# Developmental Programming: Prenatal Bisphenol A Treatment Disrupts Mediators of Placental Function in Sheep

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## Abstract

Gestational Bisphenol A (BPA) exposure is associated with low birth weight. We hypothesized that the low birth weight is the consequence of reduced placental efficiency and a function of BPA-induced inflammatory, oxidative, lipotoxic, angiogenic, steroidal and fibrotic changes involving epigenetic alterations. Placentomes were collected during early (day 65) and mid (day 90) gestation (term ~147 days) from control and BPA (gestational day 30–90)-treated pregnant sheep. BPA treatment: reduced placental efficiency and fetal weight; increased interleukin 8, lipid peroxidation marker, antioxidants, aromatase, 17 alpha-hydroxylase, estrogen receptor 2, insulin like growth factor (IGF) 2 receptor and IGF binding proteins (IGFBP), and histone deacetylase 1 and 2; reduced tumor necrosis factor alpha and IGF1 receptor at early gestation (Day 65). Gestational BPA-induced mid-gestational changes include: reduced angiogenic factor hypoxia inducible factor 1 alpha; increased IL1beta, oxidative stress markers, triglyceride, 17alpha hydroxylase, IGFBP 1, DNA methyltransferase 3A and histone deacetylase 1. These findings indicate that gestational BPA, either acting directly or by altering steroidal input, produces early/mid-gestational-specific epigenetic changes culminating in placental disruptions at several levels, in keeping with time-specific/time-lagged pregnancy-associated changes in placental efficiency and fetal weight. The reduced early-gestational placental efficiency may be a function of increased inflammation/oxidative stress and reduced IGF bioavailability with the mid-gestational restoration of placental efficiency likely driven by improved IGF bioavailability and the time-lagged response to antioxidant increase. This compensation, the result of time-lagged response to increases in negative mediators of placental function must have failed with pregnancy advancement to explain the low birthweight outcome.

## Keywords

female reproduction; Endocrine disruptors; oxidative stress; inflammation; angiogenesis; lipotoxicity; epigenetics

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## Introduction

Developmental insults resulting from disease state, stress, nutritional excess/deficiency or environmental factors, can lead to poor birth outcomes such as intrauterine growth restriction (IUGR), preterm birth and low birth weight, risk factors for adult onset diseases as posit by developmental origin of health and disease (DoHaD) hypothesis (Barker, 2004). The increase in the incidences of the non-communicable diseases coinciding with the rapid industrialization over the 20th century (Neel and Sargis, 2011) has resulted in environmental chemicals especially those with endocrine disrupting potential being viewed as major developmental insults. National Health and Nutrition Examination Survey (NHANES) conducted by Centers for Disease Control and Prevention shows that most Americans have detectable levels of many such environmental endocrine disrupting chemicals (EDCs) (CDC, 2014). Bisphenol A (BPA), one such EDC, is found in >90% of the US population including pregnant women (Calafat et al., 2005; Calafat et al., 2008). Commonly found in many household plastics, BPA in addition to having estrogenic (Vom Saal, 2016) and antiandrogenic (Lee et al., 2003a) properties has been shown to disrupt thyroid hormone action (Ahmed, 2016) and enhance insulin secretion (Alonso-Magdalena et al., 2006). Several human cohort and animal studies have linked gestational exposure of BPA to adverse birth outcomes (Savabieasfahani et al., 2006; Lee et al., 2014; Peretz et al., 2014; Veiga-Lopez et al., 2015a).

Considering that placenta serves as the conduit of nutrients to the fetus (Wooding and Burton, 2008), any disruption in placental homeostasis could affect fetal growth trajectory leading to IUGR and small for gestational age babies such as that seen under conditions of maternal malnutrition, maternal stress and exposure to environmental insults (Nugent and Bale, 2015). Disruptions in placental dysfunctions may be facilitated via reduced nutrient availability, diminished vascular supply, and/or compromised hormonal, inflammatory, oxidant and metabolic states. While evidence exists that gestational exposure to EDCs including BPA is associated with disrupted maternal/fetal cord blood inflammatory/oxidative stress states (Veiga-Lopez et al., 2015b; Watkins et al., 2015; Huang et al., 2017; Kelley et al., 2019) and altered lipid homeostasis (Geetharathan and Josthna, 2016; Shoaito et al., 2019), the extent to which this also involves the placenta is unknown.

Understanding the mechanisms by which gestational BPA disrupts placental function is critical for developing preventive strategies. Considering that placenta is a steroidogenic organ (Strauss et al., 1996), a possible mechanism of BPA action is via disruption of placental steroidal milieu. Recent demonstration that gestational exposure to phthalate, another EDC with antiandrogenic and estrogenic properties, is associated with disruption of maternal and fetal steroidal milieu in humans (Sathyanarayana et al., 2017) supports such assertions. Likewise, BPA exposure during gestation has also been shown to induce steroidal changes during pregnancy (Saadeldin et al., 2018; Kolatorova et al., 2018) and these steroidal changes were found to be associated with BPA induced alterations in expression of placental steroidogenic enzymes (Saadeldin et al., 2018). Steroids by their virtue of being powerful programming agents (Padmanabhan et al., 2010) can induce placental dysfunctions via epigenetic mechanisms (Maccani and Marsit, 2009; Robins et al., 2011; Nugent and

Bale, 2015; Marsit, 2016; Yang et al., 2019). In fact, animal studies suggest placental tissues are more susceptible to epigenetic changes than embryonic tissues (Nelissen et al., 2011).

Disruptions in any of the several mediators of placental function can compromise placental growth and processes that are important to meet the metabolic demands of the growing fetus leading to IUGR and low birth weight offspring (Sharma et al., 2016; Woods et al., 2018). Considering that BPA reduces placental cell proliferation (Benachour and Aris, 2009; Elmetwally et al., 2018), genes that regulate placental growth and development such as the members of the insulin-like growth factor (IGF) axis consisting of IGF1 and 2 and IGF binding proteins (IGFBPs) (Haywood et al., 2019) are also potential targets of BPA action. For instance, gestational exposure to polyaromatic hydrocarbons and polybrominated diphenyl ethers was found to be associated with reduced cord blood levels of IGFs (Xu et al., 2013) and BPA with reduced methylation of IGF2 promoter (Montrose et al., 2018) lending support to such possibility.

Due to ethical considerations and the risks associated with early pregnancy sampling of placental tissue, placental research in humans are generally limited to those collected at the time of delivery (Grigsby, 2016). Studies utilizing animal models are therefore of translational value and help fill this knowledge gap for understanding early placental changes associated with maternal exposures (Grigsby, 2016), critical for identifying targets for early interventions. Utilizing the sheep model of gestational BPA treatment that exhibits low birth weight (Savabieasfahani et al., 2006), we tested the hypothesis that gestational treatment with environmentally relevant levels of BPA, increases negative mediators of placental function, disrupts angiogenesis and placental steroidogenesis and contributes to reduced placental efficiency. Furthermore, we hypothesized that such placental compromises are associated with epigenetic modifications and reduced expression of genes involved in placental growth. Documentation of placental compromises resulting from gestational BPA treatment may explain the low birth weight associated with pregnancies from BPA exposures (Savabieasfahani et al., 2006; Veiga-Lopez et al., 2015a).

## Methods

### Approach:

Utilizing sheep placentae collected during early and mid-gestation coinciding with major organizational windows of fetal organ differentiation (Padmanabhan and Veiga-Lopez, 2014) this study addressed the impact of prenatal BPA treatment on mediators of placental function such as angiogenesis, inflammation, oxidative stress, lipid accumulation, fibrosis, and steroidal milieu. In addition, as many of these changes are regulated transcriptionally and EDCs can bring about epigenetic alterations (Alonso-Magdalena et al., 2016), impact of gestational BPA exposure on gene expression of enzymes that mediate epigenetic alterations were also examined.

