THE EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON PLACENTAL DEVELOPMENT AND FUNCTION

By

Jeremy Gingrich

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology–Doctor of Philosophy

2020

ABSTRACT

THE EFFECTS OF ENDOCRINE DISRUPTING CHEMICALS ON PLACENTAL DEVELOPMENT AND FUNCTION

By

Jeremy Gingrich

Endocrine disrupting chemicals (EDCs) are compounds that can interfere with normal endocrine functions. Human exposure to EDCs is particularly concerning during vulnerable times in life, such as early development and pregnancy due to the plasticity of the fetus' developing organs during the prenatal period. EDCs are pervasive, with studies demonstrating that most EDCs can cross the placental barrier, reaching fetal circulation. Chemical transfer into fetal circulation could have negative implications for the developing progeny. However, often overlooked is the effect that these chemicals may pose to the placenta. Being a transient yet complex steroidogenic endocrine organ, with abundant expression of hormone receptors, the placenta is highly sensitive to EDC exposures. Such exposures during pregnancy can alter the development and function of the placenta, resulting in adverse birth outcomes like intrauterine growth restriction, or complex obstetric disorders like preeclampsia. This dissertation will present research which advances our understanding of EDC exposures on the development and function of the placenta.

Bisphenols are a class of chemical used in the production of industrial and consumer plastics, epoxy resins, thermal receipt paper, and canned food lining. The most widely studied of these is bisphenol A (BPA), but little is known about commonly used emerging bisphenols, like bisphenol S (BPS) and bisphenol F (BPF). BPA, BPS, and BPF are the three most common bisphenols in human circulation worldwide and are detected concomitantly in biomonitoring studies. Despite broad human environmental exposures, the toxicokinetics of emerging bisphenols, particularly in mixture, and during pregnancy, remain unknown. Therefore, the first aim of this dissertation was to determine the comparative toxicokinetics of BPA, BPS, and BPF, in mixture versus a single compound, in a pregnancy

model. As a sub-aim, this toxicokinetic dataset was used to develop predictive pregnancy physiologically-based toxicokinetic models for BPA and BPS. These mathematical models were developed to be employed as risk assessment tools to better understand maternal and fetal exposures to emerging EDCs across gestation.

As previous studies have shown BPA exposure negative effects placental development in mice, we aimed to study this effect following exposure to BPS and hypothesized that chronic gestational exposure to both BPA and BPS would disrupt placental development and endocrine function. Here, we observed a novel placental defect in endocrine function and altered fused placental trophoblast populations resultant from chronic BPS, but not BPA, exposure. As a sub-aim, and for the purpose of interspecies translatability, we use *in vitro* techniques to evaluate the effect of BPS on primary isolated human placental trophoblast cell fusion; a necessary process for the development and function of the placenta.

Data from aim two are suggestive of BPS-induced repression of gap junction intercellular communication (GJIC). Because primary isolated placental trophoblast cells are unsuitable to conduct functional assays to assess this outcome, ovarian theca cells, which have a similar expression of proteins forming gap junctions were used. Therefore, the third aim of this dissertation was to evaluate the effects of *in vitro* exposure to BPA, BPS, and BPF on GJIC, and hypothesized that BPS would inhibit GJIC in ovarian theca cells. Importantly, BPS was observed to enhance GJIC through the mitogen-activated protein kinase signaling pathway in sheep and human theca cells. A sub-aim of this study was to develop a parachute assay capable of measuring GJIC in immortalized placental trophoblast cells.

Together, the data presented in this dissertation evaluates the pregnancy toxicokinetics of three prominent bisphenols, identifies a novel placental defect following chronic gestational exposure to BPS, a potential mechanisms through which this defect may be occurring, and provides an overall cohesive insight into the risk emerging EDCs, like BPS, pose to the development and function of the placenta.

To my parents, who have provided unlimited support and love throughout my life.

ACKNOWLEDGMENTS

Successful completion of this dissertation would not have been possible without the assistance of many others. I first would like to acknowledge my PI and mentor, Dr. Almudena Veiga-Lopez, who has enhanced my education by magnitudes solely through her mentorship and provided me with immeasurable support. Dr. Yong Pu, who started this journey with me, and the scientist whose work ethic I strive to emulate. The remainder of the Veiga-Lopez team for all their supporting efforts: Dr. Elvis Ticiani, Ms. Barbara Makela, Ms. Gabriela Saldana, Ms. Jiongjie Jing, and Dr. Cesar Rosales-Nieto. My graduate committee members for their professional input, career insight, and life advice: Dr. Margaret Petroff, Dr. Patricia Ganey, Dr. Stephanie Watts, Dr. James Luyendyk, and Dr. Robert Roth. My undergraduate students, who all have bright futures: Ms. Lindsay Hannah, Ms. Alysha Yoe, Ms. Madilyn Johnson, Mr. Alexander Mantey, Ms. Madison White, and Mr. Kwame Kannatey-Asibu. Finally, Michigan State University Sheep Teaching and Research Farm, Ms. Ramona Ehrhardt, and Dr. Jennifer Roberts for their help working with sheep.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AAVLD - American Association of Veterinary Laboratory Diagnosticians

ABC – ATP binding cassette transporter

ABCB1 - ATP-binding cassette transporter B1

AChE - acetylcholinesterase

ATBC - O-acetyl tributyl citrate

AUC - area under the curve

AUCfet - fetal area under the curve

AUCmat - maternal area under the curve

AUMC - area under the first moment curve

AUMCfet - fetal area under the first moment curve

AUMCmat - maternal area under the first moment curve

BCL-2 - B-cell lymphoma 2

BMP - bone morphogenetic protein

BPA - bisphenol A

BPF - bisphenol F

BPS - bisphenol S

ClTB - total body clearance

ClTB-mat - maternal total body clearance

Cmax - mat maximum observed maternal plasma concentration

Cmax - maximum plasma concentration observed

Cmax-fet - maximum observed fetal plasma concentration

COX2 - cyclooxygenase 2

CRH - corticotrophin releasing hormone

CTB - cytotrophoblast cells

CTNNAL - cadherin-associated protein

Cx32 - connexin 32

Cx43 - connexin 43

CYP - cytochrome P450

DAB - 3,3'-diaminobenzidine

DBT - dibutyltin

DCHP - dicyclohexyl phthalate 165

DCPAH - Diagnostic Center for Population and Animal Health

DDT - dichlorodiphenyltrichloroethane

DEHA - di(ethylhexyl) adipate

DEHP - di-2-ethylhexyl phthalate

DHP - dihexyl phthalate

DINCH - di(isononyl) cyclohexane 1,2-dicarboxylate

DPHP - di(2-propylheptyl) phthalate

EDC - endocrine disrupting chemicals

EGFR - epidermal growth factor receptor

ELISA - enzyme-linked immunosorbent assay

EM - exposure medium

enJSRV - envelop Jaagsiekte sheep retrovirus

EPA - U.S. Environmental Protection Agency

ER - endoplasmic reticulum

ERVW - endogenous retrovirus envelope genes

ESR1 - estrogen receptor 1

EVT - extravillous trophoblast cell

F/M - fetal to maternal ratio

FDA - U.S. Food and Drug Administration

Fet - fetal

FM - fire-master

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GCM1 - glial cell missing factor 1

GD - gestational day

GJIC - gap junction intercellular communication

hCG - human chorionic gonadotropin

HSD - hydroxysteroid dehydrogenase

HYAL2 - hyaluronoglucosaminidase 2

IS - internal standard

IUGR - intrauterine growth restriction

JNK - c-Jun N terminal kinase

LLE - liquid-liquid extraction

lncRNAs - long non-coding RNAs

LOAEL - lowest observed adverse effect level

MAPK - mitogen-activated protein kinase

Mat - maternal

MEHP - monoethylhexyl phthalic acid

miR - microRNA

MRT - mean residence time

MSU - Michigan State University

NCA - non-compartmental analysis

NDF - neutral detergent fiber

NICHD - Human Development of the National Institutes of Health

NIEHS - National Institute of Environmental Health Sciences

OH-PBDE - hydroxylated metabolites of PBDEs

PAG - pregnancy-associated glycoprotein

PAG1 - pregnancy-associated glycoprotein 1

PBDE - polybrominated diphenyl ethers

PBTK - Physiologically based toxicokinetic

PC-PLC - phosphatidyl choline-phospholipase C

PCB - polychlorinated biphenyls

PDE3a - phosphodiesterase 3a

PFBS - perfluorobutane sulfonate

PFC - perfluorinated compounds

PFHxS - perfluorohexane sulfonate

PFOA - perfluorooctonoic acid

PFOS - perfluorooctane sulfonate

PFOS - perfluorooctanesulfonic acid

PFUA - perfluoroundecanoic acid

PGE2 - prostaglandin E2

P-gp - placental glycoprotein

PKA - protein kinase A

PKC - protein kinase C

PKCα - protein kinase C-α subunit

PPARγ - proliferator-activated receptor γ

PSPB - pregnancy-specific protein B

RfD - reference dose

ROS - reactive oxygen species

rT3 - reverse 3,3’,5’-triiodothyronine

RXR - retinoid X receptor

SS - serum starvation

STB - syncytiotrophoblasts

t1/2 - chemical half-life

TBT - tributyltin

Tmax - time at which the Cmax is observed

TNF - tumor necrosis factor

USDA – U.S. Department of Agriculture

ΣDEHP - total maternal urinary metabolites for di-2-ethylhexyl phthalate

Introduction

This introduction, presented as the peer-reviewed article that follows (Gingrich et al. 2020), provides a holistic view of the current knowledge of placental effects upon endocrine disrupting chemical (EDC) exposures by reviewing epidemiological, *in vivo* and *in vitro* exposure data. EDCs chosen for this review include ubiquitous chemicals in the environment with available human biomonitoring data. These EDCs are found in daily-use cosmetics, consumer plastic goods, canned foods, dust, anti-fouling paints, industrial pesticides, and municipal, bottled and ground water. The breadth of these chemical classes is also wide, and includes bisphenols, phthalates, parabens, dichlorodiphenyltrichloroethane, organophosphates, organotins, polybrominated diphenyl ethers, polychlorinated biphenyls, and perfluorinated compounds. Placental endpoints summarized include: weight, apoptosis, steroidogenesis, spiral artery remodeling, drug-transporter expression, implantation and cellular invasion, lipid accumulation, fusion, proliferation, and oxidative stress.

Placenta disrupted: endocrine disrupting chemicals and pregnancy

Jeremy Gingrich[1,2], Elvis Ticiani[1], and Almudena Veiga-Lopez[1]

[1]Department of Animal Science, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan, USA

[2]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan, USA

Funding sources: Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institute of Health (R01ES027863 to A.V-L.), Michigan State University (MSU) General Funds, and MSU AgBioResearch. Funding for J.G. was supported in part

by the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health (NICHD) under Award Number T32HD087166. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

I.1. Introduction

Environmental chemicals and pregnancy outcomes

Over 86,000 chemicals are registered with the EPA through the Toxic Substances Control Act (EPA 2019), and many of which are considered endocrine disrupting chemicals (EDCs) as their exposure can alter normal endocrine function. Growing evidence supports the notion that these chemicals pose a risk to human health. Particularly concerning are exposures that occur during pregnancy whose effects on the developing fetus lead to long-term postnatal pathologies (Gore et al. 2015). Discrepancies between the volume of chemical produced annually and its detection in humans through biomonitoring studies (Figure I.1A) (Birnbaum and Cohen Hubal 2006, Petrovic et al. 2007, van den Berg 2009, Kelland 2010, Grube et al. 2011, Lim et al. 2011, Grun 2014, IARC 2016, Hassanzadeh 2017) highlights the fact that higher production does not necessarily translate to a higher human exposure. Chemicals used in everyday personal care products and plastics, such as phthalates, parabens, and bisphenols are detected in human circulation at the highest levels (Figure I.1B) (Whyat et al. 2003, Frederiksen et al. 2010, Li et al. 2013, Rantakokko et al. 2014, Pycke et al. 2015, Fisher et al. 2016, Yang et al. 2016, Veiga-Lopez et al. 2018, Zhang et al. 2018) compared to other high-volume production chemicals, like organophosphates, that are not as prevalent in human circulation.

