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Strain-Specific Induction of Endometrial Periglandular Fibrosis in Mice Exposed During Adulthood to the Endocrine Disrupting Chemical Bisphenol A

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Abstract

The aim of this study was to compare effects of bisphenol A (BPA) on collagen accumulation in uteri of two mouse strains. Adult C57Bl/6N and CD-1 mice were exposed to dietary BPA (0.004–40 mg/kg/day) or 17 α -ethinyl estradiol (0.00002–0.001 mg/kg/day) as effect control. An equine endometrosis-like phenotype with increased gland nesting and periglandular collagen accumulation was characteristic of unexposed C57Bl/6N, but not CD-1, endometrium. BPA non-monotonically increased gland nest density and periglandular collagen accumulation in both strains. Increased collagen I and III expression, decreased matrix metalloproteinase 2 (MMP2) and MMP14 expression, and increased immune response were associated with the endometrosis phenotype in the C57Bl/6N strain and the 30 ppm BPA CD-1 group. The association between the pro-collagen shift in increased collagen expression and decreased MMP2 expression and activity implies that strain differences and BPA exposure alter regulation of endometrial remodeling and contributes to increased fibrosis, a component of several human uterine diseases.

Keywords

BPA; endocrine disruptor; uterus; mouse; collagen; dose response; endometrial periglandular fibrosis; equine endometrosis; matrix metalloproteinase; non-monotonic

1. Introduction

Bisphenol A (BPA) is an endocrine disrupting chemical (EDC) released from polycarbonate plastics and linings of food and beverage containers that contaminates the consumed contents of the container [1–3]. Consumption of BPA-contaminated foods and beverages is the major route of exposure in humans, with additional exposure occurring through handling of thermal receipt paper, application of dental sealants, and transfer from medical equipment [4–6]. Supporting widespread and prolonged human exposure to BPA, measurable levels of

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BPA metabolites were found in over 93% of human urine samples in the 2003–2004 National Health and Nutrition Examination Survey [7]. Because of the known estrogen and androgen endocrine disrupting activity of BPA, results from numerous *in vitro* and *in vivo* studies have demonstrated that development and function of reproductive tissues, including the uterus, were sensitive to BPA exposures [8–15]. Estrogenic activity of BPA in the uterus has been established with the rodent uterotrophic assay, for example [16, 17], and numerous examples of changes in BPA-responsive gene expression have been reported from studies in a variety of animal models [18–22]. Endocrine disrupting actions of BPA have also been associated with pathological changes in the uteri of BPA-exposed rats and mice [23–26].

Outside of the reproductive axis, we recently demonstrated that lifelong exposure to as little as 4 µg/kg/day of BPA resulted in functional alterations of the collagen extracellular matrix (ECM) in the hearts of male and female CD-1 mice [27]. In the uterus, the collagen ECM plays a dynamic structural and functional role in tissue remodeling of the cycling uterus and during pregnancy [28], whereas dysregulation of ECM function and collagen accumulation contributes to endometrial adenocarcinoma, fibroids (leiomyomas), and endometriosis [29–33]. In addition to being associated with pathology of the human uterus, dysregulation of collagen accumulation and excessive fibrosis is a hallmark of equine endometriosis, one of the most important causes of infertility in mares [34]. Equine endometriosis is an age-related and irreversible degenerative disease of the uterus characterized by markedly increased endometrial stromal and periglandular fibrosis (EPF) associated with “gland nest” structures and is unrelated to human endometrioses [35–39]. The severity of EPF is inversely correlated to successful conception and gestation and is associated with increased rates of embryonic or fetal foal loss and increased susceptibility to infection [37]. The etiology of EPF is unknown, in part due to the lack of suitable laboratory animal models to facilitate experimental studies. The presence of EPF has also been noted in canines [40]; however, it is unknown whether a similar pathology characterized by excessive periglandular collagen accumulation and gland nest formation occurs in humans or laboratory rodent models.

Accumulation of collagen in the uterus during the female reproductive cycle is a dynamic and tightly regulated process involving hormonal control of collagen gene expression and matrix metalloproteinase (MMP) expression and activity [28]. The types I and III are primary collagens expressed in mammalian uterus, with lower levels of collagen types IV, V, and VI expressed variably during the cycle [41–44]. 17β-Estradiol (E2) can increase mRNA and protein synthesis of many collagen subtypes in the uterus [45–47]. Estradiol also plays a critical role in the cyclic remodeling of the endometrium through dynamic modulation of collagen degradation by tightly regulating MMP expression and activity [28, 48, 49]. The MMP9 and MMP2 proteins are key collagen degrading enzymes present in both latent and active forms in endometrial tissue of the uterus throughout the reproductive cycle [28]. Regulation of MMP2 activity during the estrous cycle of rodents and the menstrual cycle in humans is in part controlled by MMP14, which activates latent MMP2 (proMMP2) by proteolytic cleavage [28, 50, 51]. Vascular smooth muscle cells respond to increasing levels of estrogen by induction of MMP14 protein expression and a corresponding increase in MMP2 activity [52]. Control of collagen accumulation also involves inhibition of MMP activity through serum and tissue inhibitors of metalloproteinases (TIMPs) which, in response to varying levels of E2 and progesterone, are also differentially expressed during

the phases of the estrous and menstrual cycles [28, 53–56]. TIMP-1, -2, and -3 are all expressed at high levels in the uterus throughout the cycle, with TIMP-3 exhibiting the most dynamic spatio-temporal expression profile [28]. While the effects of BPA on reproductive tissues are well-studied, the impacts of exposures to BPA and other EDCs on collagens, MMPs and the ECM of most tissues, including the uterus, are poorly understood. Evidence supporting the possibility that BPA may alter MMP activity leading to dysregulation of collagen dynamics in the uterus is limited. However studies in developing mammary gland have identified an exposure related change in ECM collagen content, and BPA can increase expression of MMP2 and MMP9 expression in ovarian granulosa cells and ovarian cancer cell lines [57–59].