### Animals and prenatal treatments

All procedures were performed under the approved protocol from the Institutional Animal Care and Use Committee at the University of Michigan. Suffolk ewes aged 2–3 years

purchased locally were maintained, bred and prenatally treated as described before (Manikkam et al., 2004; Veiga-Lopez et al., 2013). Treatment groups involved mothers that received only the vehicle (corn oil; Control group) and BPA-treated animals that received 0.5 mg/kg /·day of BPA (purity 99%, cat. no. 239658; Aldrich Chemical, Milwaukee, WI) dissolved in corn oil and administered daily through subcutaneous (sc) injections. BPA administration was carried out from days 30 through 90 of gestation (term: ~147 days). This mode of administration was found to result in umbilical arterial levels of ~2.6 ng/ml of free/uncongugated BPA on gestational day 90 (Veiga-Lopez et al., 2013), well within the range observed in umbilical cord blood of human pregnancy cohorts (Gerona et al., 2013; Veiga-Lopez et al., 2015a, Lee et al., 2018) and maternal urine (Lee et al., 2018).

### Tissue collection

Sheep are characterized by cotyledonary placenta. Unlike human, where a single large area of contact exists between maternal and fetal vascular systems, sheep have numerous smaller areas of contacts called placentome. Depending on progression of placental differentiation they are classed as type A, B, C and D (Vatnick et al., 1991). All dams were anesthetized with 20–30 mL of pentobarbital iv (Nembutol sodium solution, 50 mg/mL; Abbott Laboratories, Chicago, IL). Placentomes were collected while dams were maintained under general anesthesia with isofluorane (1%–2%; Halocarbon Laboratories, River Edge, NJ). Through an abdominal midline incision, uterus was exposed and the uterine wall incised to access the fetus. Dams were administered a barbiturate overdose (Fatal Plus; Vortech Pharmaceuticals, Dearborn, Michigan) and fetuses with the placenta removed for harvest of the placentomes. Placentomes were collected, washed, weighed and the type of placentomes identified as described before (Vatnick et al., 1991). To address early perturbations, Type A placentomes, representing the earliest stage of placental differentiation, were used for the analysis in this study. Placentomes were collected from control (C) and BPA-treated fetuses on gestational day 65 (GD65; n = 8, each for C and BPA) and gestational day 90 (GD90; n = 8 and 5, respectively for C and BPA), quick frozen, and stored at −80°C until processing. One type A placentome from each animal was used in the present study. The fetal sex distribution in the control groups were male = 3 and females = 5 at both gestational time points. In the BPA group the distribution was males = 3 and female = 5 at GD65 and male = 1 and female = 4 at GD90.

### Real time PCR analysis

The markers of inflammation (proinflammatory cytokines interleukin [IL] 1 beta [IL1B], IL6, IL8, tumor necrosis factor alpha [TNF], chemokine ligand 2 [CCL2] and macrophage marker CD68), antioxidants (glutathione reductase [GSR], superoxide dismutase (SOD) 1 and 2), angiogenic markers (hypoxia inducible factor 1 alpha [HIF1A] and vascular endothelial growth factor [VEGF]), steroidogenic enzymes (aromatase [CYP19] and 17 alpha hydroxylase [CYP17]) and steroid receptors (androgen [AR], estrogen [ESR1 and ESR2] and progesterone [PGR] receptors), epigenetic enzymes (DNA methyltrasnferases [DNMT 1, 2 and 3]; histone methylases [SUV39H1 and EZH2], histone demethylase [KDM1A], histone deacetylases [HDAC1, 2 and 3] and histone acetyltransferases [EP300]) and IGF family members (IGF1 and 2, IGF receptors [IGF1R and IGF2R] and IGF binding proteins [IGFB1, 2, 3 and 4] were measured through gene expression analysis. Total RNA

was isolated using Trizol reagent (Life Technologies, Carlsbad, CA), DNAse treated and purified using RNAeasy kit (Qiagen, Germantown, MD) as per manufacturer's instructions. RNA was quantified using Nanodrop and 260/280 ratios were generally    1.8. About 1000ng of total RNA was subjected to cDNA synthesis using the SuperScript VILO kit as per manufacturer's instructions (Life Technologies). Gene expression analysis was carried using SYBRgreen based real time RT-PCR on a BioRad myiQ iCycler instrument as described before (Puttabyatappa et al., 2017). Oligonucleotide primers for the genes under study were either designed using Primer Express software (Life Technologies, Carlsbad, CA) or from previously published reports (Supplemental Table 1). The relative amount of each transcript was calculated using the    CT method and normalized to the endogenous reference gene ribosomal protein L19 (RPL19).

## Oxidative stress measures

Oxidative stress markers Thiobarbituric Acid Reactive Substances (TBARS, a lipid peroxidation marker) and oxidized protein tyrosine moieties, 3-nitrotyrosine (NY) and *o, o'*-dityrosine (DY) were quantified in placentome tissue extracts. TBARS was quantified utilizing commercially available colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Oxidized tyrosine moieties were measured through isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) as described previously (Vivekanandan-Giri et al., 2011). Briefly, the placental proteins were precipitated with ice-cold trichloroacetic acid (10% vol/vol) and then delipidated with the mixture containing water, methanol, water-washed diethyl ether (1:3:7; vol/vol/vol). Known amounts of isotopically labeled internal standards13C6-Y and 13C6-NY and 13C12-o, o'-DY were added and precipitated proteins were hydrolyzed at 110°C for 24 hours in 4M methanesulfonic acid solution saturated with 1% benzoic acid. Samples were then subjected to solid-phase extraction and the amount of oxidized amino acids was quantified by HPLC-ESI-MS/MS with multiple reactions monitoring by integrating peak areas of the labeled standards and the analytes. The levels of the oxidized amino acids in each sample were then normalized to the amino acid tyrosine content in the respective samples and expressed as the ratio of the oxidized product over the total tyrosine. The Intra- and inter-assay coefficients for TBARS assay were 5.6% and 10.2%, respectively and the measurable range is 0.0625–50 μM. Intra-assay coefficients of variation for NY and DY were 8.47% and 8.16%, respectively.

## Ectopic lipid accumulation

Lipid accumulation in liver were assessed by Oil Red O staining of placentome cryosections and triglyceride content using a commercially available assay kit as described before (Puttabyatappa et al., 2017). The measurable range for the triglyceride assay is 1.1 to 2000 mg/dL. All samples were assessed in a single assay and the intra-assay coefficient of variance for triglyceride assay was $3.53 \pm 0.08$.

## Sircol collagen assay

The acid-soluble collagen content in the placentome was measured using a sirius red dye binding (Sircol) assay following the protocol described before (Coentro et al., 2017). Briefly, the acid-soluble collagen was extracted from 100 mg placentome homogenates incubated

with 0.5 M acetic acid for 4 h at 4 °C and centrifuged to remove tissue debris. The soluble collagen was incubated with 1 mL 0.1% Direct Red 80 in saturated picric acid dye reagent solution for 1 h at room temperature. The collagen-dye complex was precipitated by centrifugation at 10,000 g for 10 min, and the supernatant was removed. The resulting pellets were dissolved in 1 mL 0.5 M sodium hydroxide, and the relative absorbance was measured in a 96-well plate at 540 nm using a microplate reader (Molecular Devices SpectraMax, San Jose, CA). Collagen content was normalized to total protein content determined as described before (Puttabyatappa et al., 2017) in placentome protein lysates prepared in parallel from 100 mg of tissue. All samples were assayed at the same time and the intra-assay coefficients of variance for Sircol assay was $2.81 \pm 1.01$. The measurable range for this assay is 31.25 to 500 μg/mL.

### Statistical analysis

Heterogeneity of variance was tested with Fisher's test and when required data were log-transformed. The means from C and BPA-treated groups at their respective time points (GD65 and GD90) were compared using two-tailed Student's t- test (Prism 6 software, GraphPad, La Jolla, CA) and the threshold for significance was set as $p$  0.05. Data were also subjected to effect size analysis (Cohen, 1992) and computed Cohen's d value  0.8 was considered large magnitude changes induced by BPA treatment.

## Results

### Effect of gestational BPA exposure on placental differentiation, weight and efficiency, and fetal weight:

There were no significant changes in number of Type A or B+C+D placentomes at GD65 or GD 90 (Figure 1A and 1B), although a trend for a large magnitude (d= 0.9; p = 0.09) increase in number of type A placentomes was evident in the BPA-treated sheep by Cohen's effect size analysis at GD65. There was no effect of gestational BPA treatment on the weight of Type A or other (B+C+D) placentomes at either gestational time points (Figure 1C and D). In contrast, gestational BPA treatment induced a significant decrease in total fetal weight at GD65 (p = 0.04) but not at GD90 (Figure 1E). Consistent with this reduction, the placental efficiency calculated as ratio of fetal/placental weights was also significantly reduced at GD65 (p = 0.04) and not at D90 in animals exposed to BPA during gestation relative to controls (Figure 1F).