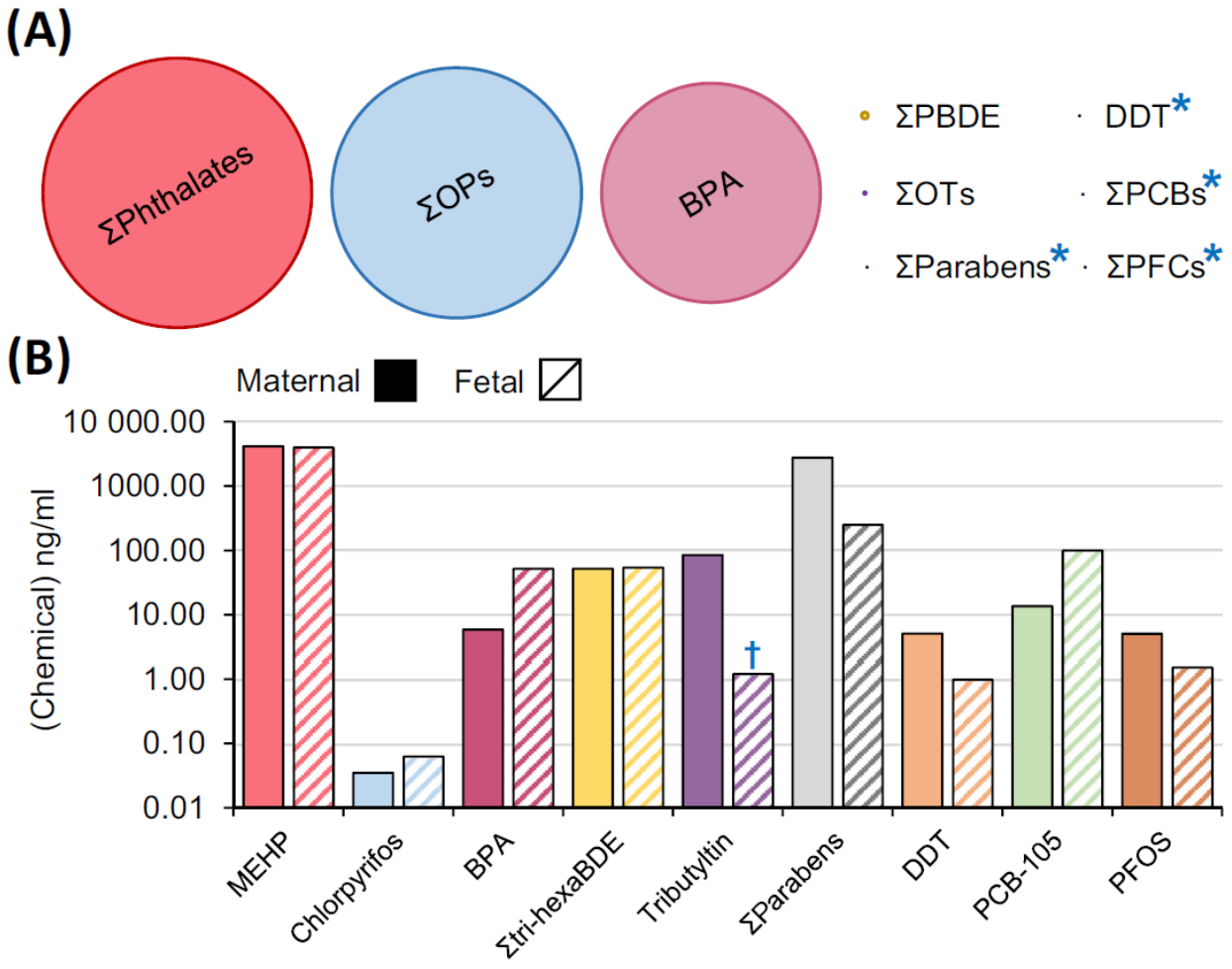

Figure I.1: Graphical representation of worldwide endocrine disrupting chemicals (EDC) production and paired maternal blood or urine and umbilical cord blood EDCs concentrations. (A) Graphical representation of worldwide endocrine disrupting chemicals (EDC) production. (B) Paired maternal (solid bars) blood or urine and umbilical cord blood (hatched bars) EDCs concentrations (B). EDCs included here have been demonstrated to alter placental function (see text for details and Figures I.2 and I.3 for a graphical summary). BPA: bisphenol A, Σtri-hexaBDE: sum of tri - hexa brominated diphenyl ethers, DDT: dichlorodiphenyltrichloroethane, PCB-105: polychlorinated biphenyl congener #105, PFOS: perfluorooctane sulfonate. Blue asterisk means that the size of the dot is the smallest visible size and

Figure I.1 (cont'd): thus larger than the total production volume, † means that concentration was taken from placental tissue due to lack of available maternal-fetal paired-matched data.

During pregnancy, women can be exposed to over 50 different chemicals in combination (Woodruff et al. 2011, Johns et al. 2017), stressing the need to understand the combined effects of the total chemical body burden during pregnancy. Epidemiological studies have begun to associate environmental chemical exposure to pathological pregnancy outcomes on birth weight, placental weight, and more recently pregnancy complications (Birks et al. 2016, Marsit 2016, Grindler et al. 2018). Whether this increased prevalence is related to the ever-increasing exposure to environmental chemicals (Skakkebaek et al. 2011) is a highly active area of research. Epidemiological evidence linking chemical exposures to hypertensive pregnancy disorders prevalence (Kahn and Trasande 2018) and pregnancy outcomes (Strakovsky and Schantz 2018) has been reviewed elsewhere. However, the evidence of chemical exposure outcomes in the context of placental dysfunction is at its infancy, which is the focus of this review.

The placenta, a transient, vulnerable organ

Pregnancy is a vulnerable period for fetal and maternal health due to the dynamic nature of the developmental and tissue remodeling processes. Pregnancy complications occur in ~19% of pregnancies (Cavazos-Rehg et al. 2015), and include disorders like gestational diabetes, gestational hypertension, preeclampsia, eclampsia, preterm birth, and placenta percreta spectrum disorders. The prevalence of pregnancy complications such as hypertension and postpartum hemorrhaging have steadily increased over the past few decades (CDC 2019), pointing to environmental exposures as one of the potential contributors to this increasing prevalence (Varshavsky et al. 2019).

The placenta is a transient multifunctional organ necessary for fetal development that facilitates cholesterol and steroid biosynthesis, and chemical metabolism and transport. Multiple factors including nutrition, stress, and maternal diseases can result in inadequate placental development (Fowden et al. 2015), causing harmful long-lasting effects to the fetus, including cardiovascular and metabolic diseases (Burton et al. 2016, Marciniak et al. 2017). Of fetal origin, the placenta develops from the trophectoderm layer of the blastocyst, comprised of stem cells known as cytotrophoblast cells (CTBs). In humans, around day 10 of pregnancy, CTBs begin to differentiate into two functionally different paths, invasion or syncytialization (Cross et al. 1994). What determines CTB differentiation to either pathway is still not fully understood, but may be due to gene regulation, epigenetic changes (Kwak et al. 2019), secretory peptides (Morrish et al. 2001), or distinct stem cell populations (James et al. 2005). Invasive CTBs, known as extravillous trophoblasts (EVTs), migrate away from the primary trophectoderm bundle, forming an anchoring villus (Figure I.2). EVTs embed themselves in the maternal uterine lining and upon invasion, act by widening the spiral arteries to increase blood flow to the endometrial space where implantation occurred. As gestation progresses, endometrial vessel remodeling is required to perfuse the main body of the placenta and the developing fetus with maternal blood (Figure I.2). Dysregulation in EVT invasion can result in placental defects such as placenta accreta, increta, or percreta, which may result in miscarriage and postpartum hemorrhaging (Shamshirsaz et al. 2019). Concurrently, CTBs begin to terminally fuse, forming a multinucleated syncytium of cells known as syncytiotrophoblasts (STBs) located above the CTB layer, which continue to proliferate and fuse. STBs line the placental villi and act as a barrier for direct maternal blood exposure to the fetus. Functionally, STBs secrete progesterone, human chorionic gonadotropin (hCG) and other proteins (Guller 2009, Kumar and Magon 2012, Yabe et al. 2016). Abnormal or poor syncytialization can impair pregnancy through the loss of progesterone production, and has been implicated in abnormal birth pathologies such as preeclampsia and intrauterine growth restriction (Wu et al. 2015). Importantly, the syncytium facilitates gas and nutrient

exchange between mother and fetus, serving as a semi-permeable barrier for fetal chemical exposures (Fowden et al. 2015).

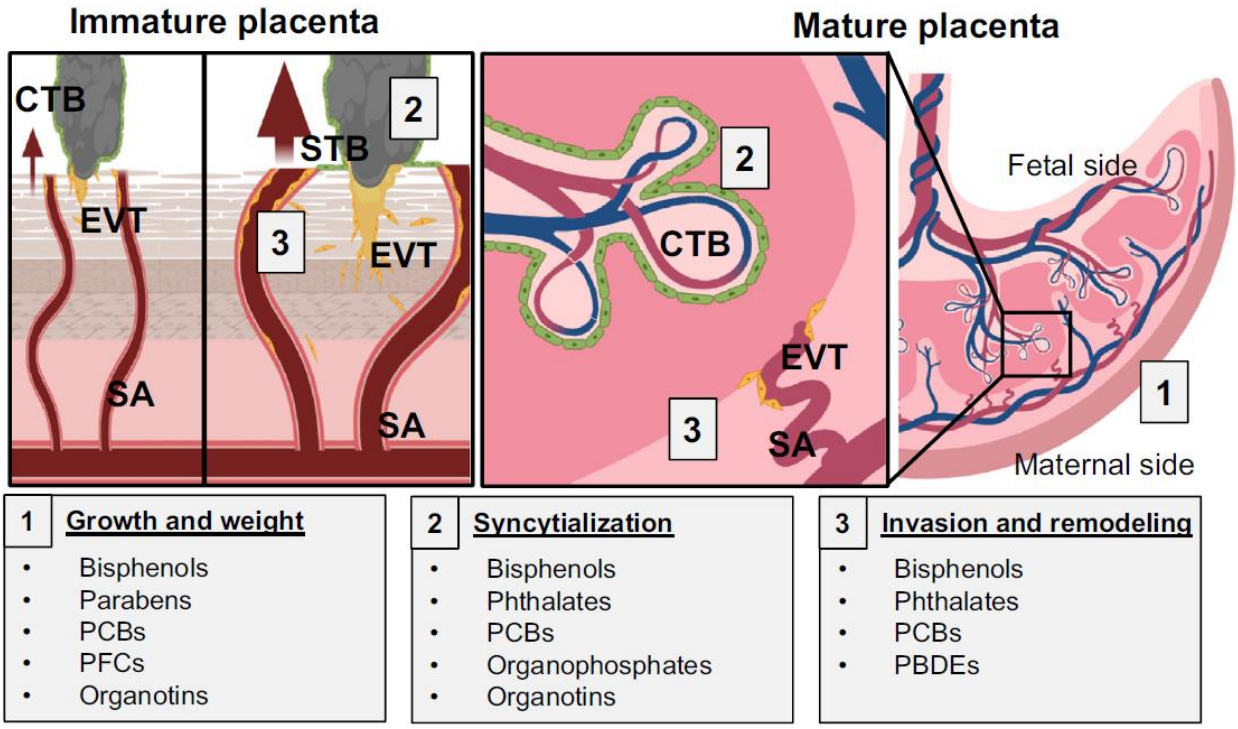

Figure I.2: Overview of developmental, anatomical, and histopathological sites in the placenta affected by endocrine disrupting chemical (EDC) exposure. Overview of developmental, anatomical, and histopathological sites in the placenta affected by endocrine disrupting chemical (EDC) exposure. In the immature placenta (left), cytotrophoblasts (CTB; grey) may differentiate into two distinct lineages: invasive extravillous trophoblasts (EVT; yellow), and barrier syncytiotrophoblasts (STB; green). EVTs invade into maternal tissues and allow increased maternal blood perfusion (arrows) towards the placenta through spiral artery (SA) remodeling. In the mature placenta (right), CTBs replenish the STB population, and EVT-remodeled SAs bathe the fetal villi in maternal blood. EDC-induced alterations include: placental gross mass/wet weight (grey box 1), CTB fusion/syncytialization (grey box 2), and EVT invasion and SA remodeling (grey box 3). PBDEs: polybrominated diphenyl ethers, PCBs: polychlorinated biphenyls, and PFCs: perfluorinated compounds.

I.2. Endocrine disrupting chemicals and the placenta

The high abundance of steroid hormone receptor expression in the placenta (Fowden et al. 2015) make it especially vulnerable to endocrine disruption. Research focused to understand the effect of EDCs on placental development has steadily increased over the past 5 years and is presented here by chemical class. Chemical classes included in this review have 1) available biomonitoring data, 2) a reported chemical-induced placental defect and 3) reported congeners that are able to cross from maternal circulation - through the placenta - into fetal circulation (Whyatt et al. 2003, Hogberg et al. 2008, Sapbamrer et al. 2008, Correia-Carreira et al. 2011, Liu et al. 2011, Chen et al. 2017, Tang and Zhai 2017, Kolatorova et al. 2018, Li et al. 2018, Zbucka-Kretowska et al. 2019). As data on heavy metal exposures, like with cadmium, on placenta-specific outcomes is extensive and has been previously summarized (Caserta et al. 2013, Geng and Wang 2019), those compounds were excluded from this review. World-wide human exposure levels to these chemicals have been previously summarized (Veiga-Lopez et al. 2018). Overviews of anatomical sites on the placenta affected by EDC exposure, and their subsequent mechanisms and outcomes are summarized in Figures I.2, I.3, and I.4, respectively.

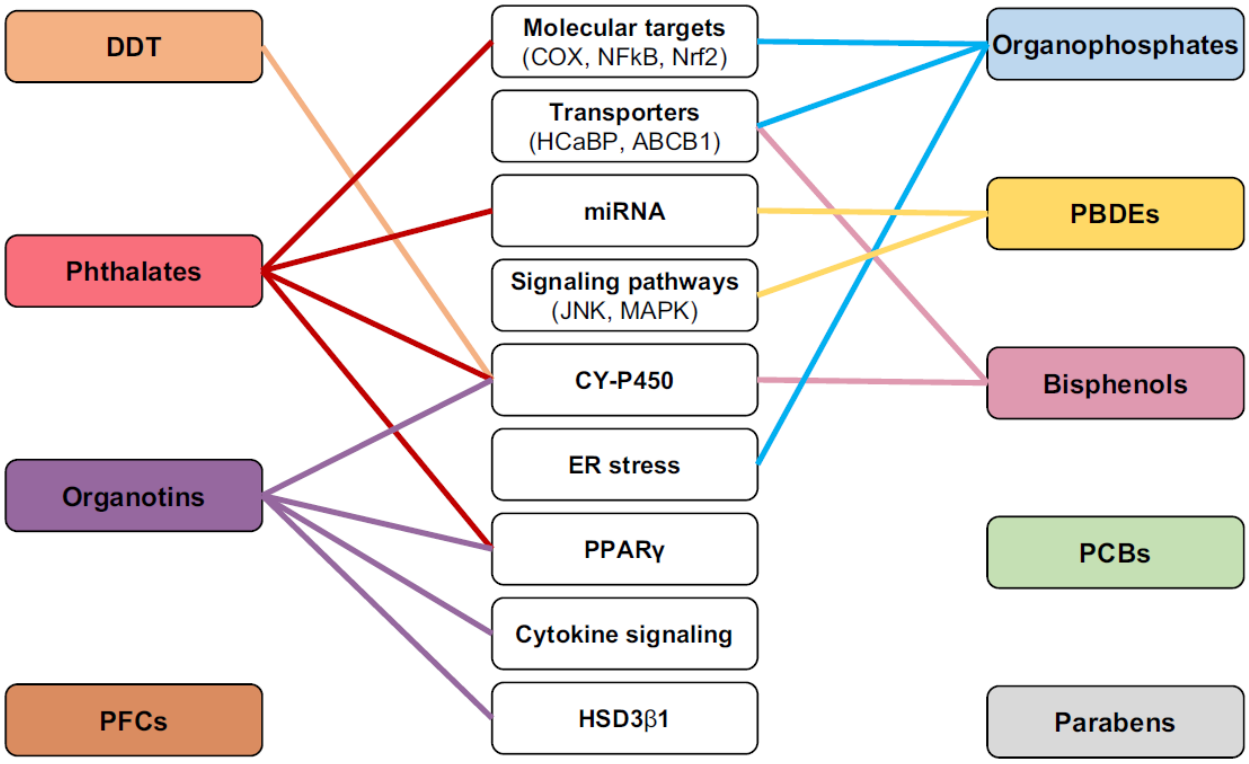

Figure I.3: Summary of mechanisms of action of endocrine disrupting chemicals on the placenta.

