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Bisphenol A impairs decidualization of human uterine stromal fibroblasts

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ABSTRACT

This study examined the effect of bisphenol A (BPA) exposure on human uterine stromal fibroblast cells (HuF) undergoing decidualization. HuF cells were isolated and cultured for eight days in the presence of a decidualization-inducing cocktail, while concurrently exposed to physiological and supra-physiologic doses of BPA (1 ng/mL, 10 ng/mL, 0.5 μg/mL, 10 μg/mL and 20 μg/mL). Decidualization markers, steroid hormone receptors and cell cycle gene expression were detected by qRT-PCR and cellular proliferation was assessed by KI-67 immunofluorescent staining and MTS assay. BPA impaired decidualization at 10 μg/mL and 20 μg/mL, but not at lower doses. Additionally, BPA at 20 μg/mL decreased progesterone receptor and estrogen receptor-alpha compared to controls. The highest dose of BPA also reduced cellular proliferation and cyclin D2 expression compared to controls. These findings demonstrate that BPA disrupts *in vitro* decidualization of uterine stromal fibroblasts by altering steroid hormone receptor expression at higher concentrations but not at lower physiological doses.

1. Introduction

BPA is a known endocrine disruptor which is an extensively used chemical in the production of epoxy resins, polycarbonate plastics and flame retardants [1]. Globally, BPA production is increasing at substantial rates. Nearly 5.5 million tons were produced in 2011, compared to 3.2 million tons in 2003 [2,3]. In 2007, the United States accounted for approximately 30% of the global production of BPA [4]. BPA can leach from these products and enter into the human body by ingestion and absorption through direct contact with BPA or contact with products containing BPA such as metal food cans, dental sealants and plastic drinking containers [1]. Therefore, humans are constantly exposed to BPA and a recent study detected BPA in 95% of human urine samples tested [5]. As an endocrine disruptor, BPA can bind to estrogen receptor-alpha and −beta and can exhibit weak estrogenic activity, but it is potency ranges from 10 to 10,000 fold less than estradiol [6,7]. Due to this action and other significant evidence, BPA was recently classified as a female reproductive toxicant by a panel of experts in the field [8].

Epidemiological studies have linked BPA exposure to pregnancy loss and a higher incidence of polycystic ovarian syndrome, endometriosis and implantation failure in women [9–12]. Furthermore, BPA exposure has been extensively tested in rodent models and *in vitro* studies which confirm BPA's role as a selective estrogen receptor modulator [8].

The female reproductive system and specifically the uterus is highly sensitive to changes in sex hormone concentrations as the endometrium proliferates and differentiates each month in response to estrogen and progesterone, respectively. During the proliferative stage, estrogen receptor-alpha and progesterone receptor are expressed in the endometrial epithelium and stromal compartments. However, around the mid- to late-secretory stage, estrogen receptor-alpha and progesterone receptor expression are absent in the epithelium, but retained in the stroma [13]. Progesterone receptor A is the predominant progesterone receptor isoform in the uterus and is also highly expressed throughout pregnancy [14]. Aberrant expression of estrogen receptor-alpha and progesterone receptor in the female uterus can lead to infertility, recurrent pregnancy loss and endometrial hyperplasia [15].

Decidualization is the process of stromal cell differentiation in the endometrium that must occur during a successful pregnancy. The decidual cells create an environment that facilitates embryo attachment and placental development [16]. In humans, this process is under maternal control, meaning that decidualization occurs

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every cycle in the mid- to late-secretory phase due to endocrine and paracrine regulation. Decidual cells are characterized by rounded nuclei, a large cytoplasmic area, significant extracellular matrix production and gap junction formations [17]. Prolactin and IGFBP-1 are produced and secreted by decidual cells and are considered markers to assess the status of stromal cells decidualizing *in vitro* [16].

Effective decidualization relies upon a harmonized coordination of hormone, hormone receptor and paracrine factors and the perturbation of any of these factors may lead to failed decidualization and ultimately pregnancy loss [16].

Previous studies demonstrated that BPA reduced proliferation and altered expression of hallmark genes associated with decidualization in human stromal fibroblasts [18,19]. One study tested doses of BPA above known physiological exposure levels [18] and the other study tested a range of physiological and supra-physiological doses, but only observed significant effects of decidualization at doses above physiological exposure levels [19]. In the current study, we questioned whether exogenous BPA exposure would impair the process of decidualization in uterine stromal cells, as previous studies only assessed BPA's effect on stromal fibroblasts which were pre-decidualized. To address this question, we treated HuF cells (human uterine stromal fibroblasts) during the process of decidualization with vehicle, physiological or supra-physiological doses of BPA and examined the expression of genes associated with decidualization and cell cycle regulation and evaluated several markers of proliferation.