### Effect of gestational BPA treatment on early placental inflammatory status:

Gestational BPA treatment significantly increased interleukin (IL) 8 (*IL8*; p = 0.005) but decreased tumor necrosis factor alpha (*TNF*) mRNA expression at GD65 (p = 0.04) (Figure 2A). In contrast at GD90, gestational BPA significantly increased *IL1B* mRNA (p = 0.04) and showed a trend for a large magnitude decrease (d= 2.0; p = 0.06) in *TNF* mRNA expression (Figure 2A). No changes at either time points were evident for the expression of cytokines chemokine ligand 2 (*CCL2*) and *IL6* and macrophage marker *CD68* between C and BPA-treated groups.

### Effect of gestational BPA treatment on early placental oxidative stress markers:

Gestational BPA treatment significantly increased oxidative stress markers dityrosine (p = <0.001) and nitrotyrosine (p = 0.04) at GD90 but not at GD65 (Figure 2B). No change in lipid peroxidation marker TBARS product malondialdehyde was evident at although a trend for a large magnitude increase (d = 1.0; p = 0.06) in TBARS in the BPA-treated group was evident at GD65 by effect size analysis.

Gestational BPA treatment significantly increased the expression of antioxidant genes glutathione reductase (*GSR*; p = 0.001), superoxide dismutase (SOD) 1 (p = 0.007), and *SOD2* (p = <0.01) at GD65 but not at GD90 (Figure 2B).

### Effect of gestational BPA treatment on early placental lipid and collagen accumulation:

Gestational BPA treatment significantly increased triglyceride accumulation in the placentome by GD90 (p = 0.003) but not at GD65 (Figure 3A). Consistent with this, there was a trend for a large magnitude increase (d = 1.1; p= 0.07) in collagen accumulation in the placentomes of BPA-treated animals at GD90 and not at GD65 (Figure 3B).

### Effect of gestational BPA treatment on early placental angiogenesis:

The effect of gestational BPA treatment on placental angiogenesis was examined by examination of genes that promote vascularization. There was a significant reduction in hypoxia inducible factor 1 alpha (*HIF1A*) at GD90 (p = 0.01) but not at GD65 (Figure 3C). There was no effect of gestational BPA treatment on vascular endothelial growth factor (*VEGF*) expression at either time point (Figure 3D).

### Effect of gestational BPA treatment on early placental steroidogenic enzyme and steroid receptor expression:

The effect of gestational BPA treatment on steroidogenic components was examined by assessing the gene expression of steroidogenic enzymes and steroid receptors. Gestational BPA treatment significantly increased expression of steroidogenic enzymes 17α-hydroxylase, 17,20-lyase (*CYP17;* p = < 0.001) and aromatase (*CYP19*; p = 0.02) at GD65 (Figure 4A and B). In contrast at GD90, gestational BPA treatment significantly increased *CYP17* expression only (p = 0.02) (Figure 4A). Relative to steroid receptors, BPA treatment had no impact on androgen receptor (*AR*) expression at both time points (Figure 4C). In contrast, gestational BPA treatment resulted in a significant increase in estrogen receptor 2 (*ESR2*) (p = <0.001) and a non-significant large magnitude increase (d= 0.8; p = 0.14) in estrogen receptor 1 (*ESR1*) as well as progesterone receptor (*PGR*) (d = 0.8; p = 0.15) at GD65 but not GD90 in the BPA-treated animals (Figure 4D–F).

### Effect of gestational BPA treatment on placental expression of IGF family members:

Gestational BPA treatment induced non-significant large magnitude increases in *IGF2* expression at both GD65 (d = 0.81; p = 0.11) and 90 (d = 0.92; p = 0.13) and *IGF1* (d = 1.1; p = 0.08) only at GD90 (Figure 5A and B). As for the expression of receptors for IGFs, BPA treatment resulted in a significant reduction in the expression of IGF1 receptor (*IGF1R*) at GD65 (p = 0.02) with only a non-significant large magnitude increase (d = 0.9; p = 0.12) at

GD90 (Figure 5C). In contrast, the IGF2 receptor (*IGF2R*) was significantly increased at GD65 (p = 0.01) with a trend for a large magnitude increase (d = 1.2; p = 0.07) at GD90 (Figure 5D). Relative to the expression of proteins that regulate IGF bioavailability, gestational BPA treatment increased significantly the expression of *IGFBP1* (p = 0.004) and *IGFBP3* (p = 0.03) at GD65 and expression of *IGFBP2* (p = 0.02), *IGFBP3* (p = 0.04) and *IGFBP4* (p = 0.04) at GD90 (Figure 5E–H).

**Effect of gestational BPA treatment on early placental epigenetic machinery:**

Epigenetic alterations affect expression of key genes required for placental function and usually involves covalent modulation of DNA and histones through methylation and acetylation (Kouzarides, 2007; Smith and Meissner, 2013). These changes are driven at the level of DNA by the enzymes belonging to the DNA methyltransferase (DNMT) family of which DNMT1, DNMT3A and DNMT3B are important (Smith and Meissner, 2013). Histone modification through methylation involves methyltransferases SMYD3, SUV39H1 and enhancer of zeste 2 (EZH2) and demethylase KDM1A. Another major histone modification involves acetylation that enhances gene expression and is driven by histone acetyltransfease EP300 (Roth et al., 2001). On the contrary reduction of gene expression is driven by histone deacetylases (HDAC) (Marks et al., 2003). The effects of EDC exposure including BPA in enhancing DNA methylation and histone modification in various bodily systems including placenta have been studied (Kundakovic and Champagne, 2011; Robins et al., 2011; Hogg et al., 2012; Nahar et al., 2015; LaRocca et al., 2016; Barouki et al., 2018). In the current study, the impact of gestational BPA treatment on placental epigenetic machinery was determined by examining the expression of enzymes that bring about modification of DNA and histones. Among the DNA methyl transferases (DNMTs), gestational BPA treatment significantly increased *DNMT3A* at GD90 (p = 0.02) but not GD65, while having no effect on *DNMT1* and *DNMT3B* at both time points (Figure 6A–C). Gestational BPA treatment did not have any effect on histone methyl transferases *SUV38H1* and *EZH2* and histone demethylase *KDM1A* (Figure 6D–F) at both time points. Among the histone deacetylase (HDAC) enzymes, the expression of *HDAC1* was significantly elevated by BPA treatment at both GD65 and GD90 (p = 0.004 and 0.005, respectively), while *HDAC2* was only significantly increased at GD65 (p = 0.003) (Figure 6G and H). Cohen's effect size analysis revealed large magnitude non-significant increase in *HDAC2* (d = 1.1; p = 0.14) at GD90, a trend for an increase in *HDAC3 at* GD65 (d = 1.2; p = 0.055) and a non-significant large magnitude increase in HDAC3 at GD90 (d = 1.2; p = 0.18) (Figure 6H and I). In line with the changes in HDAC but only at GD65, BPA treatment, by effect size analysis, also induced a large magnitude non-significant increase (d = 1.0; p = 0.09;) in expression of histone acetyl transferase enzyme EP300 (Figure 6J).

## Discussion

The findings from this study indicate that gestational BPA treatment increases several negative mediators of placental function and that the impact on these negative mediators differ between mid – (day 90) and early (day 65) gestation. Changes in steroidogenic enzymes and receptors, altered IGF bioavailability, inflammatory and oxidative stress markers, lipotoxicity and epigenetic enzymes induced by gestational BPA-treatment are all

possible mechanisms through which placental function and fetal growth is compromised. The finding that placental efficiency and fetal weight are reduced at day 65 but not at day 90 suggests some compensatory mechanism such as latency in response to day 65 increase in antioxidants may be in place to overcome the disruptive effects of BPA. The implication of these findings relative to BPA-associated placental dysfunction is discussed below.