Summary of mechanisms of action of endocrine disrupting chemicals (EDCs) on the placenta. EDCs reviewed are listed in left and right-side boxes. Potential effectors are shown in center boxes (transporters, signaling pathways, transcriptions factors, enzymes, endoplasmic reticulum stress, and microRNA). PFCs, PCBs, and parabens have no identified mechanisms of action in the context of placental outcomes. See text for additional details. ABCB1: ATP-binding cassette sub-family B member 1, COX: cyclooxygenase, CY-P450: cytochromes P450, DDT: dichlorodiphenyltrichloroethane, ER: endoplasmic reticulum, HCaBP: calcium-binding protein, HSD3β1: hydroxy-Δ-5-steroid dehydrogenase 3-β and steroid Δ-isomerase 1, JNK: c-Jun N terminal kinase, MAPK: p38/mitogen-activated protein kinase, miRNA: microRNA, NFκB: nuclear factor κ-light-chain-enhancer of activated B cells, Nrf2: nuclear factor erythroid 2-related factor 2, PBDEs: polybrominated diphenyl ethers, PCBs: polychlorinated biphenyl, PFCs: perfluorinated compounds, PPARγ: peroxisome proliferator-activated receptor γ.

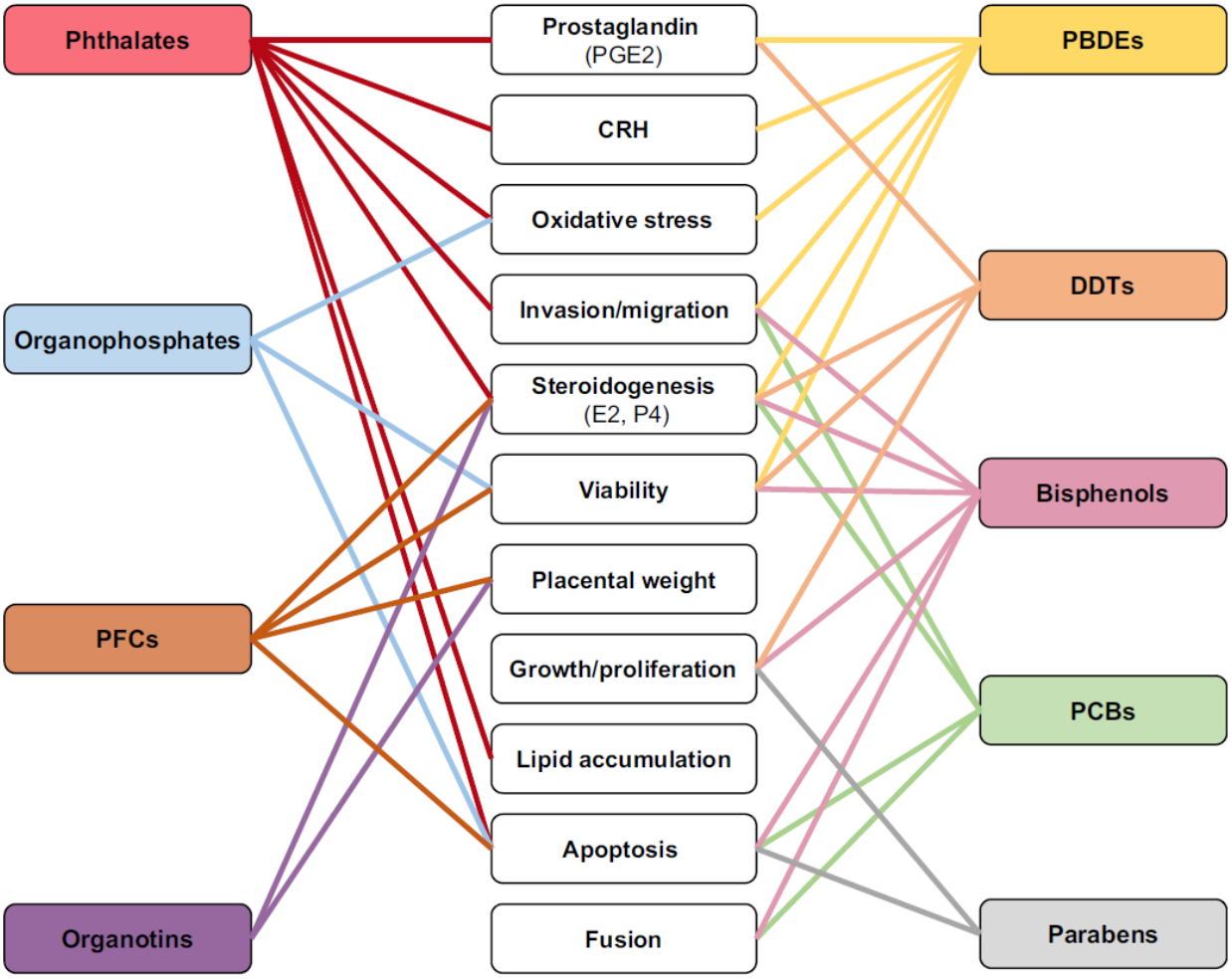

Figure I.4: Summary of functional placental disruptions linked to endocrine disrupting chemical exposures on the placenta. Summary of functional placental disruptions linked to endocrine disrupting chemical (EDC) exposures on the placenta. EDCs reviewed are listed in left and right-side boxes Potential outcomes are shown in center boxes (hormones production, oxidative stress, invasion/migration, viability, placental weight, cell proliferation, lipid accumulation, apoptosis, and fusion). See text for additional details. DDT: dichlorodiphenyltrichloroethane, PBDEs: polybrominated diphenyl ethers, PCBs: polychlorinated biphenyl, PFCs: perfluorinated compounds, CRHs: corticotropin-releasing hormone, E2: estradiol, P4: progesterone, PGE2: prostaglandin E2.

Bisphenols

Bisphenols are man-made chemicals widely used in the production of polycarbonate plastics, epoxy resins, and thermal receipt paper (Konieczna et al. 2015, Eckardt and Simat 2017). With the exception of bisphenol A (BPA), the effect of bisphenol exposure on the development of the placenta is not well established. Although BPA biomonitoring exposure levels may have been underestimated to date (Gerona et al. 2019), epidemiological data demonstrate a positive association between total BPA concentration in the placenta and placental global methylation (Nahar et al. 2015). Lower birth weight has also been linked to a higher ratio of BPA concentration in the amniotic fluid *vs*. maternal plasma in pair-matched samples (Zbucka-Kretowska et al. 2019), suggesting that an individual's placental permeability to bisphenols may be one of the defining factors driving exposure levels and subsequent outcomes.

In vivo exposure to BPA during pregnancy has been studied in doses ranging from 0.002 to 200 mg/kg/day across pregnancy. Much of these data have been already reviewed (Vrooman et al. 2016, Strakovsky and Schantz 2018), and highlights BPA's impact on inducing placental cell apoptosis, labyrinth layer loss, and altered expression of nuclear hormone receptors. Importantly, both intrauterine growth restriction (IUGR)-like (Muller et al. 2018) and pre-eclampsia-like phenotypes (Ye et al. 2019) have been reported, and were hypothesized to result from aberrant spiral artery remodeling (Figure I.2). At higher doses (BPA: 0.5 mg/kg/day), an IUGR-like phenotype accompanied by placental inflammatory changes has been recently reported in sheep (Song et al. 2019). Studies regarding placenta-specific outcomes following exposure to BPA-analogues, or "replacement" chemicals remain scarce. A single study has reported a placental defect following exposure to bisphenol S (BPS: 0.5 mg/kg/day) (Gingrich et al. 2018) with a reduction in binucleate cells, the sheep homologue of human STBs and hypothesized to occur through a cell fusion defect (downregulation of e-cadherin) (Gingrich et al. 2018). Despite lower world-

wide exposures to BPS than BPA (Lehmler et al. 2018), a recent toxicokinetic study reported a prolonged half-life in fetal circulation (Gingrich et al. 2019).

In vitro, BPA exposure at 1,000 µM can either reduce (Wang et al. 2015) or increase (Ponniah et al. 2015) cell proliferation in the choriocarcinoma cell line BeWo - an *in vitro* model of syncytialization. Functionally, BPA exposure in human metastatic choriocarcinoma-derived JEG-3 cell line (doses: 0.1 - 50 µM) reduced estrogen synthesis (Huang and Leung 2009, Xu et al. 2019) and altered cytochrome P450 (CYP) enzymatic activity (Nativelle-Serpentini et al. 2003) and protein expression (CYP11A1 (Chu et al. 2018); CYP19 (Marqueno et al. 2019); and CYP1A1 (Xu et al. 2019)) (Figure I.3). These exposure conditions disrupted hormone signaling via a reduction in corticotropin gene expression (Basak et al. 2018). Of the emerging BPA-analogue compounds (Chen et al. 2016), only BPS exposure has been studied for placenta-specific outcomes. BPS reduces the activity of the transport protein ATP-binding cassette transporter (ABC) B1 in CRL-1584 cells, a transformed placental epithelial cell line (0.5 nM) (Speidel et al. 2018). In contrast, BPA can directly stimulate ABCB1 expression in the choriocarcinoma cell line BeWo (10 µM) (Jin and Audus 2005), leading to an increase in drug efflux. *In vitro* BPA's effect on EVTs invasiveness and apoptosis has been reviewed (Yang et al. 2019). However, the mechanism of action of BPA on EVT invasiveness has not been fully elucidated (Yang et al. 2019). BPA has also been shown to have epigenetic effects, increasing microRNA expression at relatively high doses (25 ng/µl BPA) in the EVT cell-line HTR8-SVneo (Avissar-Whiting et al. 2010). Despite this breadth of *in vitro* data demonstrating that bisphenols can have placenta-specific effects, epidemiological evidence supportive of such bisphenol-induced placental dysfunctions in humans remains lacking.

Phthalates

Phthalates are ubiquitous chemicals present in a myriad of consumer and personal care products, pesticides, and solvents. Found in ~100% of humans tested (Silva et al. 2004, Marsee et al. 2006, Hogberg et al. 2008) phthalates tend to be higher in females compared to males (James-Todd et al. 2012). Total maternal urinary metabolites for di-2-ethylhexyl phthalate (ΣDEHP) have been inversely associated with placental weight at term in U.S. and E.U. cohorts, suggestive of placental insufficiency (Mustieles et al. 2019, Philippat et al. 2019). ΣDEHP urine concentrations are also higher in IUGR pregnancies (Zhao et al. 2015), and associated with lower expression of trophoblast differentiation genes (Adibi et al. 2010) and various long non-coding RNAs (lncRNAs) with unknown placental function (Machtinger et al. 2018). Phthalate exposure during the first trimester has also been negatively associated with the expression and methylation of the epidermal growth factor receptor (EGFR) in placental tissue (Grindler et al. 2018). Given that EGFR is most abundant in placental tissue compared to any other tissue and the role of EGFR in human placental development (Whigham et al. 2019), the implications of these epigenetic modifications on placental pathology should be investigated.

In vivo data on the effects of phthalate exposure on placental function is limited to rodents and oral exposure to the most historically produced phthalate (vom Saal et al. 2008), di-2-ethylhexyl phthalate (DEHP), although other phthalates such as dihexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) have also been investigated (Ahbab et al. 2017). Phthalate exposure regimens span gestation, in most cases starting at vaginal plug detection, and use doses ranging from 20 to 1,500 mg DEHP/kg/day. Data from these studies has been previously reviewed (Vrooman et al. 2016, Strakovsky and Schantz 2018). In brief, all histopathological observations reported a reduction in the placental labyrinth layer which is analogous to the human syncytium, containing spongiotrophoblast cells similar in function to human STBs (Bradbury 2008). Reduction in the STB population in human placentas has been linked to the

pregnancy complication preeclampsia, and is a noted a defect in the placentas from IUGR pregnancies (Wu et al. 2015). Gene expression changes in the placenta following DEHP exposure reflect altered fatty acid homeostasis, apoptosis, and angiogenesis which is accompanied by irregular vessel formation in the labyrinth resulting in an IUGR-like phenotype (Yu et al. 2018) (Figure I.2). Importantly, dosing regimens in the above-mentioned studies exceed not only the estimated population daily intake (Koch et al. 2003), but both the U.S. Environmental Protection Agency's (EPA) reference dose (RfD) and the E.U. Scientific Committee for Toxicity, Ecotoxicity and the Environment's the tolerable daily intake for DEHP by, in some cases, over three orders of magnitude (Koch et al. 2003). This highlights the need to conduct studies that better reflect environmentally relevant exposure levels in human pregnancies.