There are well known differences in the sensitivity of rodent strains to pathologic effects of estrogenic compounds, including E2, diethylstilbestrol (DES) and BPA. Differences in sensitivity of a variety of hormonally regulated responses, among them male reproductive development, prolactin release, oocyte development, and expression of estrogen receptors (ERs) in uterine epithelium, have been characterized [60–65]. Strain-specific responses to estrogen-like EDCs vary and are dependent on the responsive tissue of interest and the phenotypic or physiological response being examined. Even substrains derived from the C57Bl/6 line have been shown to have important genetic and phenotypic differences [66, 67]. In the uterus specifically, numerous strain-specific differences in sensitivity of uterine and female reproductive responses to estrogenic compounds have been reported in regards to uterine weight alterations, development of pyometra, immune cell infiltration, and onset of puberty, including findings from our previous work which demonstrated that C57Bl/6N mice were more sensitive to the actions of 17 α -ethinyl estradiol (EE) and BPA than CD-1 mice [26, 62, 68–70]. Based on that differential sensitivity we hypothesized that the sensitivity to developing collagen-related pathology of the uterus would likewise differ between the CD-1 and C57Bl/6N strains and that dietary BPA exposure would alter the regulation of collagen. To examine whether BPA differentially altered collagen accumulation in the adult uterus, uterine pathology was analyzed in young adult female C57Bl/6N and CD-1 mice that were exposed for 12–15 weeks to dietary BPA at doses extending below the accepted safe exposure level in humans (0.05 mg/kg/day) to the defined no observed adverse effect level (NOAEL) of 50 mg/kg/day [17, 26, 71, 72]. To characterize the differential impacts of these BPA exposures, histological examination was performed to examine collagen-related uterine pathology, including presence of EPF, gland nest formation, and collagen accumulation, with phenotypes compared across exposure groups and between strains. Immunohistochemistry was also used to compare differences in the distribution of MMP2, MMP14, F4/80, α -smooth muscle actin (α -SMA), and ER α in the uterus. MMP expression and activity were also assessed to examine the whether changes in collagen degradation were contributing to strain- and exposure-related differences in collagen accumulation.

2. Materials and Methods

2.1 Animal Husbandry and Necropsy

All animal procedures were performed in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee and followed

recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Six to seven week old C57Bl/6N Hsd and Hsd:ICR (CD-1 Swiss) mice were received from Harlan Laboratories, Inc. (Indianapolis, IN), group housed five per cage and randomly assigned to control or dietary exposure groups. Animals were housed in a polycarbonate-free housing system with single-use BPA-free polyethylene cages and water bottles (Innovive, San Diego, CA). Sanichip bedding (Irradiated Aspen Sani-chip; PJ Murphy Forest Products Corp, Montville, NJ) was used to prevent contamination from mycoestrogens in corncob bedding. Sterile drinking water containing <1% of oxidizable organics was produced from a water purification system designed to eliminate organic chemical contamination (Millipore Rios 16 with ELIX UV/Progard 2, Billerica, MA). Animals were fed a defined case in-based phytoestrogen-free diet (Product # D1010501, Research Diets, Inc.; New Brunswick, NJ) unsupplemented (control or 0 ppm) or supplemented with either BPA (2,2-bis(4-hydroxyphenyl)propane; CAS No. 80-05-7; Lot 11909; USEPA/NIEHS standard) or EE (1,3,5(10)-estratrien-17 α -ethinyl-3,17 β -diol; CAS No. 57-63-6; Batch No. H923; Steraloids Inc.; Newport, RI) that was homogenously incorporated in the diet at desired concentrations. Final dietary BPA concentrations (0.03, 0.3, 3, 30, and 300 ppm) resulted in doses of 4, 50, 500, 5000, and 50,000 μ g/kg/day, and concentrations of EE (0.0001, 0.001, and 0.01 ppm) resulted in a doses of 0.02, 0.2, 1 μ g/kg/day [17, 26]. Following a 2 week acclimation to the BPA-free system, study animals were maintained on assigned control or test diet *ad libitum* until necropsy at 19 to 23 weeks of age, for a total exposure period of 12 to 15 weeks. During this time, animals were bred and those that produced a litter were included in this study. Progression of estrous cycles was monitored by morphological analysis of vaginal lavage and necropsy was performed while in estrus. Estrous stage was confirmed by histological assessment of uterine tissues.

2.2 Histology and Immunohistochemistry

Tissue isolation, fixation and preparation were described in detail previously [17, 26]. Formalin fixed tissues were blocked for sectioning with a sterile razor blade and washed several times in 70% ethanol prior to automated tissue processing and embedding in paraffin (Histoplast IM; Richard Allen, Kalamazoo, MI). Uteri were placed in the block longitudinally to examine the entire length of the uterine horn, as well as the dorsal and lateral sides of the uterus. Microtome sections were cut at 5 μ m thickness from blocks at 4 °C and placed on positively charged slides for hematoxylin and eosin (H&E) staining and immunohistochemistry. Standard H&E staining was performed in order to examine tissue structure and morphology and to conduct histopathological analysis. For immunohistochemistry, sections were dewaxed in xylene and rehydrated through graded series of ethanol concentrations into phosphate buffered saline (PBS) for MMP2, F4/80, α -SMA, and ER α immunostaining or Tris-buffered saline with 0.1% Tween 20(TBST) for MMP14. Heat-mediated antigen retrieval was performed at 100°C in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% H₂O₂ in PBS. Sections were incubated at room temperature(RT) in 3% normal goat serum in PBS for 1 hour and then incubated overnight at 4°C with either rabbit polyclonal anti-MMP2 (3.33 μ g/mL (1:300); NB200-193, Novus Biologicals; Littleton, CO), rabbit monoclonal anti-MMP14 (0.5 μ g/mL (1:250); ab51074, Abcam; Cambridge, MA), rat monoclonal anti-F4/80 (C1:A3-1; 4 μ g/mL (1:250); ab6640, Abcam; Cambridge, MA), rabbit polyclonal anti- α -SMA (1 μ g/mL