### Gestational BPA exposure on placental inflammation

As placenta forms the maternal-fetal interface where maternal cells encounter fetal cells which are akin to an allograft, the establishment and maintenance of pregnancy involves preserving a balance between placental immune reactivity and suppression (Redline, 2007). Any disruption of this balance would compromise the normal inflammatory cascade contributing to the poor birth outcomes (Trevisanuto et al., 2013) such as that seen in pathological conditions like preeclampsia and gestational diabetes (Kumar et al., 2013; Syngelaki et al., 2016; Rambaldi et al., 2019). EDCs including BPA have been shown to alter maternal and neonatal cord blood inflammatory cytokines (Ferguson et al., 2015b; Huang et al., 2017; Kelley et al., 2019). The finding of increased secretion of IL6 by placental explants following treatment with tetrabromobisphenol A (Arita et al., 2018), a brominated BPA derivative with similar endocrine disrupting action as BPA (Borghoff et al., 2016), is supportive of BPA-induced disruptions in placental inflammatory processes as well. The significant increase in inflammatory cytokines *IL8* at early and *IL1B* at mid-gestation in BPA-treated animals (this study) is consistent with this premise. The increase in the expression of *IL8* may have contributed to the reduced placental efficiency and fetal weight evidenced during early pregnancy. On the contrary, the significant large magnitude decrease at GD65 and non-significant large magnitude decrease in *TNF* expression in the BPA-treated group is not consistent with a pro-inflammatory role of TNF (Idriss and Naismith, 2000).

### Gestational BPA exposure on placental oxidative stress

Normal placental function is associated with balanced production of reactive species and antioxidants (Wang et al., 1991) that helps prevent induction of oxidative stress. In pregnancies associated with poor placental function such as pre-eclampsia, fetal growth restriction and preterm labor this balance is disrupted leading to development of oxidative stress (Al-Gubory et al., 2010). In general, conditions associated with proinflammatory state are usually accompanied by oxidative stress (Chatterjee, 2016). In human, high BPA levels are associated with Increase in maternal and fetal oxidative stress markers, di and nitrotyrosine (Veiga-Lopez et al., 2015b). Gestational BPA treatment also increases oxidative stress markers in sheep and rats (Veiga-Lopez et al., 2015b). Furthermore, BPA treatment has been found to increase oxidative stress in studies with placental explants (Basak et al., 2018) as well as JEG3 and BeWo cells (Ponniah et al., 2015; Perez-Albaladejo et al., 2017). Our findings at the placental level of large magnitude albeit non-significant increase in TBARS at GD65 and significant large magnitude increases in di- and nitro-tyrosine at GD90 in gestational BPA-treated sheep are consistent with these other findings. However, the reason behind these time-dependent changes with increase in lipid peroxidation marker at early gestation, and nitrative and oxidative stress markers at mid gestation is not clear. The significant large magnitude increase in antioxidants only at GD65

by BPA treatment may be reflective of compensatory response to overcome the increase in oxidative stress evident at that time and may have contributed to the normalization of fetal weight by GD90. Lagged response to increase in oxidative stress markers coupled with unchanged antioxidant levels at GD90 may result in failure of this compensatory process as pregnancy progresses and contribute to the low birth offspring of gestational BPA treated animals (Savabieasfahani et al., 2006).

### Gestational BPA exposure on placental lipotoxicity and fibrosis

While lipids are essential for maintenance of placental structure and function (Delhaes et al., 2018), increase in placental lipid accumulation in conditions such as obesity (Calabuig-Navarro et al., 2017; Delhaes et al., 2018) can lead to adverse programming. Abnormal placental lipid metabolism and lipid accumulation have also been observed following: 1) in vitro treatment of rat trophoblast cells (Xu et al., 2006) and in vivo treatment of rats (Xu et al., 2008) with phthalates metabolites and 2) treatment of JEG3, a placental cell line with bisphenol A diglycidyl ether, a synthesis product of BPA (Marqueno et al., 2019). Therefore, it is not surprising that gestational BPA treatment produced a significant large magnitude increase in placental lipid accumulation at mid-gestation.

Chronic activation of cellular repair mechanisms due to various insults can lead to increased production of extracellular matrix such as collagen resulting in placental fibrosis such as that seen with placental vaculopathies and IUGR (Chen et al., 2005; Ohmaru-Nakanishi et al., 2018). Insults that can lead to fibrosis include inflammation, oxidative stress and lipid accumulation (Zeisberg and Kalluri, 2013), all of which have been observed in gestational BPA-treated sheep (this study). Consistent with this premise, a large magnitude albeit non-significant increase in collagen accumulation was observed at mid- gestation in BPA-treated sheep, a finding requiring further validation with larger sample sizes. These emerging fibrotic changes at mid gestation may amplify with progression of pregnancy and contribute to the low birth weight offspring of BPA-treated sheep (Savabieasfahani et al., 2006).

### Effect of BPA treatment on placental angiogenesis

Placental angiogenesis and vasculature changes markedly with the progression of gestation and pathogenesis associated with placental insufficiencies such as preeclampsia are associated with compromised angiogenesis and vascular functions (Charnock-Jones et al., 2004; Kaufmann et al., 2004; Mayhew et al., 2004). That endocrine disruptors such as phthalates and BPA are also associated with reduced angiogenic markers in maternal circulation (Ferguson et al., 2015a) suggest that they have the potential to affect placental angiogenesis. In support, gestational BPA-treatment alters placental vasculature in mouse placenta (Tait et al., 2015). The significant large magnitude decrease in angiogenesis promoter *HIF1A* found at mid-gestation in the BPA-treated sheep is in agreement with compromised angiogenesis. Considering estrogens promote placental angiogenesis (Albrecht and Pepe, 2010), the adverse impact of BPA - an estrogen mimic - is surprising and may relate to other properties of BPA. The lack of impact of gestational BPA treatment on *VEGF* expression was unexpected considering pregnancies associated with IUGR have reduced levels of VEGF transcripts (Ravikumar et al., 2019). A possibility to consider is that

the *VEGF* disruption may be manifest during later stage of gestation, since HIF1A is known to exert its proangiogenic role by increasing *VEGF* expression (Zimna and Kurpisz, 2015).

### Effect of BPA treatment on placental steroidal milieu

Apart from its function as a conduit between the mother and fetus, placenta is also a steroidogenic organ as well as a target of steroid action (Strauss et al., 1996; Miller and Auchus, 2011). Conditions associated with placental dysfunction are associated with altered steroidogenic enzyme expression in humans (Hogg et al., 2013). Maternal exposure to plasticizers such as phthalates are associated with alterations in maternal and fetal steroidal milieu (Sathyanarayana et al., 2017). While BPA treatment reduces aromatase activity in placental cell line JEG3 (Perez-Albaladejo et al., 2017), there is no consensus on its effect on *CYP19* expression with one study showing no effect (Nativelle-Serpentini et al., 2003) and another showing reduced expression (Chu et al., 2018). BPA treatment has been shown to reduce placental *CYP19* expression in term trophoblast cultures (Chu et al., 2018). The finding of a significant large magnitude increase in placental *CYP19* expression in gestational BPA treated sheep is inconsistent with these *in vitro* findings from human placenta. These discrepancies may be a function of species differences (human vs. sheep), time of placental collection (early in sheep vs term in human), treatment duration (chronic *in vivo* in sheep vs acute *in vitro* in human) or their organizational (this study) vs. activational (in vitro studies in human) effects.

In contrast to the report that human placenta does not express *CYP17* (Miller and Auchus, 2011), sheep placenta not only expresses *CYP17* but BPA treatment induces a significant large magnitude increase in *its* expression at both GD65 and GD90 time points. While effects of BPA in increasing *CYP17* have been shown in other cell types (Zhou et al., 2008), to our knowledge this is the first report of such induction in placental level. Effects of BPA on placental expression of steroidal receptors have not been previously examined. The significant large magnitude increase in *ESR2* and non-significant large magnitude increases in *ESR1* and *PGR* at early gestation by BPA treatment is supportive of paracrine actions of steroids at the placental level. Similar changes in placental steroid receptor mRNAs were seen in rats following treatment with the estrogen precursor, testosterone (Sun et al., 2012). Since placenta can aromatize testosterone to estradiol, the similarity in outcomes in terms of steroid receptor expression in both situations suggest that the regulation may be via estrogenic actions in both instances. The findings from this study point to BPA-induced disruption of placental steroidal biosynthesis and action. Whether the compromised steroid milieu is a contributor to the placental dysfunctions discussed above or vice versa remains to be determined.