Although non-cytotoxic at doses up to 500 µM (Perez-Albaladejo et al. 2017), *in vitro* studies for the DEHP metabolite monoethylhexyl phthalic acid (MEHP) can induce apoptosis, and increase reactive oxygen species production and DNA damage in HTR-8/SVneo cells (Strakovsky and Schantz 2018) (Figure I.4). Of note, H_2O_2-induced oxidative stress alters the expression levels of miRNAs and mRNA expression of genes involved in placental development (Cross et al. 2015), an effect also observed with miR-16 (Meruvu et al. 2016b), which plays an important role in MEHP-induced trophoblast cell apoptosis by decreasing B-cell lymphoma 2 (BCL-2) expression (Meruvu et al. 2016b). Additionally, in human CTBs, MEHP exposure inhibited hCG production (Shoaito et al. 2019), but enhanced mRNA expression of corticotrophin releasing hormone (*CRH*) (Wang et al. 2016). Marked accumulation of glycerolipids and glycerophospholipids in the rat trophoblast cell line HRP1 (Xu et al. 2006) coupled with altered lipid metabolism in JEG-3 cells (Petit et al. 2018) also points to a potential phthalate-induced placental lipid imbalance. Although the effect of an altered placental lipidome is not yet understood (Petit et al. 2018), glycerolipids and glycerophospholipids can inhibit receptor binding of progesterone and estrogen (Pulkkinen and Hamalainen 1995). An enhanced inflammatory response following MEHP exposure was

also noted in primary isolated placental macrophages (180 μM) (Tetz et al. 2015) and human CTBs (Wang et al. 2016) through an increase in cyclooxygenase 2 (*COX2*) mRNA expression and protein abundance (Figure I.3). Even though the mechanisms underlying most of these phenotypes remain elusive, MEHP-induced inflammatory responses appear to be driven through peroxisome proliferator-activated receptor γ (PPARγ) activation (Adibi et al. 2010), of which MEHP has been shown to be a high affinity ligand (Hurst and Waxman 2003). Furthermore, it has been demonstrated that HTR-8/SVneo cell invasion is reduced upon MEHP exposure (Strakovsky and Schantz 2018).

Similar to other EDCs, the occurrence of phthalates in humans is in a mixture (Adibi et al. 2017). Phthalates in mixture have been shown to act through PPARγ in a sex-specific manner, and are hypothesized to be PPARγ agonists in females and antagonists in males using primary isolated CTBs (Adibi et al. 2017). Although phthalates are among the best studied chemicals in the context of placental function, emerging chemicals in DEHP-free plasticizers, such as di(isononyl) cyclohexane 1,2-dicarboxylate (DINCH), di(2-propylheptyl) phthalate (DPHP), di(ethylhexyl) adipate (DEHA), and *O*-acetyl tributyl citrate (ATBC) (Salthammer 2020) have yet to be evaluated.

Parabens

Generally recognized as safe by the U.S. Food and Drug Administration (FDA), parabens are used as antimicrobial agents in personal care products (Halla et al. 2018). To our knowledge, a single epidemiologic study has evaluated placenta-specific outcomes, detecting a placental growth defect via a positive association between total maternal urinary paraben levels and placental weight (Philippat et al. 2019) (Figure I.2). *In vivo* pregnancy exposure studies are restricted to a pharmacokinetic study in pregnant rats where a 3-fold higher concentration of ethylparaben was observed in the placenta *vs*. the fetal liver (Frederiksen et al. 2008), suggestive of placental accumulation. Additionally, in humans, the

negative association observed between cord blood ethylparaben and testosterone concentrations points to a potential risk for prenatal development (Kolatorova et al. 2018). A recent *in vitro* study using HTR-8/SVneo cells reported that butylparaben exposure inhibits cell proliferation and induces both apoptosis and endoplasmic reticulum stress (200 µM) (Yang et al. 2018) (Figure I.4). However, the specific molecular mechanism(s) of how paraben exposure results in these outcomes remains unexplored. As world-wide exposure to parabens during pregnancy is second most only to phthalates (see Figure I.1B), the large discrepancy between known gestational exposure outcomes between EDC classes is likely driven by the fact that parabens are generally recognized as safe. This provides a gap in knowledge worth evaluating, especially in combination with other common chemical exposures.

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are man-made organic chemicals used in the production of electrical equipment and building materials, and contain over 200 known congeners (EPA 2003). Despite being banned in the U.S. since 1979, many products manufactured earlier still contain PCBs and thus, exposures continue to occur to this day (Mulle et al. 2019). Because PCBs were used in the form of chemical mixtures (trademark examples include: Arochlor, Clorphen, or Phenochlor) that included several PCB congeners, PBCs were among the first chemicals evaluated as EDC mixtures (Baker et al. 1977). The 10-15 years half-life estimated for PCBs results in long-term exposure in humans. Given that PCBs can cross the placental barrier (Correia-Carreira et al. 2011) the developing fetus is at risk of PCB exposure. Epidemiologically, PCBs concentrations in the placentas from the Japan Environment and Children's Study cohort have been associated with a decrease in syncytiotrophoblast volume in the placenta and elevated placental growth factor (PIGF) expression, which stimulates placental vessel branching and spiral artery remodeling (Tsuji et al. 2013). Importantly, birth weight has been inversely

correlated with placental PCB concentrations in a Chinese cohort (Wu et al. 2011), an effect that could be attributed to placental disruption.

Gestational exposure to over two magnitudes of the EPA's RfD for PCBs ((20 ng/kg BW/day; (EPA 2017)) disrupts the placental labyrinth layer in rats (20 µg/kg BW/day, PCB-126 (Ahmed et al. 2018)) and minks (0.65 mg/day in feed, Clophen A50 (Backlin et al. 1998)). In minks, this effect was combined with altered spiral artery remodeling resulting in fetal growth retardation or demise (Backlin et al. 1998). However, despite the estrogenic, antiestrogenic, or androgenic effects of PCBs (Drenth et al. 1998, Svobodova et al. 2009), human studies have reported no association between PCB exposure and the risk of spontaneous abortion and/or stillbirth (Small et al. 2007). *In vitro* models using BeWo cells have demonstrated placental transfer of PCBs with transfer speeds differing across PCB congeners (i.e. PCB-180 transfers more rapidly than PCB-52) (Correia-Carreira et al. 2011). PCB mixtures can induce trophoblast cell apoptosis through upregulation of the adaptive immune response (PCB mixtures #77, #126, and #169: 40-120 µmol/l (Gu et al. 2012)), disrupt invasion in HTR-8/SVneo cells (10 µg/ml Aroclor 1254 (Kalkunte et al. 2017)), and induce anti-angiogenic effects at the maternal-fetal interface (Aroclor 1254 (Kalkunte et al. 2017)) (Figure I.4). Bovine placental explants exposed to different doses of a PCB mixture (PCB-153, PCB-126, and PCB-77; 1-100 ng/ml) report increased connexin 43 (Cx43) and 32 (Cx32) expression (Wojciechowska et al. 2018); of which Cx43 is involved in the intercellular communication required for placental cell fusion (Cronier et al. 2003). Most PCB congeners are formulated for use as a mixture. This, coupled with the fact that PCBs have an accumulative environmental persistence, identifying any placenta-specific mechanisms responsible for exposure outcomes remain amongst the most challenging.

Perfluorinated compounds

Produced since the 1950s, perfluorinated compounds (PFCs) are used in the production of antifouling paints, non-stick cookware, and waterproof clothing (Corsini et al. 2014). While perfluorooctonoic acid (PFOA) and perfluorooctane sulfonate (PFOS) are the most commonly studied PFCs, others persist in the environment (Dorman F 2012). Using pair-matched maternal blood, cord blood, breast milk, and placental samples, epidemiological studies have demonstrated that all PFCs examined (PFOS, PFOA, PFDA, and PFTrDA) are able to enter fetal circulation both prenatally through placental transport, and postnatally during lactation (Liu et al. 2011, Chen et al. 2017). Recently, umbilical cord blood levels of PFCs perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS) and perfluoroundecanoic acid (PFUA) from a Chinese birth cohort have all been positively associated with preeclampsia (Huang et al. 2019). Because of PFCs cumulative nature and long half-lives (~3.4 and ~2.7 years for PFOS and PFOA, respectively (Li et al. 2018)), pregnancy exposures are of particular concern. Limited animal studies include mice (Lee et al. 2015) and rat (Li et al. 2016) PFOS exposure through oral gavage (8 - 20 mg/kg/day) during mid-to-late gestation resulting in reduced fetal and placental weights accompanied with placental necrosis (Lee et al. 2015) (Figure I.2), an increase in fetal serum corticosterone (Li et al. 2016), and an inhibition of placental 11-β-hydroxysteroid dehydrogenase (HSD) activity (Li et al. 2016). However, doses used were 6 magnitudes higher than EPA's RfD (20 ng/kg BW/day) for both PFOS and PFOA (EPA 2016, EPA 2016b) .

In vitro, PFOS modulates steroid hormone signaling by suppressing aromatase production, estradiol secretion, and progesterone production in a concentration-dependent manner in primary isolated human CTBs with effects noted at doses as low as 0.001 µM (Zhang et al. 2015) (Figure I.4). Aromatase inhibition was also observed in JEG-3 cells after PFOS, PFOA and PFBS exposure (IC50: 57 - 80 µM (Gorrochategui et al. 2014). PFOS additionally led to decreased cell viability (Gorrochategui et al. 2014,

Zhang et al. 2015) and induction of apoptosis (Zhang et al. 2015) in the same cell lines (Figure I.4). A perfusion model using human placental explants has reported a negative correlation between the organic anion uptake transporter OAT4 and fetal PFOA transfer (Kummu et al. 2015), demonstrating the protective potential of placental OAT4 against fetal PFC exposure. Most *in vivo* data use rodents, which have been shown to eliminate PFCs more rapidly than humans (Filgo et al. 2015), making them a less than ideal animal model for gestational PFC exposures. Despite the concerns raised from *in vitro* experiments using human cell lines, studies that focus on lower, more physiologically relevant dosing strategies are necessary to further the toxicological evaluation of PFCs.

Organophosphates

Organophosphates are esters of phosphoric acid used as insecticides due to their direct interference with the neurotransmitter acetylcholinesterase (AChE), causing systemic muscle paralysis (Colovic et al. 2013). To our knowledge, no epidemiologic studies exist reporting placenta-specific outcomes in association with organophosphates exposure. However, the assessment of life-long or past organophosphate exposures appears to be a crucial limitation to epidemiologic studies because organophosphates are not persistent in the human body (Blanc-Lapierre et al. 2013). Despite the existence of over 40 organophosphates, placenta-specific outcomes evaluated are restricted to chlorpyrifos and methyl parathion. Limited mid-to-late gestation exposures (GD 9 - 21) in rats, high dose exposures (10 - 30 mg/kg) to either chlorpyrifos, methyl parathion, or a mixture of the two, inhibited placental AChE activity (Abu-Qare et al. 2001). Gestational exposure to only methyl parathion (1 - 2 mg/kg) reduced the trophoblast giant cell population, and increased phagosome vacuoles in the labyrinth layer (Levario-Carrillo et al. 2004) (Figure I.2). To note, these cytotoxic findings occurred at doses within two magnitudes of the EPA established RfD for human exposure to organophosphates (0.025 - 100 µg/kg/day (NCBI 1999)).

Chlorpyrifos exposure, even at micromolar concentrations, is also cytotoxic in human placental choriocarcinoma cells (JEG-3 (Guinazu et al. 2012)) (Figure I.4) and can induce apoptosis in JAR cells through tumor necrosis factor (TNF) modulation (Saulsbury et al. 2008) (Figure I.3). However, not all studies have reported cytotoxicity, even with the same cell line (JEG-3) (Ridano et al. 2012). Chlorpyrifos also altered expression of pregnancy maintenance markers such as the ABC transporter ABCG2, the transcription factor GCM1 (glial cells missing transcription factor 1) and hormone subunit β-hCG (Ridano et al. 2012), but not progesterone or estradiol production (Rieke et al. 2014). Additionally, enhanced reactive oxygen species (ROS) production (Chiapella et al. 2013) and upregulation of endoplasmic reticulum (ER) stress-related proteins (Reyna et al. 2017) occurred after chlorpyrifos exposure in JEG-3 cells. Attenuation of chlorpyrifos-induced oxidative stress (Chiapella et al. 2013) and ER stress (Reyna et al. 2017) occurs through adaptive activation of the Nrf2-antioxidant response element signaling pathway.

One major limitation in the study of the effects of organophosphates exposures is that they are commonly found in mixtures, such as the flame-retardant mixture fire-master (FM) 550, for which the exact composition and RfD are not available. FM 550 accumulates in the placenta to a greater extent in males than females (Baldwin et al. 2017), but no association with developmental outcomes has yet been reported. Dosing regimens used in these studies (300 or 1,000 µg/kg/day FM 550 (Baldwin et al. 2017, Rock et al. 2018)) are both within the RfD range for organophosphates. Due to the sex-specific accumulative nature of FM 550 in the placenta, outcomes following exposure to other organophosphates chemical mixtures as well as long-term fetal exposure outcomes should be further evaluated.