(1:200); ab5694, Abcam; Cambridge, MA), or rabbit polyclonal anti-ER α (MC-20; 1 μ g/mL (1:200); sc-542, Santa Cruz Biotechnology; Dallas, TX). Immunoreactivity was visualized with 0.05% 3'-diaminobenzidine by the avidin-biotin peroxidase complex method (Vector Laboratories; Burlingame, CA). Sections were counterstained in Hematoxylin 2 (Richard Allen; Kalamazoo, MI) for 30 seconds, dehydrated, and coverslipped using Permount (Fisher Scientific; Fair Lawn, NJ). Mouse lung tissue was used as a positive control for MMP2 and MMP14, mouse spleen tissue was used as a positive control for F4/80, mouse heart tissue was used as a positive control for α -SMA, and rat mammary tumor was used as a positive control for ER α . Negative staining controls included replacement of primary antibodies with non-specific serum. Stained sections were examined on a Nikon Eclipse 55i microscope using a DS-Fi1 CCD camera controlled with Digital Sight Software (Nikon; Melville, NY). Final figures were generated using Adobe Photoshop (San Jose, CA).

2.3 Picrosirius Red Stain and Collagen Assessment

Paraffin-embedded uterine sections were stained with Picrosirius Red (Polysciences; Warrington, PA) to visualize total collagen (red) with bright field illumination and by polarization birefringence to assess the thickness and packing density of collagen fibers [73–75]. Briefly, tissue sections were deparaffinized, rehydrated, and stained for 8 minutes with Weigert's hematoxylin (American MasterTech; Lodi, CA). Stained sections were rinsed with tap water for 5 minutes, incubated for 2 minutes in 0.2% phosphomolybdic acid hydrate, and rinsed in deionized H₂O for 30 seconds. Slides were then stained for 1 hour in Picrosirius Red F3BA solution (1.3% 2,4,6-trinitrophenol, 0.4% Direct Red 80), transferred to 0.1 N hydrochloric acid solution for 2 minutes, washed in 70% ethanol for 45 seconds, dehydrated and then coverslipped. Stained sections were examined on a Nikon Eclipse 80i microscope equipped with a polarized light attachment and a DS-Fi1 CCD camera controlled with Digital Sight Software (Nikon; Melville, NY). Final figures were generated using Adobe Photoshop.

2.4 Quantitative RT-PCR

Frozen uterine tissue 30 mg from CD-1 control and 30 ppm BPA exposure groups were homogenized for RNA isolation using an RNeasy Fibrous Tissue Mini Kit (Qiagen; Valencia, CA). Uterine RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies; Grand Island, NY). Predesigned TaqMan RT-PCR assays (Applied Biosystems; Grand Island, NY) were used for the following genes: *Col1a1* (Mm00801632_gH), *Col3a1* (Mm00802296_g1), *Mmp2* (Mm00439498_m1), *Timp1* (Mm00441818_m1), *Timp2* (Mm00441825_m1), and *Timp3* (Mm00441826_m1). PCR amplification was performed in a final volume of 20 μ L containing 1x TaqMan expression assay primers, Universal Master Mix (Applied Biosystems; Grand Island, NY), and 10 ng of cDNA. Amplification was performed in triplicates on a Step One Plus Real-Time PCR System (Applied Biosystems; Grand Island, NY) under the following fast conditions: 20 seconds at 95°C for one holding stage, then 1 second at 95°C and 20 seconds at 60°C for the cycling stage (60 cycles). Relative expression was quantified using the Ct method, using *18s* as the endogenous control for normalization.

2.5 SDS-PAGE and Immunoblotting

Uterine lysates from frozen tissue were generated by homogenization in 20 mM Tris-HCl (pH 7.5) with 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton containing protease (Roche; Nutley, NJ) and phosphatase (Sigma-Aldrich; St. Louis, MO) inhibitors. Protein concentrations of cleared lysates were determined using the Bio-Rad Dc protein assay (Bio-Rad; Hercules, CA). Protein lysate samples (30 µg) were mixed 3:1 with sample buffer (0.25 M Tris-HCl, 40% glycerol, 8% SDS, 0.01% bromophenol blue, pH 6.8) containing 0.04% β-mercaptoethanol and incubated at 96°C for 5 minutes. Protein was loaded onto 10% polyacrylamide gel and resolved by electrophoresis. Proteins were electrotransferred to an Odyssey[®] nitrocellulose membrane (Li-Cor Biosciences; Lincoln, NE) for 16 hours at 4°C. Membranes were stained with 0.1% Ponceau S in 1% acetic acid to confirm homogenous protein transfer, destained in water and then blocked with 5% non-fat dry milk (Blotting Grade Blocker, Bio-Rad; Hercules, CA) in TBST for 1 hour at RT. Membranes were incubated overnight at 4°C with either rabbit polyclonal anti-MMP2 (0.25 µg/mL (1:4000); NB200-193, Novus Biologicals; Littleton, CO) or rabbit monoclonal anti-MMP14 (0.066 µg/mL (1:2000); ab51074, Abcam; Cambridge, MA). Blots were coincubated with a rabbit monoclonal anti-β-actin antibody (0.02 µg/mL (1:2000); 8457, Cell Signaling; Danvers, MA) for normalization of loading. Membranes were washed and incubated with IRDye[®] 800 CW goat anti-rabbit IgG (0.2 µg/mL (1:5000); Li-Cor Biosciences; Lincoln, NE) for 1 hour at RT. Membranes were imaged using an Odyssey[®] CLx Infrared Imaging System (Li-Cor Biosciences; Lincoln, NE) and immunoreactive bands were visualized and analyzed with Image Studio Lite Software (Li-Cor Biosciences; Lincoln, NE).

