AND METHODS

Animals and BPA dosing. The adult female rhesus macaques (*Macaca mulatta*) used in these studies have been described in detail previously (Taylor *et al.*, 2011). Procedures for maintenance and handling of animals were reviewed and approved in advance by the Institutional Animal Use and Care Administrative Advisory Committee at the University of California at Davis. Animals were maintained at the California National Primate Research Center (CNPRC) and housed individually in stainless steel cages with a 0600–1800 h light cycle and at a controlled temperature of 25–27°C. Purina Monkey Chow and water were provided for ad libitum consumption. Supplements consisting of seasonal produce, seeds, and cereal were offered as environmental enrichment. Females aged 6–13 years were naturally mated according to standard CNPRC procedures. Gravid females were sonographically screened (Vom Saal *et al.*, 2014) by 40 days of gestation to identify female fetuses. Screening accuracy was 100% and all fetuses were confirmed to be female at the time of tissue collection.

Two treatment protocols were used for BPA dosing, as described previously (Calhoun *et al.*, 2014). Briefly, the first cohort was given small pieces of fruit containing 400 μg/kg body weight deuterated BPA (dBPA; CDN isotopes) or vehicle control once per day (3 control, 2 BPA treated). A second cohort received continuous dBPA exposure via Silastic tubing implants demonstrated to produce serum levels ranging from 2.2 to 3.3 ng/ml of unconjugated dBPA in nonpregnant test females (2 control, 6 BPA treated). Animals were dosed from GD 100 to term. Levels of bioactive BPA in maternal serum were measured using isotope-dilution liquid chromatography-mass spectrometry as described previously (Taylor *et al.*, 2011).

Histology and immunohistochemistry. Fixed fetal oviduct tissues (5 controls and 8 BPA exposed) were paraffin embedded, sectioned at 6 μm, and stained with hematoxylin and eosin for histological assessment as described previously (Van Winkle *et al.*, 2013). For immunohistochemistry, after deparaffinization, paraffin sections were labeled with anti-beta-tubulin 4 to stain cilia (Sigma Aldrich, St. Louis, Missouri; 1:1000 dilution) or anti-CC10 to label uteroglobin/Clara Cell Secretory Protein (CCSP) (BioVendor, Asheville, North Carolina; 1:500 dilution) as described previously (Coppens *et al.*, 2007). Controls included the substitution of primary antibody with phosphate-buffered saline, resulting in loss of specific staining. Images were taken using an Olympus BX61 microscope with an Olympus DP72 color camera (Olympus America Inc., Center Valley, Pennsylvania) and MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices, Sunnyvale, California).

To quantify the expression of uteroglobin, fluorescence density measurements were taken and analyzed using the freehand tool to draw the outline of the selected epithelium using ImageJ software (http://rsb.info.nih.gov/ij/). The measurements were subtracted from that of their corresponding negative controls (without anti-C10 antibody), and data were presented as fluorescence intensity per area measured.

Mucin staining with Alcian blue and periodic acid-Schiff (AB/PAS). Alcian blue and periodic acid-Schiff (AB/PAS) staining (American MasterTech, Lodi, California) was performed as described previously, with modifications (Coppens *et al.*, 2009). Briefly, after deparaffinization, selected paraffin sections were treated with 3% acetic acid for 3 min then stained with Alcian blue for 30 min. After a brief tap water rinse, the sections were treated with 0.5% periodic acid for 7 min, quickly rinsed with tap water, and stained with Schiff's reagent for 8 min. Slides were rinsed with running deionized water for 5 min before mounting with Cytoseal mounting media (Richard-Allan Scientific, Kalamazoo, Michigan). Images were taken using an Olympus BX61 microscope with an Olympus DP72 color camera (Olympus America Inc., Center Valley, Pennsylvania) and MetaMorph Microscopy Automation & Image Analysis Software (Molecular Devices, Sunnyvale, California).