Dichlorodiphenyltrichloroethane

Dichlorodiphenyltrichloroethane (DDT) is an insecticide whose use has been banned in the U.S. since 1973 due to its environmental persistence (biological half-life: ~7 years). Despite concerns over its estrogenic properties, accumulation in adipose tissue, potential carcinogenicity, and developmental neurotoxicity (Turusov et al. 2002), DDT continues to be used for malaria outbreaks in developing countries (Bouwman et al. 2011, van den Berg et al. 2017). DDT crosses the placenta and enters fetal circulation (Ando 1978, Sapbamrer et al. 2008). Epidemiologically, exposure to DDT has been associated with maternal hypertensive disorders (Murray et al. 2018). Associations of DDT and birth weight are cohort dependent with an inverse correlation in a Saudi Arabian cohort (Al-Saleh et al. 2012), and a positive (Kezios et al. 2013) or no correlation (Farhang et al. 2005) in two U.S. cohorts. Despite a breadth of knowledge on human health effects following DDT exposure (Longnecker et al. 1997, Merida-Ortega et al. 2019), to our knowledge, no available *in vivo* studies exploring gestational DDT exposure focus on placenta-specific outcomes. *In vitro*, DDT exposure results in decreased placenta cell viability at doses higher than 25 μM (HTR-8/SVneo (Dominguez-Lopez et al. 2012)) but did not alter proliferation at lower doses (1 nM) (Derfoul et al. 2003) (Figure I.4). In bovine placental explants, DDT increased the explant's expression of prostaglandin E2 (PGE2) synthase, 3β-HSD, and CYP11A1 (doses: 1, 10 or 100 ng/ml (Wojciechowska et al. 2017)) (Figure I.3). Alterations in enzyme expressions were accompanied by an increase in oxytocin, estradiol, and progesterone secretion (Wojciechowska et al. 2017). These effects were also observed in human placental explants (Wojtowicz et al. 2007, Wojtowicz et al. 2008) along with inhibition of aromatase activity (Wojtowicz et al. 2007). Given the role of intercellular communication in placental syncytium formation (Cronier et al. 2003), DDT exposure on connexin protein expression was tested, but no effect was observed (Wojciechowska et al. 2018). Overall, DDT exposure reduces the secretory activity of the placenta, and given its carcinogenic effect (Harada et al.

2016) and continued commercial use (van den Berg et al. 2017), further understanding of its effect upon gestational exposure - specifically on the placenta - is warranted.

Polybrominated diphenyl ethers

Similar in chemical structure to PCBs, polybrominated diphenyl ethers (PBDEs) are persistent chemicals used as flame retardants in paints, plastics, electrical equipment, and textiles (Siddiqi et al. 2003). Of the 209 known PBDE congeners (Accustandard 2017), less brominated congeners such as tetra- and penta-BDEs have a high affinity for lipids and tend to accumulate in animals, suggestive of a greater toxic potential (Siddiqi et al. 2003). PBDEs can be found in human breast milk, cord blood, and placental tissue (Tang and Zhai 2017) where they tend to bioaccumulate (Leonetti et al. 2016) and get transferred to the fetus (Frederiksen et al. 2010, Frederiksen et al. 2010b, Zhao et al. 2013). Interestingly, PBDE concentrations have been reported to be up to two-fold higher in the placentas of males than females (Leonetti et al. 2016b). Cord blood concentrations of PBDEs have been negatively correlated with birth weight (Zhao et al. 2017), and inversely correlated with placental DNA methylation changes in human pair-matched samples (Zhao et al. 2016, Kim et al. 2018), with changes specific to PBDE congener and methylation site (tetraBDE-66, *LINE1*; hexaBDE-153, *NR3C1* and *IGF2*; decaBDE-209, *IGF2*) (Zhao et al. 2016). Placenta PBDE concentrations have also been positively associated with changes in microRNA (miR)-188-5p and miR-1537 expression (decaBDE-209, (Li et al. 2015)). Both miRs have unknown roles in the placenta, but miR-188-5p is abnormally upregulated in pre-eclamptic placentas (Yang et al. 2015), providing with a potential biomarker for early detection of pregnancy complications. Despite the fact that PBDE accumulates in the fetal portion of the placenta (Ruis et al. 2019), no animal studies exploring placental effects of PBDEs are available. Gestational PBDE exposure in rats results in reduced weight at birth, and has been linked to a loss in maternal triiodothyronine production (Ruis et al. 2019).

PBDE exposure is cytotoxic in second trimester human CTBs at doses over 10 mM (BDE-47 and BDE-99 (Robinson, et al. 2019)), significantly reducing cell viability and leading to apoptosis (Robinson et al. 2019) (Figure I.4). BDE-47 at the same dose also reduced the migration and invasion of CTBs, and altered lipid and cholesterol metabolism (Robinson et al. 2019). BDE-47 is the most studied PBDE in the context of placental function, with reported effects on oxidative stress (Park and Loch-Caruso 2014) that result in an increase in PGE2 production in HTR-8/SVneo cells (Lee et al. 2015). Exposure to PBDE mixtures (congeners: 47, 99, and 100) also resulted in higher PGE2 production in second trimester placental explants (Peltier et al. 2012). In JEG-3 cells, from doses as low as 0.5 nM and in a dose dependent manner, BDE-47 increased CRH production (Zhu et al. 2017), which has been associated with premature delivery in humans. This same dose-dependent effect was observed in the mRNA expression of signal transduction proteins like protein kinase C-α subunit (*PKCα*), c-Jun N terminal kinase (*JNK*), and p38/mitogen-activated protein kinase (*MAPK*) phosphorylation (Zhu et al. 2017) (Figure I.3). Considering that only a fraction of >200 PBDEs have been tested for placenta-specific outcomes and that less studied hydroxylated metabolites of PBDEs (OH-PBDE) can inhibit CYP19 (Canton et al. 2008), research into the effect of PBDEs on reproductive and placenta-specific outcomes is merited.

Organotins

Organotin compounds are chemicals with a central tin (Sn) atom and hydrocarbon substituents that are commonly used as polyvinyl chloride stabilizers and biocides (Sousa et al. 2014). Organotins can cross the placental barrier, resulting in the accumulation of Sn in the conceptus and decidual mass in rat pregnancies (Furukawa et al. 2017). Organotins have been shown to lead to embryonic lethality in non-human primates (Ema et al. 2009) and rats (Emai et al. 1992), but just recently have been shown to lead to fetal mortality, conceptus apoptosis and malformations, lower placental weight, thinner labyrinth and basal placental layers (dibutyltin (DBT) chloride, 20 mg/kg, (Furukawa et al. 2017)) (Figure I.2). Although

these *in vivo* studies were conducted with higher doses than those observed in human exposures, a prospective Danish cohort on cryptorchidism (58 male placental homogenates) found an inverse association between the sum of placental organotins and reverse 3,3',5'-triiodothyronine (rT3), the third most common iodothyronine (Li et al. 2018). This association was more pronounced in samples with higher a tributyltin (TBT) concentration. However, these findings have yet to be reproduced in an animal study and other human cohorts. Out of the many organotins, only TBT and TPT have been studied extensively in the context of placental dysfunctions *in vitro*. TBT has been shown to either decrease in micromolar dosages (JEG-3, (Cao et al. 2017)) or increase at nanomolar concentrations (placental explants, (Arita et al. 2018); JAR, (Hiromori et al. 2016)) progesterone production (Figure I.4). This alteration in steroid hormone production was accompanied by a reduction in 3β-HSD activity (JEG-3, (Cao et al. 2017)) or an increase in 3β-HSD expression (JAR; (Hiromori et al. 2016)) (Figure I.3). Higher hCG production was also observed in JAR and JEG-3 cells (Nakanishi et al. 2002). These endocrine changes have been shown to be mediated either by PPARγ (Hiromori et al. 2016) or retinoid X receptor (RXR) (Nakanishi et al. 2006). TBT exposure also resulted in gene expression changes associated with cytokine signaling in non-human primate trophoblast stem cells (Midic et al. 2018). Importantly, TBT, a known obesogenic chemical (Veiga-Lopez et al. 2018), can also increase di- and tri-acylglycerol in JEG-3 cells (Gorrochategui et al. 2014). Despite the global ban in TBT use for anti-fouling paints in 2008, the use of organotin chemicals continues to be widespread (Sousa et al. 2014) and therefore, studies investigating the effects of organotins on placental function at environmentally relevant doses are needed.

I.3. Concluding remarks

This review provides a holistic overview of the current knowledge on placental outcomes following EDC exposures by reviewing epidemiological, *in vivo* and *in vitro* data. Despite the seemingly large body of

literature, there are many limitations to these studies which are summarized in Box 1. One of the main limitations is the use of supraphysiological dosing regimens, which are often magnitudes higher than human exposures and report directly cytotoxic outcomes. Human biomonitoring and *in vivo* pharmacokinetic data should be used to develop predictive physiologically-based toxicokinetic mathematical models to establish environmentally relevant dosing strategies. These doses should be coupled with *in vitro* models that better resemble the *in vivo* placental microenvironment, such as the use of 3-dimensional models that recreate cell-to-cell interactions and incorporate tissue shear stress and extracellular matrix components (Blundell et al. 2018, Fry et al. 2019) (see summary of Future Directions in Box 2). These alternative *in vitro* models should be used in combination with *in vivo* studies that integrate relevant animal models to capture more human-representative toxicokinetic profiles or anatomy. For instance, two well established models of studying the placenta for translatability into humans are guinea pig and sheep whose advantages in human placental translatability have been previously summarized (Grigsby 2016). However, few studies have adopted either as animal models.

Additionally, to date, most placental studies focus on single chemical exposures with mixture studies predominately restricted to rodent models investigating organophosphate mixtures. Using relevant, complex mixtures in toxicology studies will improve our understanding of the potential pharmacokinetic and pharmacodynamic interactions between chemicals and their effects on placental development and function. Additional complexity to placental toxicological studies is fostered by the lack of EPA guidelines for placenta-specific outcomes. Another aspect that is often unrecognized is that, derived from the embryonic trophoblast layer, the placenta has a defined sex. However, sex-specific effects are most often not reported. *In vitro* studies are also limited by sex, as commercially available placental cell lines are derived from male pregnancies, or do not account for sex. Recent studies reporting sex-specific associations showing PBDE, organophosphate, and paraben accumulation in the male placenta, with no

reported placenta-specific outcomes, highlight the need to further explore this gap in knowledge. Overall, significant advances in risk assessment of EDCs, particularly on understanding exposure effects on the development and function of the placenta, can be made by addressing these limitations and working towards the proposed future directions.

I.4. Thesis Introduction

This dissertation aims to fill some of the current gaps in knowledge described in this introductory chapter. These specific objectives focused on the evaluation of outcomes from bisphenolic EDCs with particular attention to emerging BPA-analog compounds like BPS and BPF during pregnancy, one of the most vulnerable developmental periods. The following chapters work through a series of aims to: 1) understand the pregnancy toxicokinetics of BPS, and evaluate the comparative toxicokinetics of BPS in mixture with two other common bisphenols (BPA and BPF), 2) investigate chronic gestational exposure to BPS on placenta-specific outcomes, and 3) determine the effect of BPA, BPS, and BPF on *in vitro* cell to cell communication. Altogether, these findings add to the growing body of literature in support of EDC-induced placental defects.

Chapter 1: Pregnancy Toxicokinetics of Bisphenol A, Bisphenol S, and Bisphenol F

Bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) are among the most abundant bisphenols concomitantly detected in humans through biomonitoring studies. Gestational exposure to these chemicals can result in metabolic, reproductive, and neurologic disorders in the progeny. Despite the multitude of available toxicokinetic data for BPA in multiple species and during pregnancy, there exists a large gap in our understanding of other bisphenols, and toxicokinetic and/or toxicodynamic interactions that may occur between these chemicals in mixture. This leads to toxicokinetic inference between BPA and emerging bisphenols like BPS and BPF. The novel toxicokinetic study presented below (Gingrich et al. 2019) achieves a better understanding of the absorption, distribution, metabolism and excretion for BPA, BPS and BPF, in mixture, and during pregnancy. Using sheep and an animal model, and a fetal catheterization technique, high quality pair-matched plasma samples were obtained over a 72 hour period from both mother and fetus following a single subcutaneous injection of either BPS (n=3, 0.5 mg/kg) or a combination of BPA, BPS, and BPF (n=3, 0.5 mg/kg per chemical) to the mother. Here, we observed clear toxicokinetic differences among bisphenols in the maximum plasma concentration reached, the time it took to reach that concentration, elimination half-life, area under the curve, area under the first moment curve, mean residence time, and total body clearance. Importantly, this publication demonstrates the ability of BPS to accumulate in the fetal compartment. These identified toxicokinetic differences among bisphenols caution against assuming similar intraclass chemical toxicokinetics, which is often the case for EDCs. Understanding these toxicokinetic differences, especially in the context of pregnancy where chemical exposures can be particularly detrimental, can aid in regulatory decisions towards the use of safer chemicals in consumer product manufacturing.

Toxicokinetics of bisphenol A, bisphenol S, and bisphenol F in a pregnancy sheep model

Jeremy Gingrich[1, 2], Yong Pu[1], Richard Ehrhardt[1, 3], Rajendiran Karthikraj[4], Kurunthachalam Kannan[4], Almudena Veiga-Lopez[1]

[1]Department of Animal Science, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan 48824, USA

[2]Department of Pharmacology and Toxicology, College of Natural Sciences, Michigan State University, East Lansing, Michigan, 48824, USA

[3]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824, USA

[4]Wadsworth Center, New York State Department of Health, and Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509, USA

Funding sources: Research reported in this publication was supported the National Institute of Environmental Health Sciences (NIEHS) of the National Institute of Health (NIH) (1K22ES026208 and R01ES027863 to A.V-L.), Michigan State University (MSU), MSU AgBioResearch, and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (Hatch MICL02383). J.G. was supported by the BioMolecular Sciences graduate program at MSU, a doctoral fellowship award through the Environmental and Integrative Toxicological Sciences (EITS) of the Institute for Integrative Toxicology at MSU, and by MSU Office of the Vice President for Research and Graduate Studies, AgBioResearch, College of Graduate Studies, and College of Human Medicine.