2.6 Gelatin Zymography

Methods for gelatin zymography were adapted from previously described protocols [76, 77]. Frozen uterine tissue was homogenized in 0.025 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1% Nonidet P-40 containing EDTA-free Protease Inhibitor Cocktail (Roche; Nutley, NJ) and cleared by centrifugation at 4°C. Protein concentration was determined using the Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA). Equal amounts of total protein (30 µg) were mixed 3:1 with sample buffer (0.25 M Tris-HCl, 40% glycerol, 8% SDS, 0.01% bromophenol blue, pH 6.8) and incubated at RT for 15 minutes. Protein was resolved onto native 10% polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, gels were washed with gentle agitation in 2.5% Triton X-100 at RT for 30 minutes, rinsed in dH₂O, and then washed for an additional 30 minutes at RT with gentle agitation in developing buffer (0.05 M Tris-HCl (pH 7.8), 0.2 M NaCl, 0.005 M CaCl₂). Washed gels were then incubated in fresh developing buffer at 37°C for 16 hours, stained with 0.5% Coomassie Brilliant Blue R-250 in 5% methanol and 10% glacial acetic acid at RT for 3 hours, and then destained at RT for consecutive washes of 15 minutes, 30 minutes, and 1 hour with 5% methanol and 10% acetic acid. Conditioned media from the human brain tumor cell line DAOY (ATCC HTB-186) was used as a positive control for MMP activity. In control experiments, the addition of EDTA (1 mM final concentration) to lysates or developing buffer resulted in loss of proteolytic activity at the expected molecular weights. Gels were imaged using an Alpha Innotech Multiimage II (Alpha Innotech; San Leandro,

CA) and optical density of cleared bands denoting areas of proteolytic activity were quantified using Image Studio Lite Software.

2.7 Statistical Analysis

Pathology was assessed by an observer blinded to exposure and strain with statistical differences analyzed using Fisher's exact test. Gland nest density was analyzed using a two-way ANOVA. Differences between control and exposure groups were analyzed using Dunnett's multiple comparison test and a Student's t-test was used for analysis of mRNA and protein expression and protein activity. Significance between differences in values was defined as $p < 0.05$. All data was analyzed using GraphPad Prism® v5/v6 software (GraphPad; La Jolla, California).

3. Results

3.1 Uterine Pathology: Gland Nests and Periglandular Fibrosis in C57Bl/6N and CD-1 Mice

A pathological assessment of a longitudinal section of the uterine horn between the oviduct and the cervix was conducted. The number of animals presenting with each pathology is summarized in Table 1. The uteri of control C57Bl/6N and CD-1 mice were characterized by a low incidence of uterine cysts. Uterine cysts were defined as hyperplastic glands surrounded by a thin layer of cuboidal epithelial cells. Distension of the glands and increased stromal proliferation was commonly observed (Table 1). Gland nests, characterized by multiple uterine glands surrounded by collagen producing stromal cells with resulting periglandular fibrosis and marked collagen accumulation (Fig. 1 arrows), were frequently observed in C57Bl/6N control and in each of the C57Bl/6N BPA and EE exposure groups (Table 1; Fig. 1). Compared to control, an increased intensity of red/yellow and decreased green polarization birefringence, characteristic of increased collagen fiber thickness and density, was associated with periglandular regions of stromal cells in the 30 ppm BPA and 0.001 EE exposure groups in C57Bl/6N mice (Fig. 1B; D; F). In contrast to C57Bl/6N controls, only a single gland nest-like structure was observed in one uterus from the CD-1 control group (Table 1). A significant increase in gland nests (Table 1; Fig. 2C, arrows) and extensive periglandular fibrosis was observed in the CD-1 30 ppm BPA exposure group (Fig. 2D) compared to control (Fig. 2A). In contrast to controls, intense red/yellow polarization birefringence, forming net-like patterns in areas between gland nest structures, was characteristic of the CD-1 30 ppm BPA exposure group (Fig. 2D). Gland nests were not present in the phenotypically estrogenized uteri of CD-1 mice exposed to 0.01 ppm EE (Fig. 2E–F), and notably lower levels of red/yellow and higher levels of green polarization birefringence were observed, suggesting a less dense packed collagen (Fig. 2F).

The density of gland nests in the endometrium for each strain and exposure group was calculated (Figure 3). Two-way ANOVA of gland nest density indicated significant main effects of both strain [$F(1,77) = 26.99$, ($p < 0.0001$)] and exposure [$F(5,77) = 4.411$, ($p = 0.0014$)] with no significant interaction between factors [$F(5, 77) = 1.493$, ($p = 0.2019$)]. The mean density of gland nest in the endometrium of control C57Bl/6N uteri ($M = 0.895 \pm SD 0.927$) was 9 times greater than the density observed in the CD-1 strain ($M = 0.101 \pm SD 0.349$; Fig. 3A). Gland nest density of neither C57Bl/6N [$F(3, 15) = 1.568$, ($p = 0.2385$)] nor

CD-1 uteri [$F(3, 38) = 0.6947$, ($p=0.5610$)] was affected by EE exposure. Exposure to 30 ppm BPA significantly increased endometrial gland nest density in the CD-1 strain (Fig. 3B) and significant increases in density were detected in 0.03 ppm BPA group and in the two highest BPA groups in the C57Bl/6N strain (Fig. 3C). The increased density detected in the lowest exposure group and the dose dependent increase in gland nest density observed across the 0.3 to 300 ppm dose range is suggestive of a non-monotonic dose response relationship. The significant increase in gland nest density in the 30 ppm BPA CD-1 mice correlated with the increased number of animals observed to have gland nests present in Table 1. In C57Bl/6N mice, however, gland nest density also increased in response to BPA whereas the number of animals with gland nests was not changed (see Table 1).