### Effect of BPA treatment on placental expression of IGF family members

Placenta also expresses insulin-like growth factors 1 and 2, its receptors and binding proteins (Han et al., 1996; Forbes and Westwood, 2008). While fetal IGF1 stimulates fetal growth by promoting anabolic events and DNA synthesis, placental IGFs influence fetal growth by promoting normal placental development/function and allowing proper transfer of nutrients to the fetus (Klauwer et al., 1997; Forbes and Westwood, 2008). Consistent with its key role, decreased placental expression of IGF1 accompanies pregnancies with fetal growth

restriction (Koutsaki et al., 2011; Martin-Estal et al., 2016). Studies in mice indicate over expression of IGF2 leads to placental and fetal overgrowth, while its deletion, the opposite (DeChiara et al., 1990; Gicquel and Le Bouc, 2006). Exposure to EDCs such as polyaromatic hydrocarbons and polybrominated diphenyl ethers during pregnancy is associated with low levels of cord blood IGFs (Xu et al., 2013), possibly reflecting impaired placental expression. However, in the current study, such a decrease in IGF ligand expression was not observed at the early time points studied. An obvious possibility is that the large magnitude albeit non-significant increase in placental *IGF2* expression observed at gestational day 65 in the present study is countered by the significant decrease in *IGF1R* expression and significant increase in expression of *IGFBP1* and *3* leading to reduced IGF bioavailability, which would be consistent with the reduced placental efficiency and fetal weight evident in BPA-treated sheep at this time point. In contrast, the large magnitude non-significant increases in *IGF1*, *IGF2*, *IGF1R* and *IGF2R* at fetal day 90 may in unison have countered the significant increase in *IGFBP 2–4* found at this time leading to increased IGF bioavailability, supportive of a compensatory response to overcome the fetal growth retardation seen at fetal day 65.

### Effect of BPA treatment on placental expression of epigenetic modifying enzymes

The disruption in the mediators of placental function such as steroidogenic enzymes, steroid receptors and IGF family members evident in this study involves disruption of normal gene expression. Regulation of gene transcription involves epigenetic modifications and facilitated via changes in DNA methylation, histone modification and/or non-coding RNAs. Exposure to BPA has been shown to induce epigenetic modifications in several systems (Kundakovic and Champagne, 2011; Robins et al., 2011; Hogg et al., 2012; Nahar et al., 2015; LaRocca et al., 2016; Barouki et al., 2018; Strakovsky and Schantz, 2018). Findings from the current study with the gestational BPA-treated animals that manifest increase in DNMT3A – a *de novo* methyltransferase - and HDACs that removes the acetyl group from histone proteins and reduces accessibility of DNA to transcription factors, is consistent with BPA's ability to induce epigenetic modifications. Studies with animal models have provided evidence that placental DNA methylation plays an important role in placental and fetal growth (Koukoura et al., 2012; Vrooman et al., 2016). In pregnancies affected by IUGR, placental IGF1 mRNA is decreased and the IGFBPs expression increased and these changes are associated with hypermethylation of IGF1 and hypomethylation of IGFBPs and IGF1R (Desgagne et al., 2014; Nawathe et al., 2016; Jia et al., 2017). Preeclampsia is also associated with hypermethylation of IGF1 promoter which is mediated by DNMT1 (Ma et al., 2018). In stark contrast, the large magnitude increase in IGF mRNA seen in the current study is not consistent with the observed changes in epigenetic enzyme. One possibility is that in BPA-treated sheep this regulation is exerted at the receptor level with the increase in HDAC1 contributing to BPA induced reduction in placental IGF1R during early gestation. Such an association between HDAC1 and IGF1R has been demonstrated in vascular smooth-muscle cells (Kavurma et al., 2007). These findings raise the possibility that the epigenetic regulation of IGF1R expression may be a function of changes in histone acetylation rather than DNA methylation.

Contrary to the dissociation in epigenetic enzyme changes and IGF, the reduced *TNF* and *HIF1A* expression observed at mid gestation in this study is in line with the observed increases in epigenetic enzymes and their predicted regulation. The findings from this study are also consistent with the decreased expression of TNF (Hermsdorff et al., 2013) and HIF1A (Walczak-Drzewiecka et al., 2010) found to accompany the increased DNA methylation of their promoters shown in other cell types.

While available evidence indicates that BPA exposure is associated with changes to placental DNA methylation, micro-RNA expression, and genomic imprinting (Strakovsky and Schantz, 2018), no reports exist regarding histone modifications at the placental level. Findings from the current study showing significant increase in histone deacetylase *HDAC1* and *HDAC2* and non-significant large magnitude increase in *HDAC3* and histone acetylase *EP300* indicate that effects of BPA on placental epigenome involve histone modifications. Since histone acetylation is viewed to be a prerequisite for TNF expression in some cells (Lee et al., 2003b) the decrease in *TNF* observed in this study maybe the result of increased HDACs that reduce acetylation of histones.

### Linking BPA induced placental changes with placental efficiency and fetal growth

The effects of BPA, a xenoestrogen, may be exerted by direct action at the placental level or mediated via changes in placental steroidal milieu and involve changes in the epigenetic machinery. Steroids are classic programming agents (Padmanabhan et al., 2010). Available evidence suggests that nuclear hormone receptors through which steroid hormones signal and epigenetic regulatory enzymes can regulate each another and cooperatively modulate expression of downstream genes (Kato et al., 2011; Vilahur et al., 2014; Kappil et al., 2016; Vrooman et al., 2016). A possibility to consider is that the plethora of placental disruptions seen in gestational BPA-treated animals - summarized in Figure 7 - may all be mediated by altered steroidal input, the result of changes at the steroidogenic biosynthetic level as well as steroid receptor level or alternatively all the other BPA-induced placental compromise may underlie the disruption in steroid homeostasis with both scenario involving changes in the epigenome. Importantly the effects of BPA on the placenta appear to vary depending on the stage of the pregnancy and consistent with a latency in final outcomes. During early gestation, BPA induced increase in inflammation and oxidative stress, reduced levels of TNF, a cytokine with role in promoting placental function (Jaattela et al., 1988; Basu et al., 2016; Pavlov et al., 2016), and reduced bioavailability of IGFs may be the main drivers for reduced placental efficiency and fetal weight. During the mid-gestation, lag in action of increased expression of antioxidants observed during early-gestation and large magnitude albeit non-significant mid-gestational increase in both IGFs and its receptors may have aided in circumventing the increase in IGFBPs leading to restoration of placental efficiency and fetal weight. However, the mid-gestational increases in inflammatory, oxidative and lipotoxic state, reduced angiogenesis and increased fibrosis is suggestive of failure to sustain such compensation with pregnancy progression and could be the basis for poor birth outcomes in prenatal BPA-treated sheep (Savabieasfahani et al., 2006), a premise that needs to be tested in future investigations.

## Strengths and limitations

The major strengths of this study include: 1) the use of an precocial large animal model with similar *in utero* developmental time line as humans, 2) treatment with environmentally relevant levels of BPA leading to achievement of fetal levels of BPA comparable to that observed in human pregnancy cohorts (Gerona et al., 2013; Lee et al., 2018), 3) focus on early and mid-gestation placenta, which is difficult to accomplish in humans, and 4) integrative assessment of plethora of mediators of placental function in unison. Notwithstanding these, the data presented here should be interpreted considering some of the limitations. These include small sample size that did not allow achievement of statistical significance with some key variables (eg. IGF), consideration of fetal sex as a variable in view of the sexual dimorphism existing at the placental level (Kalisch-Smith et al., 2017) and only gene expression analysis of inflammatory mediators, antioxidants, IGF family members, and angiogenic, steroidogenic and epigenetic markers. While further studies with larger sample size and protein and/or activity assessment of inflammation, angiogenesis, steroidal and epigenetic changes are required to fully delineate the final outcome, the coordinated directionality of changes amongst mediators of the placental phenome emphasize compromised placental dysfunction.