1.1. Introduction

Worldwide annual production of plastics has reached 8,300 million metric tons with ~80% accumulating in the environment (Geyer et al., 2017) and many of which are considered to contain endocrine disrupting chemicals (EDCs) (Gore et al., 2014). Bisphenols are EDCs used in the manufacturing of plastics, epoxy resins, and a variety of plastic and paper consumer products and food (Liao et al., 2012; Liao and Kannan, 2013). Among the leading bisphenols present in humans are bisphenol A (BPA), bisphenol S (BPS), and bisphenol F (BPF) (Liao et al., 2012; Philips et al., 2018). Depending on the cohort, BPA, BPS, and BPF can be detected in up to in 95.7, 89.4 and 66.5% of U.S. adults, respectively, of human urine samples (Liao et al., 2012b; Xue et al., 2015; Ye et al., 2015; Asimakopoulos et al., 2016; Lehmler et al., 2018; Philips et al., 2018; Rocha et al., 2018). BPS and BPF concentrations in humans are found in the same order of magnitude, but lower to that of BPA (Liao et al., 2012; Ye et al., 2015). Despite the vast information available for BPA, much remains to be known regarding emerging BPA analogues (Chen et al., 2016; Wu et al., 2018). The detection of BPS, and to a lesser extent BPF, in human fetal cord blood (Kolatorova et al., 2018), the potential association between BPS exposure and gestational length (Wan et al., 2018a), and the fact that in mammals, fetal exposure to BPS can alter mammary gland development (Kolla et al., 2018), and that BPS and BPF can alter behavior (Catanese and Vandenberg, 2017; Ohtani et al., 2017), calls for the investigation of the materno-placental transfer of these bisphenols into the fetal compartment.

Recent studies have demonstrated that BPS can be acutely toxic in *D. magna* (Chen et al., 2002), induce neuronal dysfunction in zebrafish (Kinch et al., 2015) and *C. elegans* (Mersha et al., 2015) and impair fertility in aquatic species (Naderi et al., 2014; Qiu et al., 2015). In mammalian species, gestational or perinatal exposure to BPS induces obesity in mice offspring in a sex specific manner (Ivry Del Moral et al., 2016) and alters mammary gland development (Kolla et al., 2018). We have also recently

demonstrated that gestational exposure to BPS reduces the endocrine secretory capacity of the placenta (Gingrich et al., 2018). Given the critical role of the placenta in pregnancy maintenance, oxygen and nutrient exchange with the developing fetus, and the detrimental effects that perturbations in placental homeostasis have on progeny outcomes (Gabory et al., 2013; Rosenfeld, 2015), understanding the toxicokinetics of BPS during pregnancy is much warranted. Although to a lesser extent, the effects of exposure to BPF have also been studied *in vivo* demonstrating that gestational BPF exposure alters neuronal behavior in mice (Ohtani et al., 2017).

Thus far, only one toxicokinetic study is available for BPS after oral administration in humans (Oh et al., 2018). However, this study was conducted in non-pregnant female and male individuals. Physiologically based toxicokinetic (PBTK) mathematical models integrate chemical absorption, distribution, metabolism, and excretion, and help generate health risk assessment for chemical and pharmaceutical exposures due to their predictive capabilities (Zhuang and Lu, 2016; Ke et al., 2018). Recent work focusing on the development of a PBTK model for emerging bisphenols, such as BPS and BPF, have explicitly called for *in vivo* toxicokinetic data for BPS, BPF, and BPAF using biomonitoring or animal studies (Karrer et al., 2018). However, this novel PBTK model has only been developed in a non-pregnancy model. Given that toxicokinetics of BPA can be modulated by pregnancy (Corbel et al., 2013), we aimed to study the toxicokinetics of BPS and BPF during pregnancy. Our work will enable future development of precise pregnancy PBTK models for emerging bisphenols. Toxicokinetics during pregnancy are far more complex with the inclusion of the maternal, placental and fetal compartments (Ke et al., 2018). However, ethical constraints do not allow for these toxicokinetics studies to be conducted in pregnant women. The use of refined fetal surgery techniques in a sheep animal model represents a unique opportunity to monitor the maternal, amniotic, and fetal compartments, key elements of pregnancy PBTK models (Ke et al., 2018). Sheep are excellent models to study placental

function (Fowden et al., 2015; Mourier et al., 2017) and have been used for the study of feto-maternal transfer of drugs (Krishna et al., 2002; Ngamprasertwong et al., 2016) and EDCs (Corbel et al., 2013; Corbel et al., 2015), as they allow for the simultaneous and longitudinal characterization of the pregnancy multi-compartment model in real time. The objective of this study was to comparatively determine the toxicokinetic profile of BPS, BPF and BPA in a pregnancy model by evaluating the maternal and the fetal compartments.

1.2. Materials and Methods

Chemicals

Bisphenols used included BPA (purity ≥ 99%, Cat#: 239658; Lot#: MKBQ5209V; Aldrich Chemical Co., Milwaukee, WI, USA), BPS (4,4'-sulfonyldiphenol, purity 99.7%, Cat#: 146915000, Lot#: A0337011, Acros Organics, Geel, Belgium), and BPF (purity 98%, Cat#: B47006; Lot#: 05712ME; Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil. The internal standards $^{13}C_{12}$-BPA (99%), $^{13}C_{12}$-BPS (98%) and $^{13}C_{12}$-BPF (99%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). β-glucuronidase (≥100,000 units/mL β-glucuronidase; ≤ 7,500 units/mL sulfatase) from *Helix pomatia* (Type HP-2) was purchased from Sigma-Aldrich (St. Louis, MO, USA)

Animals

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Michigan State University (MSU), are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals and meet the ARRIVE guidelines for reporting animal research (Kilkenny et al., 2010). The study was conducted at the MSU Sheep Teaching and Research Facility (East Lansing, MI; 42.7° N, 84.4° W) using animals selected from an in-house flock of Polypay x Dorset cross-bred sheep. Six female sheep at second or third parity were bred using a time mated pregnancy strategy

using three vasectomized rams as previously described (Pu et al., 2017). After estrus detection by vasectomized rams, females were moved with a fertile ram previously tested using a breeding soundness exam. Pregnancy and number of fetuses was confirmed at gestational day (GD) 45. Only singleton pregnancies were used in this study. Maternal and fetal characteristics are shown in Table 1.1. Animal diets have been previously described (Pu et al., 2017). In brief, starting 3 weeks preconception and continuing until 30 days after breeding, females were fed a total mixed ration of a diet providing an energy concentration of 3.2 Mcal/kg of digestible energy and crude protein concentration of 12%. From days 30 to 110 of gestation, energy concentration was reduced to 2.8 Mcal/kg of digestible energy. After GD110, females were fed a total mixed ration designed to maximize dry matter intake (40% neutral detergent fiber [NDF] and 70% NDF digestibility) ; 3.2 Mcal/kg digestible energy and 14% crude protein).

Table 1.1: Maternal and fetal characteristics at time of euthanasia.

	Mean ± SEM
Maternal weight (kg)	76.3 ± 3.6
Gestational age (days)	121.8 ± 0.7
Fetal weight (g)	4,389.7 ± 112.3
Fetal biparietal diameter (cm)	70.1 ± 0.8
Fetal Sex (n = Female:Male)	3:3

Note: F:M ratio for BPS only group = 2:1 and for mixture group = 1:2.

Experimental design

Experimental design is shown in Figure 1.1. Pregnant females underwent fetal catheterization surgery at GD 114.8 ± 0.8 days. After a week of recovery, females (n = 3) were injected with a single s.c. dose of BPS (0.5 mg/kg) or a combination of BPA, BPS, and BPF (n = 3; 0.5 mg/kg for each chemical), at a dosing volume of 1.9 ± 0.1 ml. The dose and route of exposure used to calculate the pharmacokinetics of bisphenols stem from previous work in which a s.c. injection of 0.5 mg/kg BPA resulted in detectable

BPA fetal concentrations (Veiga-Lopez et al., 2013) comparable to that of the median level of BPA measured in maternal circulation of U.S. women (Veiga-Lopez et al., 2015). The same doses of BPS and BPF were used to compare with the BPA dose used in the study. Although main exposure route of bisphenol exposures is oral (Liao and Kannan, 2013), the use of sheep as a model, a ruminant species, requires the use of an alternative exposure route to avoid confounding factors from ruminal metabolism.

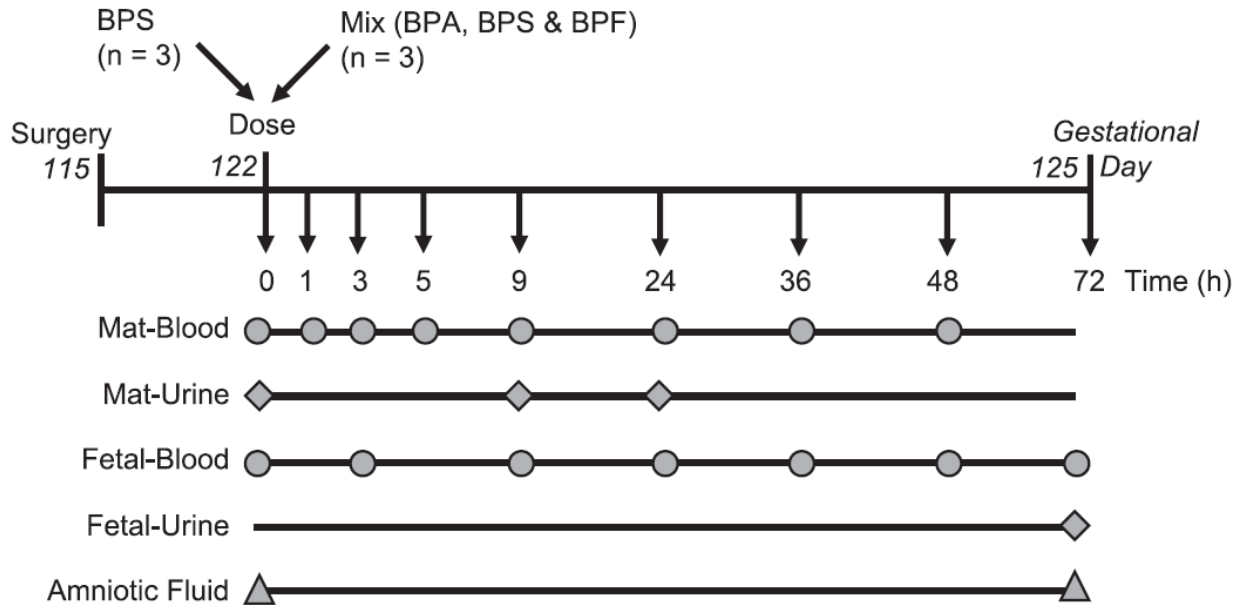

Figure 1.1: Experimental design and sample collection scheme. Experimental design and sample collection scheme for blood (*circles*), urine (*diamonds*), and amniotic fluid (*triangles*) over 72 h after bisphenol administration. Mat: maternal.

Fetal catheterization surgery

All experimental animals underwent surgery to place a catheter in the fetal descending aorta and inferior vena cava as previously described (Ehrhardt et al., 2002). In brief, fasted animals were sedated using xylazine (0.2 mg/kg), anesthetically induced with ketamine (8 mg/kg), and inhaled isoflurane (2%

vaporized) was used to maintain anesthesia throughout the procedure. A midline laparotomy was performed to isolate the uterine horn where the fetus was positioned. A hysterotomy was then performed in the uterine horn, through which the fetal limb was isolated and exposed past the tibio-tarsal joint. The cranial tibial vein was isolated with fine forceps and catheterized with a polyvinyl catheter (Saint-Gobain Performance Plastics, Beaverton, MI, USA; inner diameter: 0.86 mm, outer diameter: 1.37 mm). The catheter was threaded through the cranial tibial vein into the inferior vena cava (20 cm) and secured laterally onto the fetal hind limb using a non-absorbable suture (silk, USP 3-0). An additional catheter (Saint-Gobain Performance Plastics, Beaverton, MI, USA; inner diameter: 1.59 mm, outer diameter: 3.18 mm) was secured to the hind limb to provide access to amniotic fluid samples. The uterine incision was closed with an absorbable monofilament suture (polyglycolic acid, USP 0) using an inverted pattern finished with a single Cushing suture to avoid peri-uterine adhesion. Catheters were exteriorized through a small incision (~1 cm) in the flank of the animal nearest to the hysterotomy. The abdominal muscle wall and peritoneum were closed using an absorbable monofilament suture material (polyglycolic acid, USP 1) in an interrupted pattern. The midline skin incision was closed using non-absorbable suture (silk, USP 0) in an interrupted mattress pattern. Catheters were flushed daily with sterile saline, followed by heparinized saline to maintain patency. Fetuses all received injections (i.m.) and amniotic infusions of gentamycin (2 ml at 100 mg/ml) at the time of surgery, and amniotic infusions every 2 days after surgery. Additionally, all ewes received penicillin G (6,000 IU/kg BW) and gentamycin sulfate (2 mg/kg BW) 24 h before surgery, at the time of the surgery, and every 12 h for 3 days after surgery. Maternal health, including body temperature, appetite, and vital signs were monitored twice daily for a week after surgery and daily thereafter. No abnormal signs were observed in any of the mothers through the termination of the experiment.

Sample collection

Except for fetal catheters and syringes (non-polycarbonate plastic) used to draw samples, all materials used for sample processing, storage, and analyses were glass. Blanks (double distilled water) for catheters and syringes were assayed for all three bisphenols to assess potential contamination from sampling collection materials. Serial maternal samples were collected before the chemical administration (Time 0 = t_0) and at 1, 3, 5, 9, 24, 36, and 48 h after the chemical administration. Serial fetal samples were collected at 0, 3, 9, 24, 36, 48, and 72 h after the chemical administration. To evaluate accumulation of bisphenols in the amniotic sac, amniotic fluid samples were collected at 0 and 72 h after the chemical administration. To evaluate the urinary excretion of bisphenol, maternal urine samples were collected before the chemical administration, and at 9 and 24 h after chemical administration. To evaluate if the bisphenols could be detected in fetal urine, samples were taken at 72 h after the chemical administration. All samples were kept frozen at -80 °C until assayed.