3.2 Analysis of Collagen and MMP mRNA expression

The extensive periglandular fibrosis and increased levels of collagen observed in the CD-1 30 ppm BPA exposure group were suggestive of increased collagen expression and/or alterations in regulation of collagen fibril accumulation. The relative expression levels for *Colla1*, *Col3a1*, *Mmp2*, *Timp1*, *Timp2*, and *Timp3* mRNA in uterine tissues from CD-1 mice in the control (0) and 30 ppm BPA (30) exposure groups were quantified using qRT-PCR (Fig. 4A). Compared to control, significant increases in *Colla1* ($p=0.0379$) and *Col3a1* ($p=0.0161$) and a significant decrease ($p=0.0162$) in *Mmp2* mRNA expression levels were detected in uteri from CD-1 mice in the 30 ppm BPA exposure group. While *Timp1* was not altered by BPA exposure, *Timp2* was significantly decreased ($p = 0.0013$) in the 30 ppm BPA exposure group compared to control. Because of its broad inhibitory spectrum and more variable regulation in the uterus [28], *Timp3* expression was also compared in the uterus of control and 30 ppm BPA exposure groups and was found to be unchanged by BPA exposure.

3.3 Analysis of Protein Expression and Activity of MMPs

Western blot analysis was used to determine if protein expression of MMP2 (Fig. 4B) and MMP14 (Fig. 4C) in uteri from the C57Bl/6N control group and CD-1 30 ppm BPA exposure group were decreased compared to unexposed CD-1 controls, because these groups displayed significant differences in gland nest formation and collagen accumulation compared to CD-1 control animals. Relative to unexposed CD-1 controls, decreased levels of proMMP2 and MMP2 protein levels were detected in both CD-1 30 ppm BPA and unexposed C57Bl/6N groups (Fig. 4B). Quantification found the decrease in expression of proMMP2 ($t(9) = 3.014$, $p=0.0073$) and MMP2 ($t(8) = 2.397$, $p=0.0073$) in CD-1 30 ppm BPA and proMMP2 ($t(7) = 3.404$, $p=0.0057$) and MMP2 ($t(6) = 1.971$, $p=0.0481$) in C57Bl/6N controls was significant (Fig. 4D). The decreased expression of MMP2 was reflected by relative decreases in MMP2 gelatinase activities of the CD-1 30 ppm BPA exposure group and C57Bl/6N control group as compared to unexposed CD-1 controls (Fig. 4E). The relative decreases in proMMP2 ($t(10) = 5.486$, $p=0.0003$) and MMP2 ($t(6) = 4.610$, $p=0.0037$) activity in the CD-1 30 ppm BPA exposure group and proMMP2 ($t(9) = 5.789$, $p=0.0003$) and MMP2 ($t(5) = 6.357$, $p=0.0007$) activity in C57Bl/6N controls were significant (Fig. 4E). As expected [28], the relative activity of MMP9 in C57Bl/6N and CD-1 uterus were not significantly different ($t(4) = 0.0906$, $p=0.9322$). Decreased levels of proMMP14 and MMP14 protein levels were also detected in both CD-1 30 ppm BPA and

unexposed C57Bl/6N groups (Fig. 4C). Expression of proMMP14 in CD-1 30 ppm BPA ($t(9) = 2.056$, $p=0.0350$) and C57Bl/6N control ($t(7) = 4.367$, $p=0.0016$) groups was significantly decreased. While it appeared that relative expression of MMP14 was also decreased, results were not statistically significant due to the high levels of variance associated with quantifying the more diffusely migrating MMP14 immunoreactive b and observed in the CD-1 controls (Fig. 4C; F).

3.4 Immunolocalization of MMP2 and MMP14 in the endometrium

In the endometrium of the CD-1 control group, intense positive staining for MMP2 was found throughout the stroma, as well as in the cytoplasmic compartment of luminal and glandular epithelial cells (Fig. 5A). Intense staining of stromal cells was more variable with moderate to high intracellular staining and more diffuse staining in extracellular regions (Fig. 5A). Expression did not appear altered in stroma closer to the glandular or luminal structures.

In C57Bl/6N controls, the cell specific staining pattern in the endometrium was similar, but comparatively less intense (Fig. 5B). Immunoreactivity for MMP2 was also weaker in the cytoplasm of glandular and luminal epithelial cells, reflecting the overall decreased expression levels of MMP2 in the C57Bl/6N uterus. Intracellular MMP2 staining of stromal cells was generally weak and further reduced to background levels in the periglandular stroma surrounding the gland nest (Fig. 5B; arrows).

Immunoreactivity for MMP14 was weak to moderate in the intracellular compartment of stromal cells in endometrium of both CD-1 and C57Bl/6N controls. In the CD-1 endometrium, mainly scattered stromal cells surrounding glands were MMP14 immunopositive, and glandular and luminal epithelial cells were immunonegative (Fig. 5F). In the C57Bl/6N endometrium, intracellular immunostaining was weak to moderate and more diffusely granular in appearance and notably less intense periglandular staining was typical (Fig. 5G; arrow). Weak to moderate immunostaining was detectable in the borders of luminal epithelial cells.

3.5 Immunohistochemical Characterization of an Endometriosis-Like Phenotype

Immunohistochemistry using antibodies against F4/80, α -SMA, and ER α was performed on uterine sections from the 0 ppm CD-1, 30 ppm BPA CD-1, and 0 ppm C57Bl/6N groups to further characterize the effects of BPA exposure or strain on the observed endometriosis-like phenotype (Fig. 6). An increase in F4/80 positive macrophages, indicative of a generally increased immune response, surrounding gland nests and throughout the stroma was found in the 30 ppm BPA CD-1 exposure group (Fig. 6D) compared to the 0 ppm CD-1 group (Fig. 6A). In the 0 ppm C57Bl/6N group higher levels of F4/80 positive macrophages were observed in the stroma surrounding gland nests (Fig. 6G) as compared to the stroma surrounding glands in the 0 ppm CD-1 group. No staining for α -SMA was observed in periglandular stromal cells surrounding gland nests in the 30 ppm BPA CD-1 exposure group (Fig. 6E) or the 0 ppm C57Bl/6N group (Fig. 6H). Endothelial cells surrounding blood vessels in the stroma were α -SMA immunopositive as expected. Staining for ER α was localized to the nuclear regions in the glandular epithelium or stromal cells and relative

levels do not appear altered by BPA exposure (Fig. 6F) or strain (Fig. 6I) compared to 0 ppm CD-1 (Fig. 6C).