In conclusion, the findings from this study demonstrate that gestational BPA exposure impairs mediators of placental function at several levels and involves changes in epigenetic mechanisms, a finding of potential relevance to humans during pregnancy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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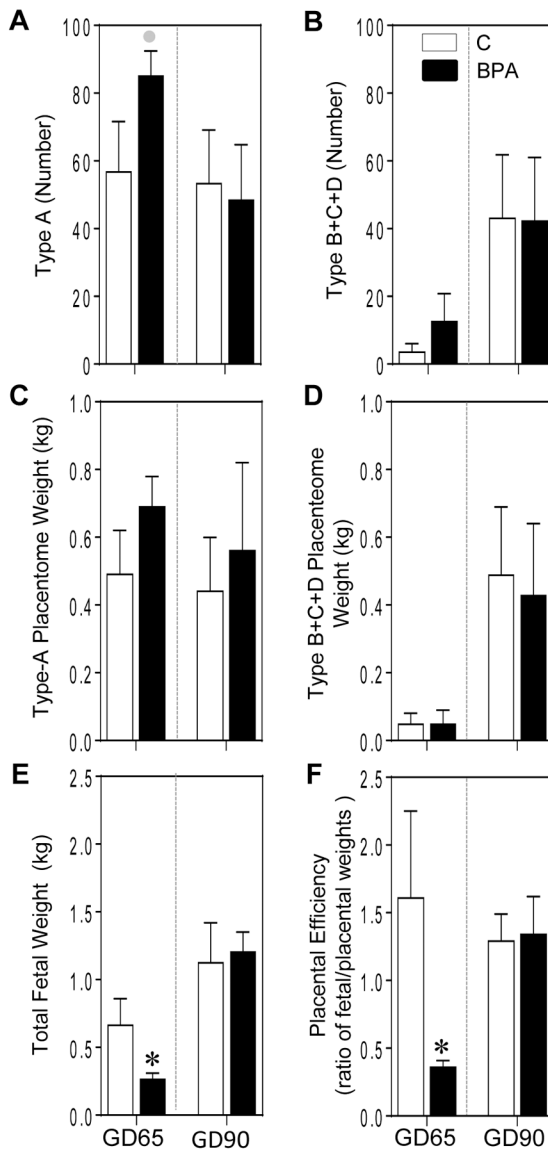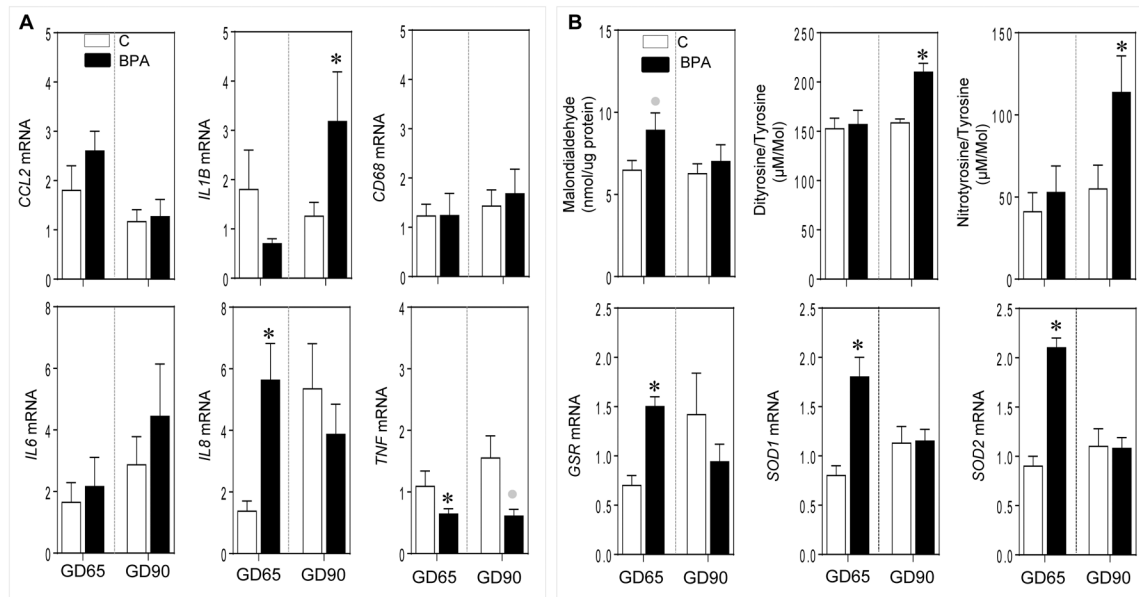
**Figure 1:**
Mean ± SEM of number (A and B) and weight (C and D) of type A and B+C+D placentomes, fetal weight (E) and placental efficiency (F) at GD65 and GD90 from control (C) and BPA-treated pregnant sheep are shown. * = p < 0.05 by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.
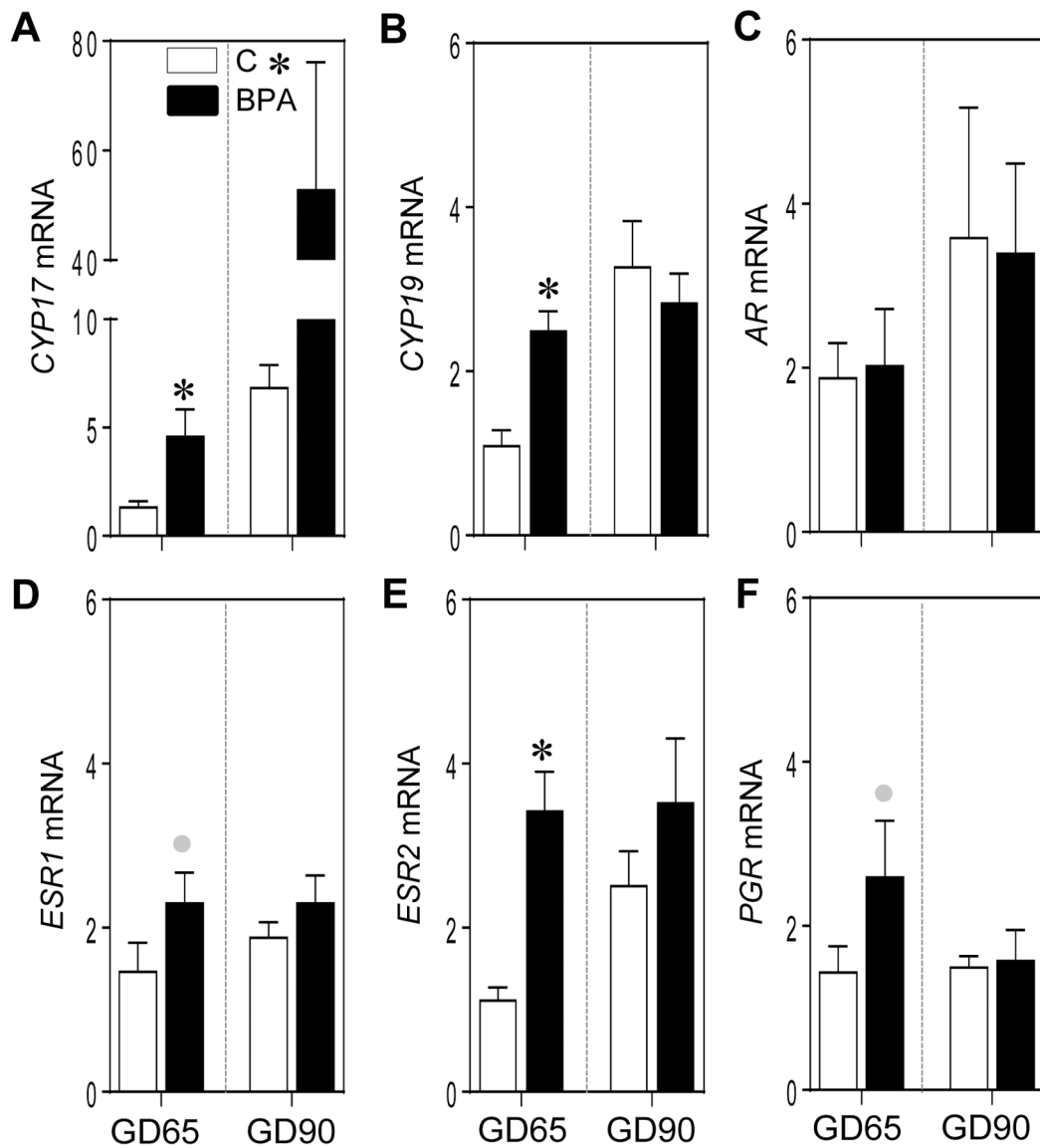
**Figure 2:**
Mean ± SEM of fold change in mRNA expression for inflammatory cytokine and macrophage marker genes (Panel A) and oxidative stress markers malondialdehyde, dityrosine and nitrotyrosine (Panel B, top) and fold change in mRNA expression of antioxidants genes (Panel B, bottom) at GD65 and GD90 from control (C) and BPA exposed pregnant sheep are shown. * = p < 0.05 by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.