HPLC-MS/MS analysis

Simultaneous extraction of total bisphenols (BPA, BPS and BPF) in the sheep biofluids was performed using a method reported earlier with slight modifications (Liao et al., 2012; Gingrich et al., 2018). Because reversion of glucuronide and sulfate forms of BPA to the unconjugated form occurs through β-glucuronidase (Ginsberg and Rice, 2009) and arylsulfatase C (Stowell et al., 2006), which are highly expressed in human tissues, such as the placenta (Andra et al., 2016); in this study, we measured total BPA to provide with a combined measure for unconjugated and conjugated forms of bisphenols in this pregnancy model. Briefly, 250 µL of sheep plasma/urine/amniotic fluid was spiked with 20 ng of the internal standard (IS) mixture. To the spiked samples, an enzymatic digestion with *β*-glucuronidase followed by liquid-liquid extraction (LLE) with ethyl acetate was performed for the extraction of the target chemicals.

Calibration curves were constructed for the target chemicals between the concentration ranges of 0.05 to 200 ng/ml with the regression coefficients > 0.998. For every 20 samples, a procedural blank, a corresponding matrix blank, 2-level matrix spike experiments (20 ng and 40 ng) and sample duplicates were performed. There was no detectable concentration of BPF found in procedural blanks. However, trace levels of BPS (0.02 ng/ml) and BPA (0.05 ng/ml) were found in blanks and they were subtracted from concentrations found in sheep biofluids. The respective mean matrix spike recoveries (at 2-levels) were 99.1% and 90.7% (BPA), 87.9% and 95% (BPS), and 92.8% and 87.4% (BPF). Duplicate analysis of randomly selected samples yielded a coefficient of variation of < 12% for the target chemicals. The instrumental level of quantification (LOQ) values for BPA, BPS and BPF were 0.33 ng/ml, 0.12 ng/ml and 0.50 ng/ml, respectively. An Agilent 1260 HPLC (Agilent Technologies Inc., Santa Clara, CA) system equipped with a Zorbax SB-Aq (150 mm x 2.1 mm, 3.5 µm; Agilent Technologies Inc., Santa Clara, CA) column was used for the chromatographic separation of target chemicals under a gradient elution method using water (A) and methanol (B) as mobile phases. An API 4500™ electrospray QTRAP mass spectrometer (ESI-MS/MS; Applied Biosystems, AB Sciex, Framingham, MA, USA) was used for the selective identification (MRM transitions) and quantification (by isotopic dilution method) of the target chemicals.

Toxicokinetic analysis

All toxicokinetic parameters were calculated using a non-compartmental analysis based on plasma concentrations of total BPA, BPS, or BPF over time for each individual. The following parameters were calculated for each chemical for the maternal ($_{mat}$) and fetal ($_{fet}$) compartments: 1) maximum observed plasma concentration ($C_{max\text{-}mat}$ and $C_{max\text{-}fet}$), 2) the time at which the C_{max} was observed ($t_{max\text{-}mat}$ and $t_{max\text{-}fet}$), 3) area under the curve (AUC_{mat} and AUC_{fet}) from 0 to 48 h for maternal and from 0 to 72 h in fetal,

and area under the first moment curve ($AUMC_{mat}$ and $AUMC_{fet}$) calculated using the logarithmic trapezoidal method (Purves, 1992), 4) mean residence time (MRT_{mat} and MRT_{fet}) calculated using the equation MRT = AUMC/AUC (Karol, 1990), and 5) chemical half-life ($t_{1/2mat}$ and $t_{1/2fet}$). Total body clearance ($Cl_{TB\text{-}mat}$) was estimated for maternal samples and calculated using the equation Cl_{TB} = dose/AUC. All parameters were calculated using the software Kinetica with a non-compartmental analysis (NCA) assistant (Adept Scientific, version 5.0, 2007). Kinetica was also used to validate AUC, AUMC, and MRT calculations. To evaluate the bioaccumulation preference in mother *vs*. the fetus, the fetal to maternal (F/M) ratio was calculated by dividing the $C_{max\text{-}fet}$ by the $C_{max\text{-}mat}$.

Statistical analysis

All data are presented as mean ± SEM. Appropriate logarithmic transformations were applied, as needed, to account for normality of data. First, maternal and fetal toxicokinetic parameters for BPS comparing females exposed to only BPS (n = 3) *vs*. the mixture (BPA, BPS, and BPF) (n = 3) was conducted using an independent T-test to evaluate if the co-administration of BPA and BPF may have affected BPS kinetics. No significant differences were observed and thus all 6 females exposed to BPS were combined and analyzed as a single group. Except for $t_{max\text{-}mat}$, comparisons of maternal and fetal toxicokinetic parameters among the three treatment groups were analyzed by a mixed model or ANOVA with Tukey posthoc tests with fetal sex as a covariate. For $t_{max\text{-}mat}$, comparisons among the three treatment groups were analyzed using a Kruskal-Wallis followed by a Dunn-Bonferroni posthoc test. In addition, to test the effect of fetal sex and interaction with treatment a general linear model was used. For comparisons within treatment group between 2 time points (i.e.: amniotic fluid) an independent T-test was used. Statistical software used was PASW Statistics for Windows release 18.0.1. Differences were considered significant at $P < 0.05$.

1.3. Results

Maternal non-compartmental toxicokinetic analysis

Maternal bisphenol concentrations after a single s.c. dose of BPA, BPS, or BPF are shown on Figure 1.2 and corresponding estimated toxicokinetic parameters using the above-referenced non-compartmental analysis are displayed on Table 1.2 (*left panel*). As described in the statistics` section, no significant differences were observed in maternal BPS toxicokinetics when comparing females exposed to only BPS (n = 3) *vs*. the mixture (BPA, BPS, and BPF) (n = 3) and thus all 6 females exposed to BPS were combined for comparison with BPA and BPF toxicokinetics. Mean $C_{max\text{-}mat}$ was statistically different among treatments with $C_{max\text{-}mat}$ for BPS (643 ± 29.9 ng/ml; $P < 0.001$) being an order of magnitude higher than for BPA and BPF (66.7 ± 1.7 and 48.8 ± 0.2 ng/ml, respectively; $P < 0.005$). $t_{max\text{-}mat}$ was achieved at the same time after BPA and BPS exposure (3.0 ± 0.0 h) and earlier (1.7 ± 0.7 h) for BPF ($P < 0.05$). Both AUC_{mat} and $AUMC_{mat}$ reported after BPS exposure were significantly higher than those of BPA and BPF ($P < 0.02$; see Table 1.2). MRT_{mat} varied among bisphenols with BPF having the longest (10.3 ± 2.5 h) and BPS having the shortest (6.7 ± 0.3 h) MRT_{mat}, although no statistical difference was observed. Mean $Cl_{TB\text{-}mat}$ was statistically different among all treatments with BPF having the fastest $Cl_{TB\text{-}mat}$ (103.8 ± 22.7 ml/h) compared to BPA and BPS (45.4 ± 5.5 and 5.2 ± 0.5 ml/h, respectively; $P < 0.02$).

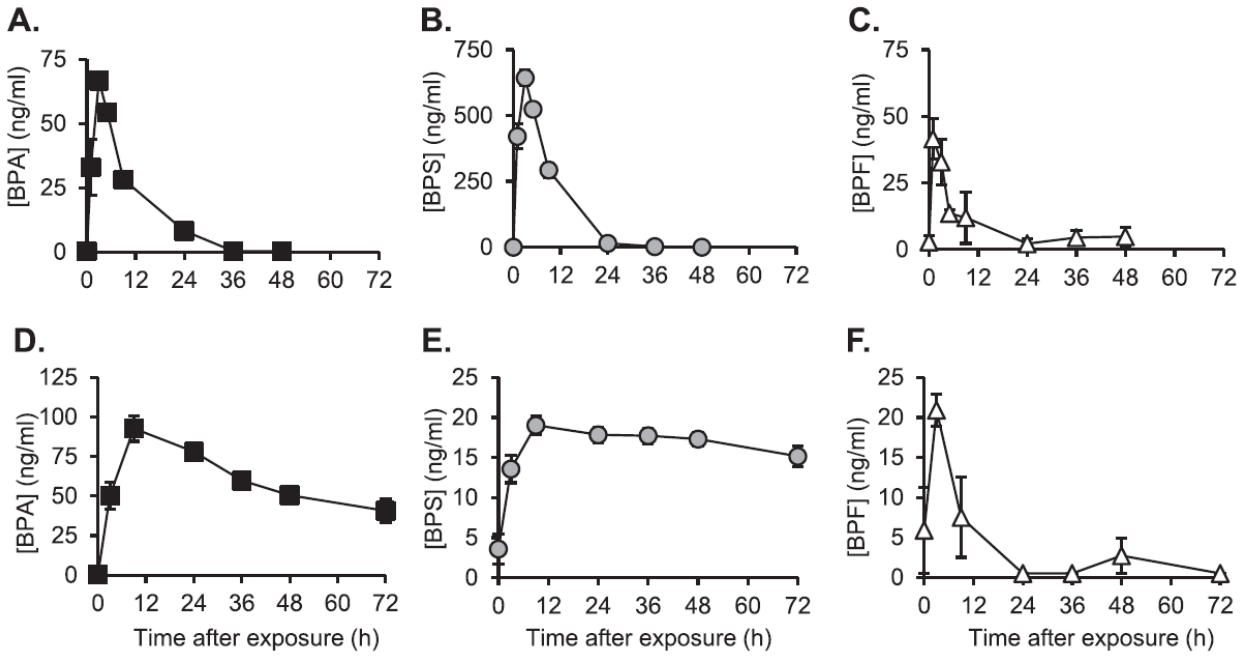

Figure 1.2: Toxicokinetic profiles for BPA, BPS, and BPF in maternal and fetal compartments.

Toxicokinetic profiles for BPA, BPS, and BPF in maternal and fetal compartments. Mean (± SEM) maternal (A-C) and fetal (D-F) plasma concentrations of BPA (A and D; closed squares; n = 3), BPS (B and E; gray circles; n = 6), or BPF (C and F; open triangles; n = 3) after a single 0.5 mg/kg s.c. dose of BPS or the combination of BPA, BPS, and BPF. Half (n = 3) of the females were only exposed to BPS while the other half (n = 3) were co-exposed simultaneously to BPA, BPS, and BPF.

Table 1.2: Toxicokinetic parameters for bisphenols in maternal and fetal plasma samples (mean ± SEM) after a single subcutaneous injection (0.5 mg/kg).

	Maternal			Fetal		
	BPA (n = 3)	BPS (n = 6)	BPF (n = 3)	BPA (n = 3)	BPS (n = 6)	BPF (n = 3)
C_{max} (ng/ml)	66.7 ± 1.7[a]	643.1 ± 29.9[b]	48.8 ± 0.2[c]	87.6 ± 6.9[a]	18.5 ± 1.1[b]	20.9 ± 2.0[b]
t_{max} (h)	3.0 ± 0.0[a,b]	3.0 ± 0.0[a]	1.7 ± 0.7[b]	14.0 ± 5.0[a]	21.0 ± 4.2[a]	3.0 ± 0.0[b]
$t_{1/2}$ (h)	5.3 ± 0.4[a,b]	3.7 ± 0.1[a]	7.7 ± 2.2[b]	52.0 ± 20.3[a,b]	402.2 ± 102.6[a]	14.2 ± 1.8[b]
AUC (µg*h/ml)	0.81 ± .07[a]	7.5 ± 0.6[b]	0.40 ± 0.1[c]	3.9 ± 0.2[a]	0.99 ± .05[b]	0.21 ± .05[c]
AUMC (µg*h^2/ml)	6.7 ± 0.4[a]	51.0 ± 5.2[b]	4.5 ± 2.3[a]	120.0 ± 17.0[a]	34.0 ± 1.5[b]	1.7 ± 0.4[c]
MRT (h)	8.3 ± 0.6	6.7 ± 0.3	10.3 ± 2.5	31.3 ± 3.6[a]	34.1 ± 1.7[a]	8.5 ± 0.2[b]
Cl_{TB} (L/h)	45.4 ± 5.5[a]	5.2 ± 0.5[b]	103.8 ± 22.7[c]			

Urinary bisphenols concentrations are shown in Table 1.3. At t_9 after bisphenol administration, maternal urine peak concentrations for BPA and BPS were 1,300 ± 210 and 3,870 ± 888 ng/ml, respectively and were reduced by one order of magnitude at t_{24}. In contrast, BPF in maternal urine was detected at much lower concentrations and with means increasing over time but with a high variability. Urinary bisphenol concentrations in maternal samples were only found to be significantly different between BPF and either BPA or BPS ($P < 0.05$; see Table 1.3), but not between BPA and BPS.

Table 1.3: Concentration of bisphenols (mean ± SEM) in maternal urine before (t0), at 9 (t9), and at 24 (t24) h after a single subcutaneous injection (0.5 mg/kg).

	BPA (ng/ml) (n = 3)	BPS (ng/ml) (n = 6)	BPF (ng/ml) (n = 3)
t_0	< LOQ[a,b]	< LOQ[a]	1.5 ± 1.0[b]
t_9	1,300 ± 210.0[a]	3,870 ± 888[a]	3.9 ± 3.4[b]
t_{24}	298 ± 104.4[a]	344 ± 50.1[a]	18.7 ± 18.2[b]

LOQ for BPA, BPS, and BPF are 0.33, 0.12, and 0.50 ng/ml, respectively.

Different superscripted letters denote statistical differences at each time point ($P < 0.05$).