2. Materials and methods

2.1. Reagents

17-β – estradiol (lot # 021M8707 V), medroxyprogesterone acetate (lot# K1293) and dibutyryladenosine cyclic monophosphate (lot # SLBK3830 V) (cAMP) were purchased from Sigma Aldrich (St. Louis, MO). Bisphenol A (99% purity) was obtained as a gift from the National Institute for Environmental Health Sciences to JF. RNA and qPCR reagents including trizol, high capacity cDNA synthesis kit and Power SYBR green PCR master mix were purchased from Life Technologies (Grand Island, NY). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI) and the KI-67 antibody was purchased from BD Pharmingen (Ref. 558616; San Jose, CA).

2.2. HuF cell isolation

Human uterine stromal fibroblasts were isolated from the decidual parietalis of the placental membrane after normal vaginal delivery. The isolation and culture of HuF cells was performed as previously described and each cell line was grown individually and treated with between passages 4–5 [20]. Each cell line (n = 4) is representative of proliferative, undifferentiated stromal fibroblasts. Placental tissues, used to harvest HuF cells, were obtained with informed consent under Institutional Review Board approved protocols at Michigan State University and Spectrum Health System.

2.3. Decidualization and bisphenol A treatment

HuF cells were cultured in RPMI1640 media supplemented with 10% charcoal dextran stripped (CDS) FBS until they reached 70–80% confluence. Subsequently, media supplemented with 2% CDS FBS was used for the eight day treatment period. During the treatment, media was changed every two days. All cells were treated with 17-β – estradiol (36 nM), medroxyprogesterone acetate (1 μM) and dibutyryl cyclic adenosine monophosphate (0.5 mM) (EPC). This hormonal cocktail treatment has been used extensively for *in vitro* decidualization in our laboratory [20–22]. HuF cells were additionally treated with vehicle or one of several doses of BPA (1 ng/mL, 10 ng/mL, 0.5 μg/mL, 10 μg/mL and 20 μg/mL or 4.38 nM, 43.8 nM, 2.19 μM, 43.8 μM and 87.6 μM, respectively) dissolved in 100% molecular grade ethanol in conjunction with the eight-day EPC treatment. The doses of BPA used in this study were selected based on previous *in vitro* studies [18,19,23,24]. The doses used were similar to and higher than reported physiological bio-fluid concentrations measured in the population [25]. The higher doses tested in this study may provide insight into the mechanisms involved with very high BPA exposure among industrial workers. In addition, it is difficult to mimic the BPA exposure scenario in humans because exposure is ubiquitous and exposure levels vary significantly within the population. BPA is also metabolized very rapidly in the body making it difficult to accurately quantify exposure [25].

2.4. RNA isolation and quantitative real-time PCR

After completion of the eight day treatment period, HuF cells were washed three times with 1X PBS and 0.5 mL of Trizol reagent was added to each well and the manufacturer's protocol was followed for the isolation of RNA. Next, 1 μg of RNA was converted to cDNA using the protocol outlined in the High Capacity cDNA synthesis kit. For qPCR, 10 ng of cDNA template was combined with primer pairs for the gene of interest (sequences listed in **supplemental** Table 1) and PowerSYBR master mix. The reaction was completed per the manufacturer's specifications and performed on Viia7 Real-time PCR machine (Life Technologies).

2.5. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay

HuF cells were cultured for eight days in 96 well plates and after the completion of treatment, 100uL of MTS reagent was added to each well and the plate was incubated for two hours at 37 ◦ Celsius. After incubation, the plate was read at an absorbance of 490 nm to determine the degree of proliferation.

2.6. Immunocytochemistry

HuF cells were cultured on 4-well chamber slides and were treated for eight days as described above. Briefly, the cells were washed three times with 1X PBS and fixed with 4% Paraformaldehyde for 15 min. After another series of washes with 1X PBS, cells were permeabilized with 0.1% Triton X-100 in 1X PBS for 10 min. Next, cells were rinsed again with 1X PBS and were blocked for 30 min with 5% NGS in 1X PBS. After blocking, 100 μL of antibody conjugated to Alexa Fluor 488 was added for 1 h. Vectashield with DAPI was added to stain the nuclei of the cells and images were captured using confocal microscopy at 488 nm.

2.7. Statistics

Data in the results and figures are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 5.0 (La Jolla, CA) One-way ANOVA was used to test the null hypothesis of differences between groups, followed by a Student's *t-test* for comparison between group pairs. $P < 0.05$ was considered statistically significant.

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