Determination of epithelial cell types in the fetal oviduct. The percentages of different epithelial cell types were assessed using a stereological approach. Briefly, for each animal, 4 sections of 6-μm paraffin-embedded oviduct tissue at least 90 μm apart were collected and stained with hematoxylin and eosin. For each section, three images of oviductal epithelium were taken at

20× using an Olympus BX61 microscope as described above, and a total of 12 images per oviduct were acquired. Two fields in each image were randomly selected, and numbers of ciliated, nonciliated, and secretory cells were counted with a cycloid grid and Stereology Toolbox (Morphometrix, Davis, California), as described previously (Vandenberg *et al.*, 2007). Cells with visible cilia were counted as ciliated cells, and cells with protruding apical membranes were counted as secretory cells. Cells were considered nonciliated if they had no visible cilia or protruding apical membranes. A range of 285–530 cells was counted for each fetal oviduct sample.

Statistical analysis. All data are expressed as mean ± standard error of mean (SEM). For all data, *N* represents the number of animals examined in each treatment group. Independent 2-sample *t*-test was used to compare data from control and BPA-treated animals. A *p*-value of less than .05 was chosen to indicate statistical significance.

RESULTS

BPA Exposure in Late Gestation Affects Fetal Oviductal Epithelium Morphology

In this study, two treatment protocols for BPA dosing were used. Oral administration of 400 μg/kg body weight of dBPA produced a peak of approximately 4 ng/ml of unconjugated dBPA in serum 1 h after dosing (Taylor *et al.*, 2011), whereas continuous dBPA exposure via Silastic tubing implants maintained serum levels of 2.2–3.3 ng/ml unconjugated dBPA in tested animals. Although the treatment protocols differed, fetuses exposed to the BPA treatments exhibited similar morphological changes in the oviductal epithelium. The data obtained from the animals of these treatment protocols were combined for analysis, and the preliminary observation revealed that oviductal epithelium in BPA-exposed fetuses had predominately ciliated cells (Figs. 1C and 1D), whereas both ciliated and secretory cells were present in the oviductal epithelium of control fetuses (Figs. 1A and 1B).

To further examine the difference in morphology between oviducts from control and BPA-exposed fetuses, we labeled cilia and ciliated oviductal epithelial cells with beta-tubulin IV antibody. In the control oviducts, ciliated cells were distributed in an even pattern throughout the oviductal epithelium, with unstained secretory cells in between (Figure 2A). However, in oviducts from the BPA-exposed fetuses, the expression of beta-tubulin IV was continuous and more extensive (Figure 2C), indicating the presence of more ciliated cells and fewer secretory cells. The expression of beta-tubulin IV was more intense near the apical membrane, suggesting that more cilia were being formed. Negative controls with beta-tubulin IV antibody omitted during immunostaining are shown in Figures 2B and 2D and did not have staining of apical cilia.

We also compared the percentage of ciliated, nonciliated, and secretory cells in the fetal oviduct in control and BPA-exposed fetuses. Ciliated (42 ± 0.05%) and secretory (36 ± 0.09%) cells were present in nearly equal abundance in the control fetuses, whereas in BPA-exposed fetuses a higher proportion of epithelial cells differentiated into ciliated cells (78 ± 0.05%) (Figure 3).

Mucosubstance-Positive Epithelial Cells Are Unevenly Distributed in Oviducts of BPA-Exposed Females

To evaluate the secretory function of the oviduct, we examined the distribution of mucins and mucosubstance-positive epithelial cells using AB/PAS staining. We did not observe a difference in mucin production in the epithelial lining (Figure 4, blue color) between oviducts from control and BPA-exposed fetuses. However, although mucosubstances were evenly expressed in the control oviduct epithelial cells (Figs. 4A and 4B, turquoise color), mucosubstances in the BPA-exposed oviduct were highly expressed in cells localized in invaginations (Figs. 4C and 4D, turquoise color, arrows) compared with the rest of the region on the fetal epithelium.