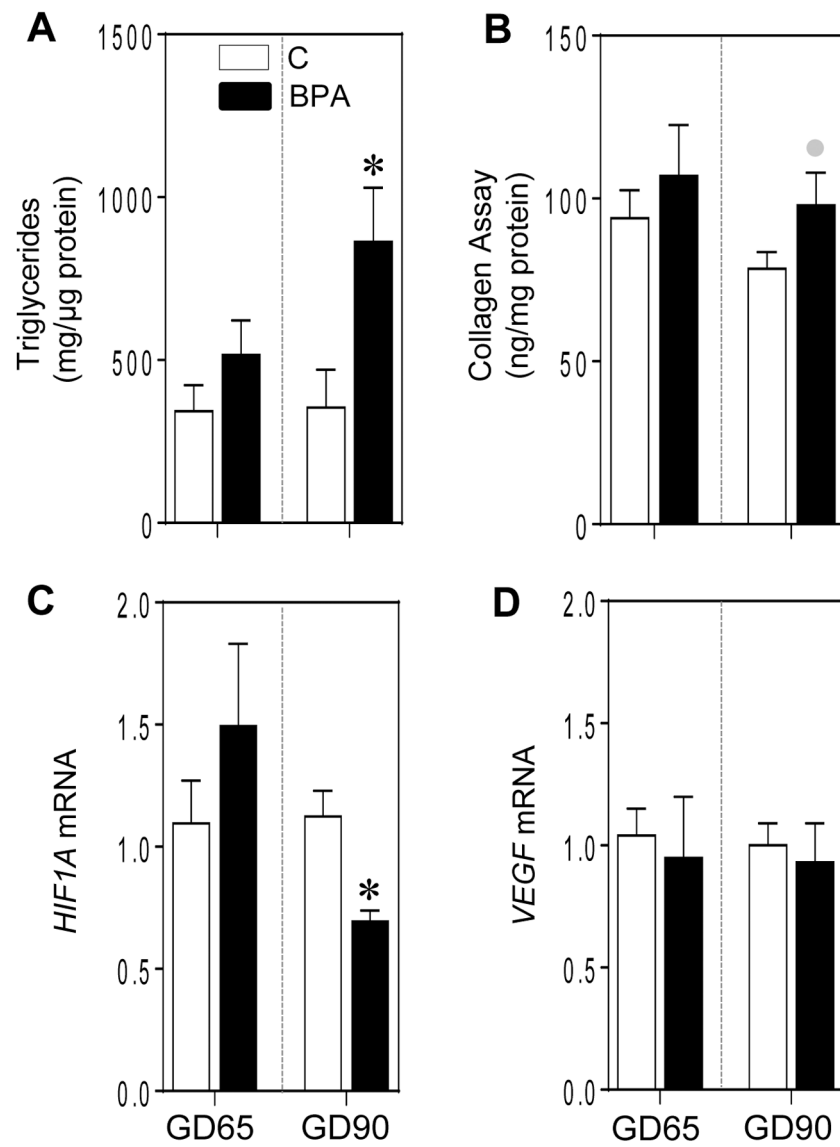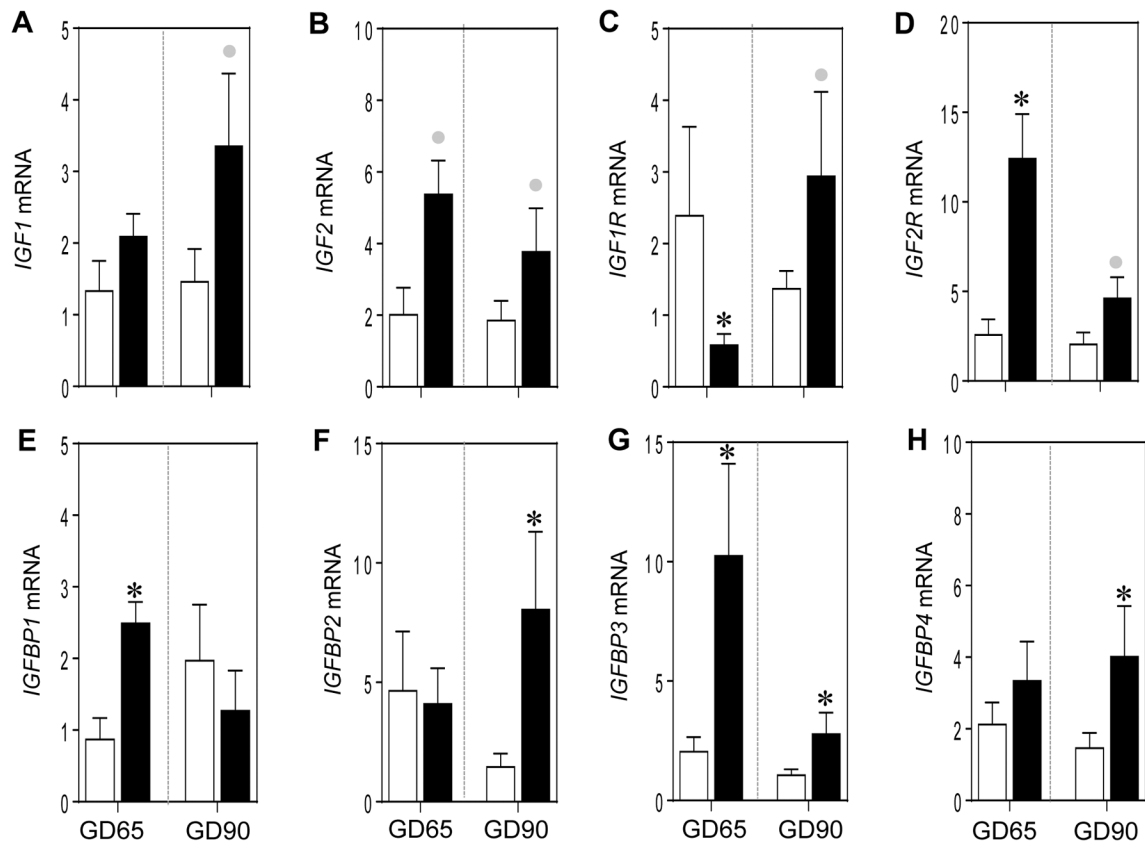
**Figure 3:**
Mean ± SEM of placentome triglyceride (A) and collagen (B) content and fold change in mRNA expression of angiogenic genes (C and D) at GD65 and GD90 from control (C) and BPA exposed pregnant sheep are shown. * = p < 0.05 by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.
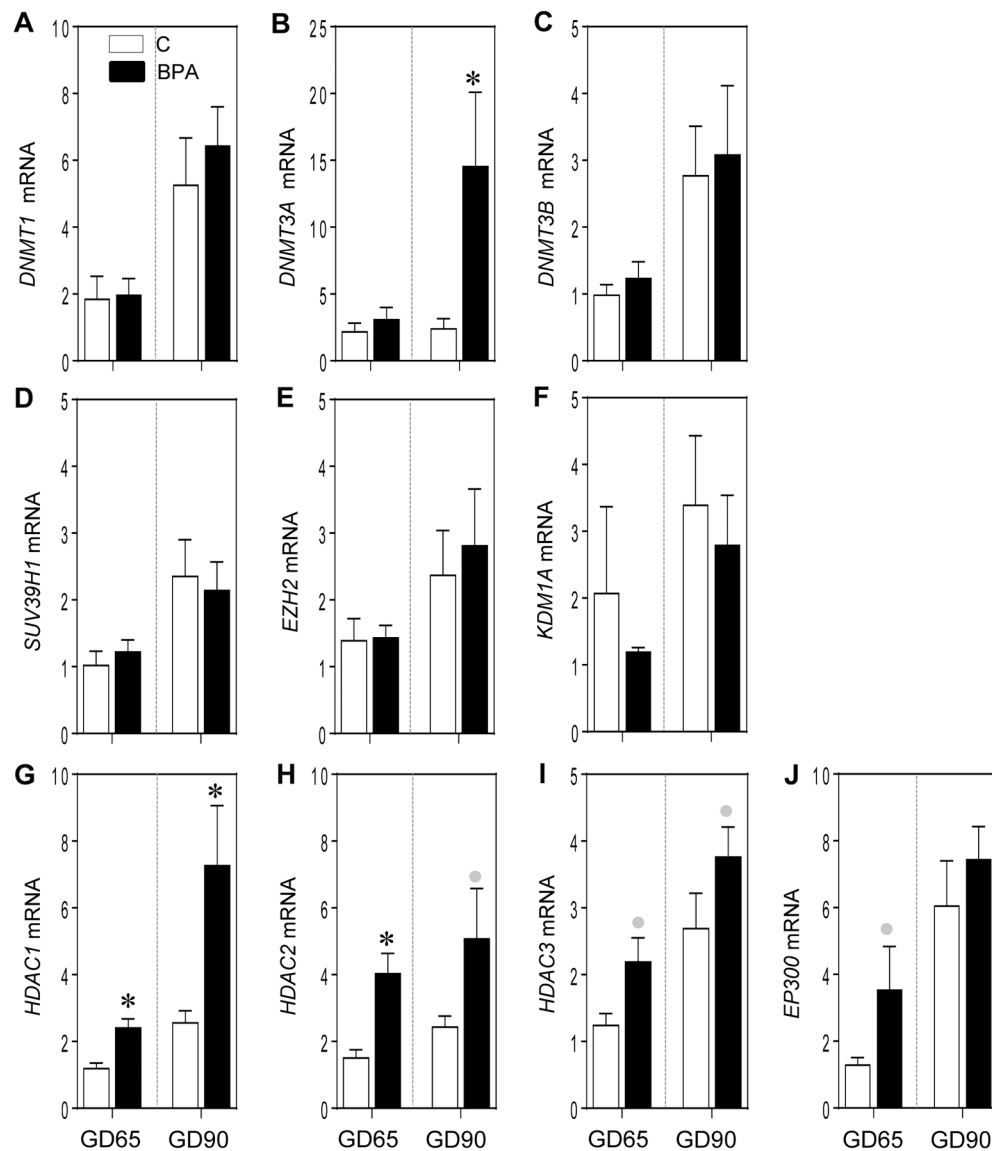
**Figure 4:**
Mean ± SEM of fold change in mRNA expression of steroidogenic genes (CYP17 and CYP19; A and B, respectively) and steroid receptors (AR, ESR1, ESR2 and PGR; C, D, E and F, respectively) at GD65 and GD90 from control (C) and BPA exposed pregnant sheep are shown. * = p < 0.05 by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.
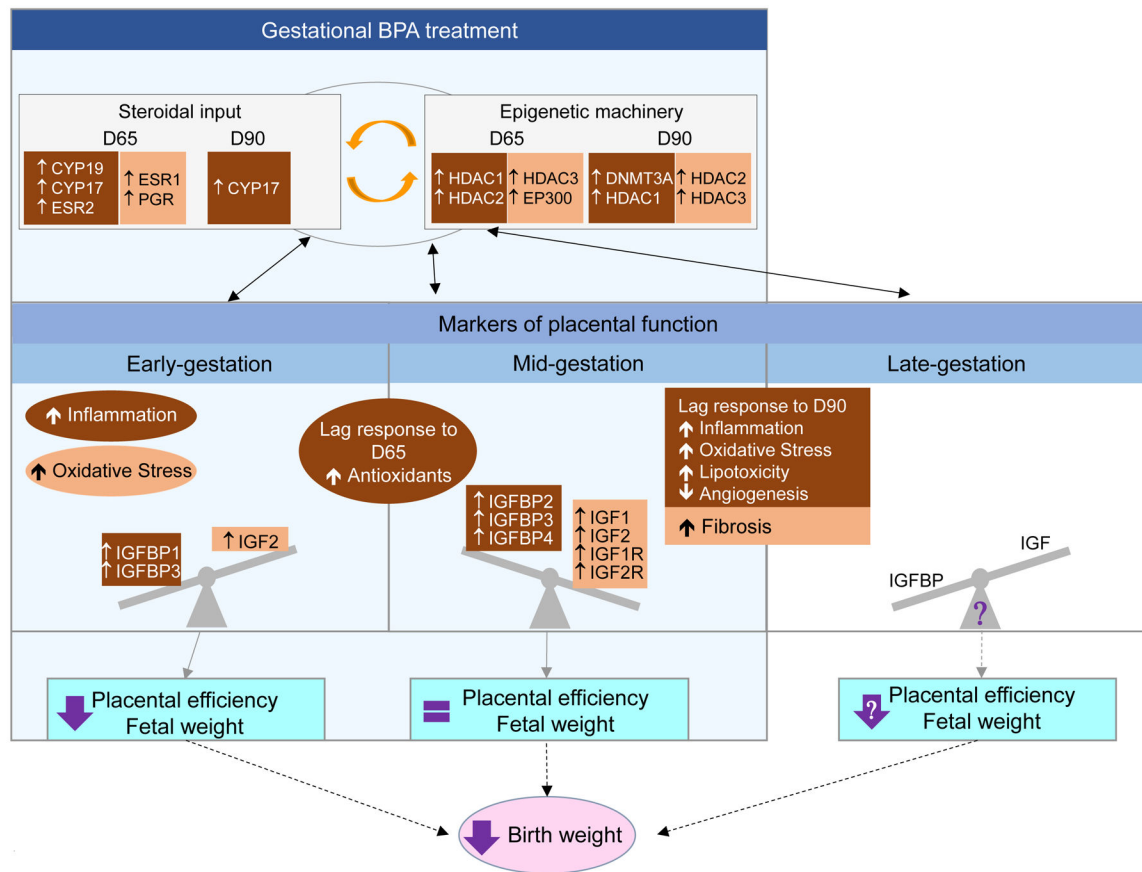
**Figure 5:**
Mean ± SEM of fold change in mRNA expression of IGF family members: IGF1 (A) and IGF2 (B), IGF receptors (IGF1R and IGF2R; C and D, respectively) and binding proteins (IGFBP1–4; E-H, respectively) at GD65 and GD90 from control (C) and BPA exposed pregnant sheep are shown. * = p < 0.05 by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.