4. Discussion

Bisphenol A is an EDC with known pathophysiologic activity in the uterus; little however is known about the relationship between BPA exposure and fibrosis, a common feature of several uterine pathologies. In this study, the presence of periglandular-associated stromal cells and fibrosis surrounding clusters or “nests” of endometrial glands, a hallmark of equine endometriosis, was found to commonly occur in the uterus of C57Bl/6N mice. To our knowledge, this is the first report of an endometriosis-like phenotype in the murine uterus. The histological features of the observed EPF and gland nest phenotype suggests that the C57Bl/6N strain may be a useful “off the shelf” experimental model for understanding the etiology and pathogenesis of progressive increases in periglandular fibrosis and chronic endometrial degeneration in horses. The decreased reproductive efficiencies and loss of fertility caused by equine endometriosis in mares suggests that the endometriosis-like phenotype may play a role in the decreased average litter size characteristic of C57Bl/6N (6.5 pups) compared to CD-1 mice (11 pups) [35, 78]. Whereas age is the most important factor related to onset of equine endometriosis in mares [36, 39], the observed increases in density and incidence of gland nests, as well as increased periglandular fibrosis resulting from BPA exposure in both strains of mice suggest that genetic and endocrine disruptive effects contribute to alterations in stromal collagen accumulation and the development of endometriosis.

The horse and the mouse have distinct similarities and differences in regards to uterine biology that need to be taken into account when considering the mouse as a model to study a disease mainly found in equine populations. Adenogenesis in both species continues postnatally and can be inhibited by progesterone treatment [79–81]. In both species, adenogenesis results in simple tubular gland structures [82]; however, glands eventually branch in mares [79], whereas they remain simple tubular structures in mouse dams. Regarding estrous cyclicity, the two species display distinct patterns. While mice are polyestrous throughout the year, cyclicity in mares is seasonal, occurring from late April to August. Outside of those months, mares remain in a state of anestrus [83, 84]. In order for parturition to occur in both species, glands and their secretions are crucial for blastocyst implantation, decidualization, and fetal survival as implantation does not occur in mares until day 35 postovulation [82, 85], with implantation occurring on day 4.5 in mice [86]. Postpartum, the uterus involutes and returns to normal cyclicity within ten days for both species [82, 87, 88]. Differences aside, the important similarities in uterine biology between the two species suggest that the mouse may be a useful model to study equine disease.

In response to BPA exposure gland nest density in both mouse strains examined here showed a non-monotonic dose response relationship commonly observed for EDCs. These complex dose-response relationships can be a result of several separate, yet simultaneously acting, mechanisms of action [89]. Estrogens and BPA are known to act with numerous receptor mediated pathways, either through classical nuclear receptor or rapid signaling mechanisms [90, 91]. While BPA has estrogen-like activity at ER α and ER β at nM concentrations [92], it

can also bind to and inhibit the androgen and thyroid hormone receptors at μM concentrations [93–96] to cause oppositional effects at low and high dose ranges. BPA can also bind to elicit multiple responses through binding of a single receptor, as shown in developing neurons and rat cardiomyocytes [77, 97] and partial agonist-like activity of BPA at $\text{ER}\alpha$ in the uterus is well established. For example BPA can increase progesterone receptor (PR) expression in the immature rat uterus, but also antagonizes E2-induced stimulation in PR expression [98]. Further study is required to elucidate the nature of the multiple regulatory mechanisms responsible for the differential effects of BPA on gland nest density that were observed at low and high BPA doses, and the nature of the baseline differences observed between the CD-1 and C57Bl/6N strains of mice.

Excessive collagen accumulation and fibrosis in endometrial tissues is associated with the progression of numerous diseases of human uterus tissues, most notably leiomyomas. Leiomyomas, or uterine fibroids, are one of the most common benign pathologies to affect women of reproductive age [99]. The connection between fibrosis and alterations in ECM signaling in uterine fibroids is well established [100]. During the proliferative phase, mRNA for collagen I and III is overexpressed in uterine fibroids compared to surrounding tissue, suggesting that ECM components are under hormonal control within the fibroid [32, 101]. Exposure to BPA and the development of leiomyomas has also been experimentally established, since exposure led to the presence of leiomyomas in murine uteri at 18 months of age [23], and *in vitro* studies in human UL uterine fibroid cells found that BPA increased proliferation and increased mRNA and protein expression of $\text{ER}\alpha$, IGF-1 and VEGF [102]. The marked increase in collagen accumulation induced by BPA in our study further supports the possibility that BPA exposures may contribute to fibrotic uterine diseases and has defined dysregulation of collagen accumulation dynamics as a plausible mechanism of BPA actions. It is notable that the doses of BPA that led to alterations in collagen accumulation observed here are below the defined NOAEL for BPA and that increases in gland nest density in the C57Bl/6N strain occurred at exposures below what is considered safe for humans [103].

Proteins involved in collagen synthesis and degradation are transcriptionally controlled throughout the estrous or menstrual cycle [28, 45]. To assess the relationship between BPA exposure and changes in collagen synthesis and degradation, we first quantified mRNA expression of representative genes involved in these processes at an exposure level (30 ppm) in CD-1 mice that led to clear increases in gland nest formation and collagen accumulation. While it is known that E2 can increase mRNA levels of collagens and MMPs in the uterus [47, 49], exposure to BPA also resulted in increased *Col1a1* and *Col3a1* expression and decreased *Mmp2* expression. In relation to collagen synthesis, BPA acted similarly to E2 [47]. However, BPA had an effect opposite of E2 on genes involved in collagen degradation as E2, a finding in support of BPA having antagonist-like actions that are interfering with the normal function of E2 in the uterus. These findings point toward an imbalance between collagen synthesis and degradation leading to an overall fibrotic phenotype.