BPA Exposure Reduces Uteroglobin Cellular Expression

We further investigated the secretory function of the fetal oviduct by examining the expression of uteroglobin secretory protein. Uteroglobin is the product of the *SCGB1A1* gene and is known as CCSP in the lung. In BPA-exposed fetal oviducts, the intensity of uteroglobin appeared significantly reduced (Figure 5C) when compared with the control oviducts (Figure 5A). Quantification of uteroglobin expression showed that the mean fluorescence intensity of uteroglobin in the control and BPA-treated oviducts were 13.92 and −0.42 (fluorescence intensity/area, arbitrary unit), respectively, indicating that there was little to no expression of uteroglobin in the BPA-treated oviducts.

DISCUSSION

In this study, we presented the first report in a primate model of BPA-induced effects on the fetal oviduct. Our data indicate that exposure to BPA during the last trimester of fetal development affects the ratio of ciliated to secretory cells and the abundance of the secreted molecules mucins and uteroglobin in the fetal oviduct. These changes occur at BPA exposure levels that are relevant to current human exposure. Because both oral and nonoral routes of exposure induced similar effects on fetal oviducts (data not shown), and because the serum and amniotic levels of BPA that result from our exposures (Calhoun *et al.*, 2014) are similar to those reported in women (Sajjad, 2010), the results in the present study have real-world implications. Importantly, although this report did not investigate the long-term effects of BPA exposure on the adult oviduct, given the importance of the oviduct for fertility, the abnormalities induced during fetal development could potentially affect adult fertility, as found in the previous mouse studies (Newbold *et al.*, 2007, 2009).

BPA was administered to pregnant rhesus monkeys during the last trimester of pregnancy, starting at about 100 days of gestation. In humans and nonhuman primates, gross morphogenesis of the female reproductive tract is largely completed during the first trimester of pregnancy (Allen *et al.*, 1982; Masse *et al.*, 2009). The oviductal epithelium continues to differentiate to form ciliated and secretory cells starting at week 22 during human fetal development; at full term, the oviductal epithelium represents the pattern of an adult oviduct (Barberim *et al.*, 1994; Konishi *et al.*, 1987). In human and nonhuman primates, both the ciliated and secretory cells of the oviductal epithelium are responsive to ovarian steroid hormones and undergo changes over the menstrual cycle (Brenner *et al.*, 1983; Patek *et al.*, 1972a; Tollner *et al.*, 2008). However, it is not known how responsive these same cell types are during fetal development, once the female reproductive tract has formed. Our study presents evidence that the differentiation of oviductal epithelial cells is altered by endocrine disruption, resulting in more ciliated cells formed in the BPA-exposed fetal oviduct.