**Figure 6:**
Mean ± SEM of fold change in mRNA expression of epigenetic modifying enzymes: DNA methyl transferases (DNMT1, DNMT3A and DNMT3B; A, B and C, respectively), histone methyl transferases (SUV39H1 and EZH2, D and E, respectively) and histone demethylase (KDM1A; F), histone deacetylases (HDAC1, HDAC2 and HDAC3; G, H and I, respectively) and histone acetyl transferase (EP300; J) at GD65 and GD90 from control (C) and BPA exposed pregnant sheep are shown. * = $p < 0.05$ by Student's t-test and grey circles indicate large effect size by Cohen's effect size analysis.

**Figure 7:**
Schematic summarizing the effects of gestational BPA treatment during early, mid and late gestation. The light blue shaded area represents the period of BPA treatment spanning early to mid-gestation (gestational day 30–90; term ~147 days). Outcomes evident during this period may be due to direct effects of BPA or indirectly mediated by the changes in steroid input – function of changes in steroidogenic enzymes and steroid receptors, epigenetic machinery and mediators of placental function. Outcomes extending into late gestation likely represent the latent effects induced by BPA during early- / mid-gestation and programmed through changes in steroidal and epigenetic changes. Significant changes are shown in brown shaded areas with white letters and non-significant large magnitude effects as determined by Cohens effect size analysis are shown in orange shaded areas with black letters.