Protein expression and activity of MMPs were quantified in order to assess the effect of exposure and strain. Exposure to BPA significantly decreased the expression and activity of latent and active forms of MMP2 and expression of the latent form of MMP14. The

C57Bl/6N strain also had significantly decreased latent and active MMP2 expression and activity and latent MMP14 expression compared to the CD-1 strain. These results coincide with the mRNA expression results, supporting that BPA decreased *Mmp2* mRNA expression leading to a decrease in protein expression. Decreased expression of proMMP14 and a trend toward decreased expression of the active form add another layer of complexity to the dysregulation of collagen degradation in the BPA-exposed CD-1 uteri or the control C57Bl/6N uteri. A lower level of MMP14 may also lead to a decrease in activation of MMP2, which would contribute to an overall increase in collagen accumulation in the uterus.

The diffuse MMP2 immunostaining in the stromal compartment is similar to previous observations in equine endometrosis [104]. One difference between equine endometrosis and the pathology observed in the C57Bl/6N and CD-1 strains was a lack of MMP2 localization to the glandular and luminal epithelial cells in the mouse endometrium. Also similar to equine endometrosis [105], an increased immune response associated with gland nests was observed, which may have a role in the development of the phenotype. In equine endometrosis, the periglandular stromal cells can differentiate into myofibroblasts that express α -SMA [106]. Differences in ER α staining and localization may also indicate an active or inactive phenotype [107]. We did not observe α -SMA staining in the periglandular stromal cells or differences in ER α staining and localization, further supporting the interpretation that gland nests present in these young adult mice represent an early stage of pathology prior to loss of fertility. Thus, the C57Bl/6N mouse may serve as a small animal model for examining the etiology and progression of equine endometrosis and also may be useful in determining the role of EDCs in this disease.

We previously found that life-long BPA exposure resulted in sex-specific pathologic changes in the ECM of the CD-1 mouse heart [27]. Along with those sex-specific responses, differential responses to BPA exposure based on strain or genetic background have also been reported [26, 68]. In the uterus, BPA or EE can significantly increase immune responses in C57Bl/6N mice resulting in development of pyometra, yet this pathology was not observed in CD-1 mice [26]. Related strain-specific responses to BPA have also been reported in other estrogen-responsive tissues [61, 65, 108]. In this study, a distinct strain difference in the response to BPA was observed, as well as differences between the control and exposure groups of mice in each strain. Strain differences may help to explain the etiology of endometrosis. C57Bl/6N control mice readily exhibit a high level of gland nests and periglandular fibrosis, signifying a potentially unrecognized underlying genetic component to progression of this phenotype, whereas BPA exposure in the CD-1 strain was required to induce gland nest development through mechanisms presumably involving dysregulation of collagen accumulation. Potential differences in baseline levels of ovarian hormones, ER and PR expression, or cellular responsiveness to E2 or progesterone could be playing a role in the differential development of an endometrotic-like phenotype observed in the CD-1 and C57Bl/6N strains [63, 64, 69, 70].

In conclusion, BPA may act as a partial ER agonist to inhibit actions of E2 and result in collagen dysregulation and potentially fibrosis in the uterus. Up until now, little was known about the impact of BPA exposure on the regulation of collagen in the uterus. This study

informs that BPA exposure increases gland nest formation and stromal and periglandular collagen accumulation in both CD-1 and C57Bl/6N mouse strains. In relation to collagen synthesis, BPA acts similarly to E2 by increasing mRNA expression levels of *Colla1* and *Col3a1*. However, BPA exposure has the opposite effect on collagen degradation as would be expected from an exposure to E2. *Mmp2* mRNA expression, protein expression levels of MMP2 and MMP14 and activity of MMP2 were decreased in BPA-exposed CD-1 mice and control C57Bl/6N mice compared to control CD-1 mice. These decreases in MMP expression and activity may result in decreased collagen degradation and lead to an accumulation of collagen in the stromal and periglandular compartments of the endometrium. Over time, this accumulation may lead to fibrosis, a component of several uterine diseases that affect humans and animals, suggesting that exposure to EDCs may play a role in the development or progression of those diseases. This study is the first report of an endometriosis-like phenotype in the murine uterus, as well as the first report of an EDC leading to the presence of this phenotype in any animal model. Based on these results, the C57Bl/6N strain may serve as an experimental animal model for studying the etiology and progression of equine endometriosis, as well as determining the effects of exposures to EDCs on this disease and other uterine pathologies.

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Abbreviations

EE	17 α -ethinyl estradiol
E2	17 β -estradiol
BPA	bisphenol A
DES	diethylstilbestrol
EDC	endocrine disrupting chemical
EPF	endometrial periglandular fibrosis
ECM	extracellular matrix
MMP	matrix metalloproteinase
NOAEL	no observed adverse effect level
TIMPs	tissue inhibitors of metalloproteinases

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Highlights

- Oral exposure to bisphenol A (BPA) in adulthood altered endometrial remodeling and pathology in the uterus of adult CD-1 and C57Bl/6N mice
- BPA increased gland nest density and periglandular collagen accumulation characteristics of an equine endometriosis-like phenotype
- The equine endometriosis-like phenotype was observed in unexposed C57Bl/6N endometrium
- Endometriosis-like phenotype and BPA's effects were associated with increased collagen I and III expression and decreased matrix metalloproteinase 2 (MMP2) and MMP14 expression and MMP2 activity

Figure 1. Effects of oral exposure to BPA or EE on uterine morphology and collagen accumulation in C57Bl/6N mice

Shown are representative photomicrographs of H&E- and Picrosirius Red-stained uterine sections from a control C57Bl/6N mouse (A–B) and from a mouse exposed to 30 ppm BPA (C–D) or 0.01 ppm EE (E–F). Similar uterine locations are highlighted in each panel. Arrows designate gland nests. Scale bar represents 50 μ m.