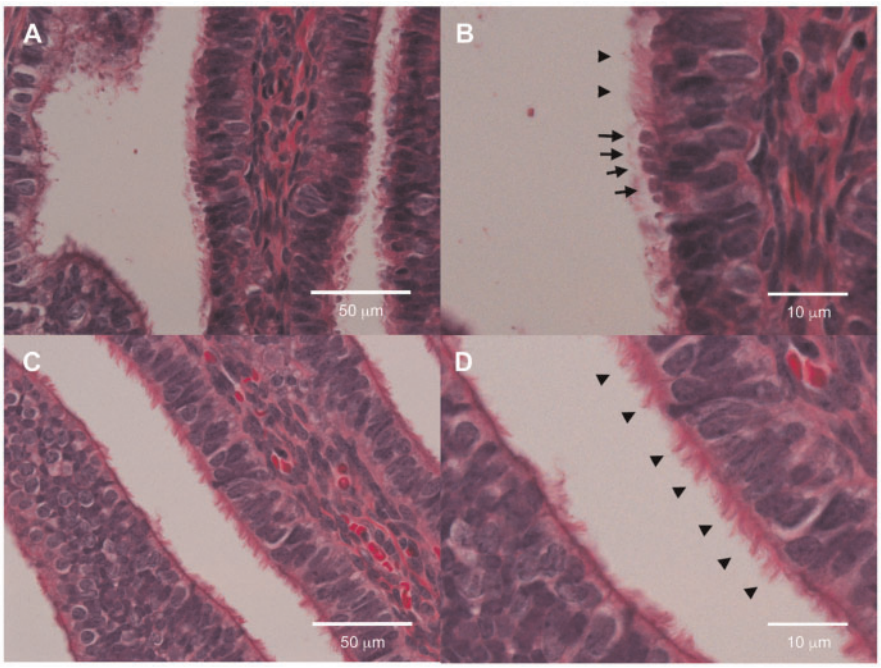

Figure 1. Morphology of oviductal epithelium (stained with hematoxylin and eosin) in control fetuses (A and B) and in late gestation BPA-exposed fetuses (C and D). The BPA-exposed fetal oviduct showed predominantly ciliated cells (arrowheads), whereas in the control oviduct, both ciliated (arrowheads) and secretory (arrows) cells were observed.

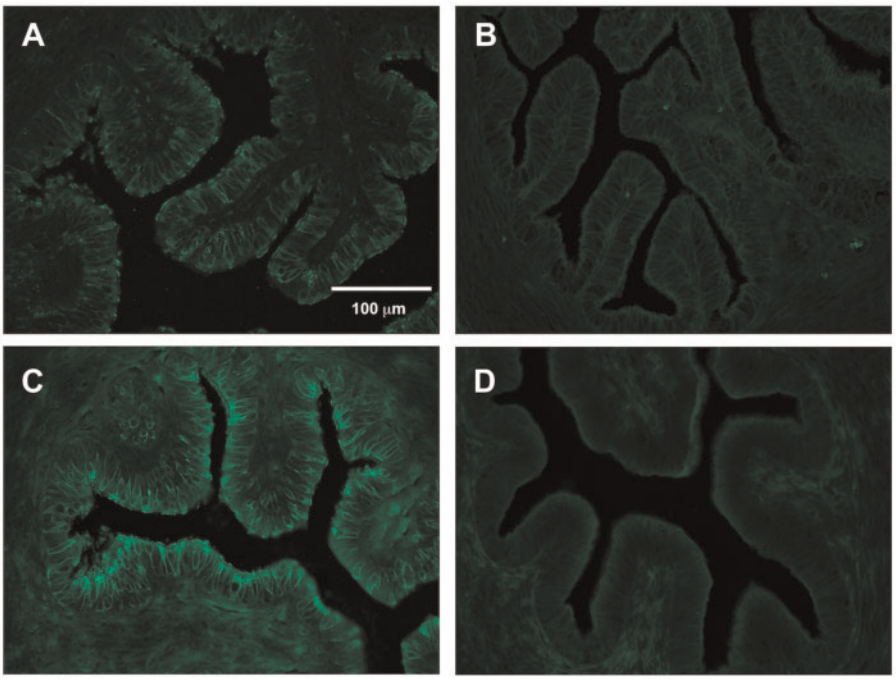

Figure 2. Localization of ciliated cells in fetal oviductal epithelium after late gestation BPA exposure. Cilia and ciliated cells were labeled with beta-tubulin IV antibody; Control (A) and BPA-exposed (C) fetal oviductal epithelium are shown. In the control oviducts, the ciliated cells were distributed in an even pattern throughout the oviductal epithelium (A); in the BPA-exposed fetal oviducts, more ciliated cells were present on the epithelium. Sections with beta-tubulin IV antibody omitted during immunostaining served as negative controls (B from control group and D from BPA group). Scale bar applies to all panels.

BPA exposure in our study began well after the initial differentiation of the oviducts. This raises the possibility that earlier exposure—for example, the chronic environmental exposure to which humans are exposed—might produce more severe effects on the female reproductive tract. Differentiation of the Mullerian ducts can be disrupted by steroid hormones, particularly estrogens acting through estrogen receptor alpha (Schrager and Potter, 2004), and is sensitive to endocrine disrupting chemicals. In humans, in utero exposure to a similar estrogenic endocrine disruptor, diethylstilbestrol (DES), increased

the incidence of both infertility and ectopic pregnancy in adult daughters of pregnant women to whom DES was prescribed during the 1940s to 1970s (Hoover *et al.*, 2011; Patek *et al.*, 1972b). In mice, prenatal exposure to BPA and DES resulted in oviductal abnormality (progressive proliferative lesion) later in life (Newbold et al., 1995, 2009). If the changes observed in the current study persist into adulthood, the differences observed in the fetal oviduct of rhesus females exposed to BPA could represent the earliest physical evidence of changes with functional consequences.

In the adult female, the oviduct is involved in modulating sperm transport, supporting fertilization, maintaining early embryo development, and transporting early embryos to the uterus (Suarez and Pacey, 2006). It is unclear how changes in the percentage of different cell types in the fetal oviduct would affect the oviductal function later in life when the animals become adults. The fact that mammalian sperm-oviduct interaction plays an important role in the success of fertilization and that mammalian preimplantation embryos spend extended time in the oviduct suggests that, the potential for disturbances in fertility seem likely. In nonhuman primates, sperm predominantly bind to the secretory or nonciliated cells of oviductal epithelium (Klug *et al.*, 2006). Human sperm appear to bind to all cell types (Baillie *et al.*, 1997), but it is not known whether they bind to all cell types with equal proportion or function. In the primate oviduct, secretory cells are more abundant relative to ciliated epithelial cells in areas of the oviduct closest to the uterus (isthmus) when compared with the more distal ampullar regions (Chapalamadugu *et al.*, 2014). Disruption of the distribution or percentage of different cell types in the oviduct can result in the perturbation of sperm transport and fertilization. If BPA-associated changes in the fetal oviduct persist, they could affect the ability of the adult oviduct to bind sperm.

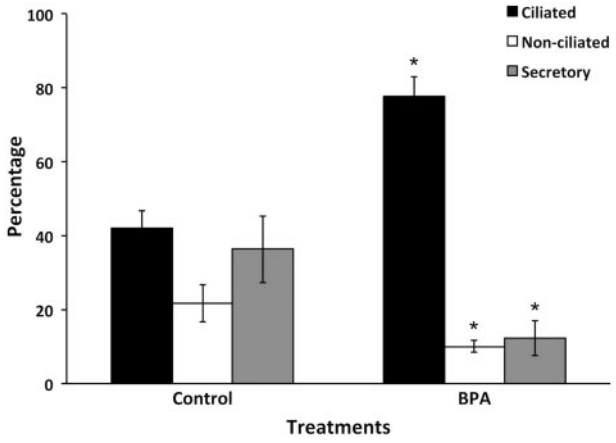

Figure 3. Percentages of ciliated, nonciliated, and secretory cells in fetal oviduct after BPA exposure. In the BPA-exposed fetal oviducts, the majority of cells were ciliated cells, whereas in the control oviducts, the percentages of ciliated and nonciliated cells were similar. Samples from 5 control and 8 BPA-treated fetuses were examined. *$p < .05$ when comparing mean differences between the same cell types in control and BPA groups.

The observed alteration in the percentage of different oviductal epithelial cell types is evidently influenced by the specific timing of BPA exposure in the prenatal period. Prenatal DES exposure causes the human and mouse female reproductive tract to become "anteriorized," a situation where the upper uterus has characteristics of the oviduct and the upper vagina has characteristics of the cervix (Jefferson *et al.*, 2011). In contrast, neonatal estrogenic chemical exposure causes "posteriorization" of the mouse female reproductive tract, producing an oviduct with uterine characteristics, and causing expression of genes characteristic of the vagina in both the oviduct and uterus (Jefferson *et al.*, 2011; Mazur *et al.*, 2014). In the current study, the increase in the percentage of ciliated cells