Figure 2. Effects of oral exposure to BPA or EE on uterine morphology and collagen accumulation in CD-1 mice

Shown are representative photomicrographs of H&E- and Picrosirius Red-stained uterine sections from a control CD-1 mouse (A–B) and from a mouse exposed to 30 ppm BPA (C–D) or 0.01 ppm EE (E–F). Similar uterine locations are highlighted in each panel. Arrows designate gland nests. Scale bar represents 50 μ m.

Figure 3. Quantification of gland nest density in the uteri of control and BPA-exposed CD-1 and C57Bl/6N mice

Comparison of gland nest density between the control groups of CD-1 and C57Bl/6N mice (A), control and BPA-exposed CD-1 mice (B), and control and BPA-exposed C57Bl/6N mice (C). The number of gland nests was counted by an observer blinded to strain and exposure group. Values indicated are the number of gland nests per mm² per animal. Error bars represent the SEM. For C57Bl/6N, $n = 3$ animals and for CD-1, $n = 8$ animals. The level of statistical significance between values was assessed using a two-way ANOVA, in which exposure and strain were the main effects. * indicates a level of difference between values is significant ($p < 0.05$); ** indicates a level of difference between values is very significant ($p < 0.01$).

Figure 4. Relative expression levels and activity of mRNA and proteins involved in collagen synthesis and degradation

Quantitative RT-PCR analysis between control (0) and BPA-exposed (30) CD-1 mice for *Col1a1*, *Col3a1*, *Mmp2*, *Timp1*, *Timp2*, and *Timp3* (A). The relative differences in gene expression between control and exposed groups were determined by the cycle threshold method with specific TaqMan assays. Representative Western blots for proMMP2 and MMP2 (B) and proMMP14 and MMP14 (C) are shown for control CD-1 and C57Bl/6N mice and 30 ppm BPA-exposed CD-1 mice. Relative proMMP2 and MMP2 protein expression levels (D) and activity (E) and protein expression levels of proMMP14 and MMP14 (F) are shown. Protein expression levels are based on Western blot analysis and activity is based on gelatin zymography as described in Methods. Results are expressed as mean of group $\pm$ SEM. Expression and activity of BPA-exposed CD-1 mice and control C57Bl/6N mice is graphed in relation to control CD-1 results for all experiments. Control CD-1 mice were compared to BPA-exposed CD-1 mice and control C57Bl/6N mice separately. For qRT-PCR, $n = 4$ animals. For protein expression and activity, $n = 4$ animals for CD-1 mice and $n = 3$ animals for C57Bl/6N mice. The level of statistical significance for differences between mean values of control and BPA-exposed groups was determined by Student's t test for all experiments and is indicated by * ($p < 0.05$).

Figure 5. Strain differences in the immunohistochemical localization of MMP2 and MMP14 in the uteri of control CD-1 and C57Bl/6N mice

Shown are representative photomicrographs of uterine sections stained for MMP2 in CD-1 (A) and C57Bl/6N (B) mice and for MMP14 in CD-1 (F) and C57Bl/6N (G) mice. Arrows in B and G point to periglandular areas with decreased staining. Control for specificity of immunostaining included the replacement of primary antibody with non-specific goat serum for MMP2 (C) and MMP14 (H). Mouse lung tissue was used as a positive control for immunostaining for MMP2 (D) and MMP14 (I), with the replacement of primary antibody with non-specific goat serum for MMP2 (E) and MMP14 (J) used to control for specificity of staining. Scale bar represents 50 μ m.

Figure 6. Immunohistochemical characterization of endometriosis-like phenotype between BPA exposure groups or strain

Shown are representative photomicrographs of uterine sections from 0 ppm CD-1 mice (A–C), 30 ppm BPA CD-1 mice (D–F), and 0 ppm C57Bl/6N mice (G–I) stained for F4/80 (A, D, G), α -SMA (B, E, H), or ER α (C, F, I). Control for specificity of immunostaining included the replacement of primary antibody with non-specific goat serum for F4/80 (A, inset), α -SMA (B, inset), and ER α (C, inset). As a positive control for immunostaining, mouse spleen tissue was used for F4/80 (D, inset), mouse heart tissue was used for α -SMA (E, inset) and rat mammary tumor was used for ER α (F, inset). Scale bar represents 50 μ m.

Table 1

Incidence of Uterine Pathology in CD-1 and C57Bl/6N Mice at 19–23 Weeks of Age

		BPA (ppm)				EE (ppm)			
		0	0.03	0.3	3	30	300	0.0001	0.001
CD-1		0	0.03	0.3	3	30	300	0.0001	0.001
n=		12	9	10	10	8	10	10	10
Gland Nests		1 (8%)	0	2 (20%)	0	6 (75%) ^a	3 (30%)	4 (40%)	0
Distended Glands		7 (58%)	7 (78%)	6 (60%)	6 (60%)	6 (75%)	8 (80%)	8 (80%)	8 (80%)
Cysts		1 (8%)	1 (11%)	2 (20%)	2 (20%)	1 (13%)	0	0	0
C57Bl/6N									
n=		7	7	7	4	6	3	4	3
Gland Nests		5 (71%)	6 (86%)	5 (71%)	4 (100%)	5 (83%)	3 (100%)	3 (75%)	2 (67%)
Distended Glands		6 (86%)	5 (71%)	4 (57%)	3 (75%)	4 (67%)	2 (67%)	1 (25%)	2 (67%)
Cysts		0	2 (29%)	3 (43%)	2 (50%)	0	1 (33%)	1 (25%)	1 (33%)

^a p = .0044 by two-tailed Fisher's exact test