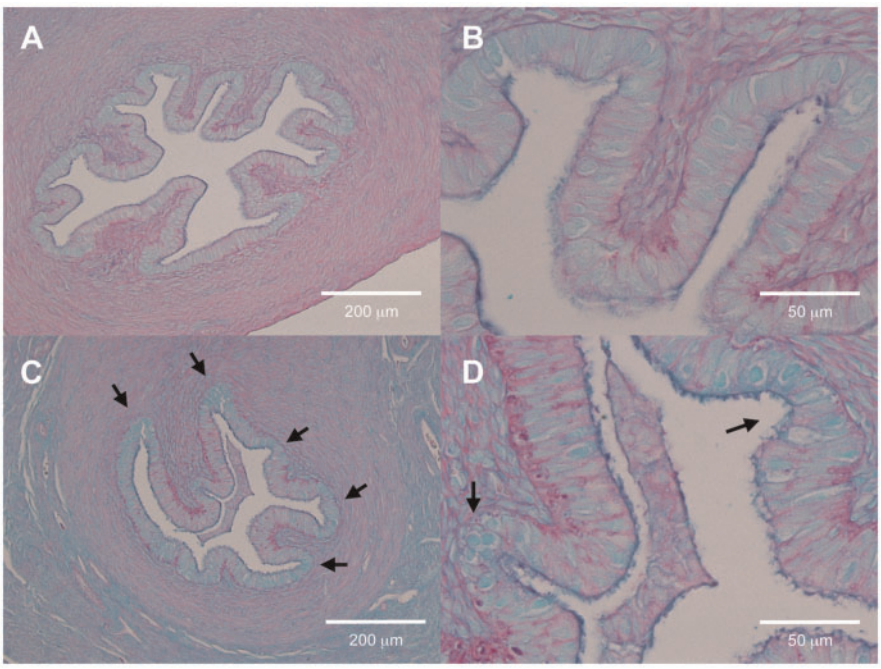

Figure 4. Localization of mucosubstances in fetal oviductal epithelium after late gestation BPA exposure. Mucosubstances were stained (turquoise) with AB/PAS in control (A and B) and BPA-exposed fetuses (C and D). The mucosubstances in the control fetal oviducts were evenly distributed. However, in the BPA-exposed fetal oviducts, aggregation of mucosubstances (arrows) in the invagination of the oviductal epithelium was observed (C and D).

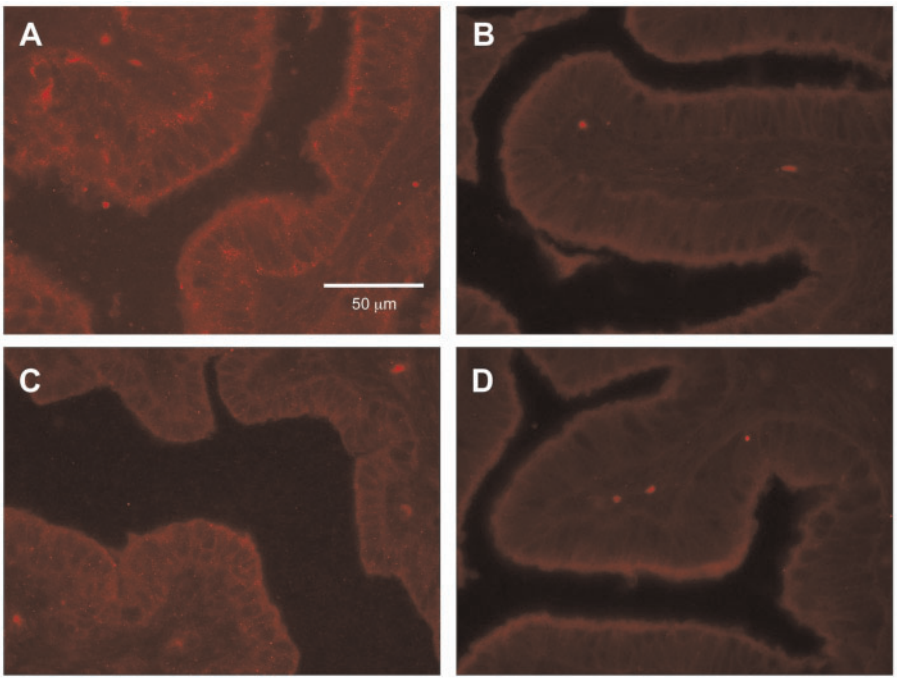

Figure 5. Expression of uteroglobin in fetal oviductal epithelium after late gestation BPA exposure. Uteroglobin was detected with CCSP antibody; Control (A) and BPA-exposed (C) oviductal epithelium are shown. In the BPA-exposed fetal oviducts, little to no uteroglobin expression was detected (C). Sections with CCSP antibody omitted during immunostaining served as negative controls (B from control group and D from BPA group). Scale bar applies to all panels.

in the oviducts of BPA-exposed fetuses could be the "anteriorization" of the differentiating oviductal epithelium.

In the adult oviduct, mucins and uteroglobin are major proteins secreted by the oviductal epithelium and are present in the oviductal fluid. The altered distribution of mucins and the decrease in uteroglobin evident in the fetal oviduct of BPA-exposed fetuses (Figs. 4 and 5) could implicate potential altered oviductal function. Mucins are large glycoproteins that generally function as lubricants and prevent dehydration of luminally disposed cell surfaces (Lagow *et al.*, 1999). In the human oviduct, the main mucin MUC1 has been found to play a protecting role in preventing ectopic pregnancy (Al-Azemi *et al.*, 2009). It is unclear whether the uneven distribution of mucins in the BPA-exposed fetal oviductal epithelium would have an impact on reproduction later in life. Uteroglobin is a secretoglobin, a family of proteins that have been found to have immunomodulary and anti-inflammatory properties (Mukherjee *et al.*, 1983). Uteroglobin has also been associated with the suppression of sperm and embryo antigenicity in the oviduct (Block *et al.*, 2000). The significantly reduced uteroglobin expression in the BPA-exposed fetal oviducts could impair fertility later in life. Further investigation is needed to evaluate the long-term effects of late gestation BPA exposure on reproductive health.

Previously, we reported that exposure to BPA in late gestation affects the development of multiple organ systems in monkeys (Elsworth *et al.*, 2013; Hunt *et al.*, 2012; Tharp *et al.*, 2012; Van Winkle *et al.*, 2013). Uneven distribution of mucosubstance positive cells and alterations in mucociliary differentiation has interesting parallels to our previous work in the lung (Van Winkle *et al.*, 2013). The lung develops on a similar timeline to the oviduct, and the distribution and maturation of ciliated and secretory cells is critical to normal function in both organs. Further, in both organs the secretory products include mucins and secretory globins and these are critical to normal physiologic function. Thus, the evaluation of similar endpoints (the ratio of ciliated/nonciliated cells) of mucins and uteroglobin (referred to as CCSP in the lung) allows for comparisons between the two organs. Interestingly, although BPA increased secretory cells and mucin production in airways (Coppens *et al.*, 2007), the oviduct exhibited opposite effects, with decreased secretory cells and increased cilia (Figure 3). The reason for the apparently opposite effects of BPA on cellular differentiation in these two tissues is not immediately obvious. We speculated that the observed discrepancy could result from differences in the specific *HOX* genes that drive anterior to posterior patterning in the lung and reproductive tract (Du and Taylor, 2016) and differential sensitivity of these transcription factors to BPA. Further study is required to investigate the molecular mechanisms of action in these two organs that respond oppositely when exposed to BPA in late gestation.

In summary, our data demonstrate that environmentally relevant levels of BPA exposure produce alterations in the oviducts of fetal rhesus monkeys and have obvious relevance for human development and fertility. BPA-induced alterations in the percentage of different cell types and secretory products in the fetal oviduct indicate changes in the tissue that may potentially impact adult fertility. A comparison of BPA effects on the fetal oviduct in the present study and the developing lung reported previously from the same fetuses demonstrates the complexity of individual tissue responses to BPA. Further, these studies underscore the need to examine the effects of endocrine disrupting chemicals on the understudied area of oviductal development and function. Taken together, our findings demonstrate the critical role of nonhuman primate studies in understanding BPA exposure effects on fetal development and raise concerns about the long-term impact of these exposures on adult health.

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