Fetal non-compartmental toxicokinetic analysis

Fetal bisphenol concentrations after a single s.c. dose of BPA, BPS, or BPF are shown in Figure 1.2 and corresponding estimated toxicokinetic parameters using the above-referenced non-compartmental analysis are displayed on Table 1.2 (*right panel*). There were no statistical differences in fetal toxicokinetics between female or male fetuses in the BPS group (data not shown) and thus data for both fetal sexes were combined. The number was too small to test this in BPA or BPF groups. Additionally, as described in the statistical section, no significant differences were observed in fetal BPS toxicokinetics when comparing females exposed to only BPS (n = 3) *vs*. the mixture (BPA, BPS, and BPF) (n = 3) (data not shown) and thus all 6 females exposed to BPS were combined for comparison with BPA and BPF toxicokinetics. Mean $C_{max\text{-}fet}$ after BPA administration (87.6 ± 6.9) was significantly higher than either BPS or BPF (18.5 ± 1.1 and 20.9 ± 2.0 ng/ml, respectively; $P < 0.001$), but within the same order of magnitude. $T_{max\text{-}fet}$ observed was bisphenol dependent with BPF being the earliest and statistically different (3.0 ± 0.0 h; $P < 0.05$), from either BPA (14.0 ± 5.0 h) or BPS (21.0 ± 4.2 h). Both, AUC_{fet} and $AUMC_{fet}$ differed significantly among bisphenols, with BPA being the highest for both variables (3.9 ± 0.2 mg*h/ml and 120 ± 17 mg*h^2/ml respectively), followed by BPS (0.99 ± 0.05 mg*h/ml and 34 ± 1.5 mg*h^2/ml respectively), and lastly BPF (0.21 ± 0.05 mg*h/ml and 1.7 ± 0.4 mg*h^2/ml respectively) ($P <$

0.001, see Table 1.2). MRT_{fet} after BPA and BPS administration was within the same order of magnitude (31.3 ± 3.6 and 34.1 ± 1.7 h, respectively), and were both significantly higher to that observed after BPF administration (8.5 ± 0.2 h) ($P < 0.001$).

Prior to maternal administration of bisphenols, BPA, BPS, and BPF concentrations measured in fetal amniotic fluid (Table 1.4) were < LOQ (< 0.33 ± 0.0), 0.3 ± 0.2, and < LOQ (< 0.5 ± 0.0) ng/ml, respectively. At t_{72}, all bisphenols were detectable in amniotic fluid, but concentrations were not different among bisphenols. At t_{72}, fetal urine total BPA was detected at 144.3 ± 46.7 ng/ml, an order of magnitude higher to that of BPS or BPF (6.6 ± 1.5 and 13.8 ± 3.4 ng/ml, respectively), however no statistical differences were detected. The F/M ratio was 1.31, 0.03, and 0.42 in BPA, BPS, and BPF, respectively.

Table 1.4: Concentration of bisphenols (mean ± SEM) in amniotic fluid and fetal urine before (t0) and 72 h (t72) after a single subcutaneous injection (0.5 mg/kg).

		BPA (ng/ml) (n = 3)	BPS (ng/ml) (n = 6)	BPF (ng/ml) (n = 3)
Amniotic fluid	t_0	< LOQ	0.3 ± 0.2	< LOQ
	t_{72}	119 ± 137.0	5.6 ± 3.0	16.4 ± 22.6
Fetal urine	t_{72}	144 ± 46.7	6.6 ± 1.5	13.8 ± 3.4

LOQ for BPA, BPS, and BPF are 0.33, 0.12, and 0.50 ng/ml, respectively.

Note: No significance was observed among groups or time points within groups.

1.4. Discussion

Fifteen different bisphenols exist, at least 8 are detectable in human urine, and concomitantly, BPA, BPS and BPF are detected in 95.7, 89.4 and 66.5% of U.S. adults, respectively (Lehmler et al., 2018). We have recently demonstrated that gestational exposure to BPS can result in placental dysfunction (Gingrich et

al., 2018), and can also result in reproductive and metabolic disorders in the progeny (Ivry Del Moral et al., 2016; Kolla et al., 2018). Pregnancy toxicokinetics of less prevalent bisphenols, such as BPS and BPF, remain unknown. The current study provides with novel insights into the toxicokinetics of these two bisphenols in a pregnancy model. We demonstrate that BPS reaches maternal circulation at a concentration one order of magnitude above that of the other bisphenols (s.c. administration) and has the shortest half-life. In contrast, in the fetal compartment, BPS has the longest half-life, persisting at a low concentration. We have also evaluated the pregnancy toxicokinetics of BPF, the first study of its kind in any model system and demonstrated that BPF has a comparatively long half-life in maternal circulation, but clears faster from fetal circulation compared to BPA or BPS. The persistence of BPS in the fetal compartment, suggestive of a higher availability compared to other bisphenols, highlights the need to conduct additional studies to understand the effects of chronic BPS exposure on the developing fetus.

In this study, sheep was used as the animal model to help understand the kinetics of three commonly found bisphenols in a pregnancy model. Although there are essential differences in placentation between sheep and humans (Hyttel, 2010; Benirschke et al., 2012), sheep are considered a valid and useful model in the understanding of human placental function (Bird, 2017). Importantly, the sheep allows for fetal catheterization during mid-pregnancy and the evaluation of the chemical transfer from the maternal into the fetal and amniotic fluid compartment (Gingrich et al., 2018). Fetal catheterization to monitor dynamic toxicokinetics is only feasible in large mammalian species, such as monkeys and sheep. However, the use of the sheep represents a challenge, as it is a ruminant species, with different digestive physiology. Thus, to avoid differences in pre-absorptive metabolism in the ruminant digestive system, s.c. was selected as the route of choice in this study. In addition to the oral route (Liao and Kannan, 2014), transdermal is also a major route of exposure to bisphenols (Bernier and Vandenberg, 2017).

Maternal toxicokinetics of bisphenols

In this study, total BPS reached the highest $C_{max\text{-}mat}$ in plasma but had the shortest $t_{1/2\text{-}mat}$ and MRT_{mat} demonstrating that despite reaching the highest circulating concentrations, BPS persisted less time in maternal circulation compared to BPA or BPF. To our knowledge, only a single study has evaluated BPS toxicokinetics after a single oral dose (8.7 µg/kg BW of d^4-BPS) (Oh et al., 2018). For total BPS, this human study reported a higher MRT (9.8 ± 1.03 h) and longer $t_{1/2}$ (6.81 ± 0.72 h) (Oh et al., 2018) compared to the current study (MRT_{mat}: 6.7 ± 0.3 h and $t_{1/2\text{-}mat}$: 3.7 ± 0.1 h). Although within the same magnitude, differences in route of exposure between studies (oral *vs*. s.c.) and lack of first pass metabolism in s.c. exposure may be accounting for a shorter $t_{1/2}$ in the present study. However, the human BPS toxicokinetic study (Oh et al., 2018) has been challenged for assumptions regarding BPS bioavailability and exclusion of contributions from first-pass metabolism (Grandin et al., 2018). Importantly, exclusion of first pass metabolism following an oral dose could result in the overestimation of MRT_{mat} and $t_{1/2\text{-}mat}$.

To our knowledge this is the first BPF toxicokinetic study in a pregnancy model. We have demonstrated that BPF kinetics during pregnancy differ to that of BPA and BPS, with BPF reaching the lowest $C_{max\text{-}mat}$ (48.8 ± 0.2 ng/ml), but having the fastest clearance rate (103.8 ± 22.7 ml/h) compared to BPS and BPA. To date, the only information on the kinetics of BPF is limited to an *in vivo* study that compared BPF excretion between pregnant and non-pregnant female rats following a high oral dose (7 or 100 mg/kg H^3-BPF) (Cabaton et al., 2006). As in rats (Cabaton et al., 2006) and humans (Lehmler et al., 2018), we were able to confirm that BPF is excreted through maternal urine (Table 1.3), although we are unable to account for BPF excretion through other routes, such as biliary, or the urinary clearance of the main BPF conjugates, glucuronide or sulfate (Cabaton et al., 2008). To note, BPF was detected in one out of three pre-exposure urine samples. As BPF has been detected in grain products (Liao and Kannan, 2013) and

because animals were housed outdoors until a week prior to surgery, it is possible the mothers were environmentally exposed to BPF prior to the start of the kinetic experiment.

The current BPA toxicokinetic results add to previous pregnancy reports in sheep and rhesus monkeys (Corbel et al., 2013; Patterson et al., 2013). Our observed $t_{1/2\text{-mat}}$ of 5.3 ± 0.4 h was similar to $t_{1/2}$ reported following oral administration in humans (6.4 ± 2.0 h (Thayer et al., 2015); 5.4 h (Volkel et al., 2002)), s.c. administration in neonatal rats (3.4 - 4.3 h (Doerge et al., 2010)), i.v. administration in pregnant female rhesus monkeys (2.8 ± 1.1 h (Patterson et al., 2013)), and i.v. administration in adult female rats (8.1 ± 6.1 h (Doerge et al., 2010)). The similar $t_{1/2}$ observed in human studies compared to our study despite the different species and route of exposure highlight the usefulness of using sheep to understand bisphenol toxicokinetics. For unconjugated BPA, a lower $t_{1/2}$ (0.76 ± 0.66 h) was reported following an i.v. administration (2 mg/kg/day) in a pregnant Lacaune sheep model when compared with the current study, despite similar MRT (9.56 ± 0.25 h) (Corbel et al., 2013) to that of our study ($MRT_{mat.}$: 8.3 ± 0.6 h). Differences in $t_{1/2}$ likely relate to form of BPA measured and the fact that Corbel *et al.* (Corbel et al., 2013) did not account for fetal number, which could blunt the $t_{1/2}$, as observed with betamethasone pharmacokinetics (Ballabh et al., 2002). Furthermore, BPA administration (s.c.) to non-pregnant female rats resulted in a t_{max} (1 - 4 h) similar to this study (3.0 h), (Pottenger et al., 2000), which may be attributed to the higher dose used (10 mg/kg) or the reproductive state of the female (non-pregnant). In humans, a single oral dose of d^6-BPA resulted in a similar but faster t_{max} (1.1 ± 0.5 h), and a C_{max} a magnitude higher (1,711 ± 496 nM) than our reported $C_{max\text{-mat}}$ value (Thayer et al., 2015). The difference in t_{max} and C_{max} between the current study and the study following an oral dose of BPA in humans may be due to differences in route of exposure, species, and/or dose administered. Unlike other studies that have reported inter-individual differences in BPA toxicokinetics (Thayer et al., 2015), in our study, all maternal and fetal BPA kinetics showed very small variability among animals. To note, BPA $t_{1/2\text{-mat}}$ was

similar to a human oral administration study (Thayer et al., 2015), while BPS $t_{1/2\text{-}mat}$ was shorter to that of another human oral exposure study (Oh et al., 2018). In addition to the pitfalls noted for the BPS human oral study, such as exclusion of first-pass metabolism in their modeling, this discrepancy may be partly explained by the MRT_{mat} calculated for each bisphenol. The faster MRT observed for BPS over BPA indicates a faster turnover BPS rate, and a larger probability that BPS toxicokinetics are altered depending on the route of exposure compared with BPA, which has a slower MRT. Additional factors that may contribute to the $t_{1/2}$ differences between our study and the two human studies may relate to pregnancy status and exposure dose, both of which differ from the current study.

Our comparative evaluation of the three bisphenols toxicokinetics demonstrated that the largest differences were evident between BPS and BPF, with BPS having the highest $C_{max\text{-}mat}$ and AUC_{mat}, and BPF the lowest, while BPF had the highest $t_{1/2\text{-}mat}$, MRT_{mat}, and $Cl_{TB\text{-}mat}$, while BPS had the lowest. With the shortest $t_{1/2\text{-}mat}$ and despite a slower clearance, BPS clears from maternal plasma by 24 h, while BPA cleared by 48 h, and BPF persisted at low concentrations and was still detected (< 9.2 ng/ml) at 48 h post-injection. Toxicokinetic differences are the result of variation in absorption, distribution, metabolism, and excretion that are directly attributed to differences in the chemical properties between BPS and BPF, including solubility, acidity constant, plasma protein binding, and molecular weight (see Table 1.5; (Cao et al., 2011; Pan et al., 2014; Pivnenko et al., 2015; Luo et al., 2016)). Chemical transport and metabolism, and competition for transport and metabolism highly depend on these properties (Griffiths and Campbell, 2015). In addition, absorption from the subcutaneous compartment into the blood circulation is not yet fully understood and factors like depth of subcutaneous adipose tissue may alter drug kinetics (Richter et al., 2012). Thus, lower water solubility and higher $logK_{o/w}$ of BPA (300 mg/l and 3.32, respectively or BPF (5.4 and 2.91, respectively compared to BPS's (1,100 mg/l and 1.65, respectively (see Table 1.5; (PubChem; Pivnenko et al., 2015)) could have contributed to larger retention

of the BPA and BPF dose in the subcutaneous adipose tissue. Further work is required to validate this hypothesis. Overall, differences in this pregnancy toxicokinetic model highlight the need to exert caution when extrapolating kinetic information from BPA, from which much is known, to other bisphenol chemicals.

Table 1.5: Chemical properties of bisphenols.

Bisphenol	Molecular weight (g/mol)	Plasma protein binding (10^4 L/mol)	pKa	Solubility in water (mg/L)	$logK_{o/w}$
BPA	228.29	2.90-3.14[1]	9.60	300	3.32
BPS	250.27	3.14-5.14[2]	8.20	1100[3]	1.65
BPF	200.24	0.36-3.52[4]	7.55	543[3]	2.91

Plasma protein binding constants are for human serum albumin (HSA). Superscripted numbers represent the following references: 1) Cao et al. 2011; 2) Luo et al. 2016; 3) Pivenko et al. 2015; 4) Pan et al. 2014; and 5) NIH PubChem compound summaries.

In this study, half of the females received only BPS, while the other half received a mixture of BPA, BPS, and BPF, similar to a co-exposure of bisphenols that humans are exposed to (> 50% of people may be exposed to the three bisphenols simultaneously in the U.S. (Lehmler et al., 2018)). Maternal or fetal BPS toxicokinetic parameters did not differ in animals exposed to only BPS *vs*. the combination of bisphenols suggesting that the co-exposure to BPA or BPF did not interfere with absorption, distribution, metabolism, and excretion of BPS. Our experimental design did not allow to address this for BPA or BPF. However, the similar BPA toxicokinetic results obtained in this study compared to previous work (Volkel et al., 2002; Doerge et al., 2010; Gayrard et al., 2013; Patterson et al., 2013; Thayer et al., 2015) suggest that co-exposure to other bisphenols did not interfere with BPF or BPA kinetics. Although, a recent study has demonstrated that the co-administration of BPA (oral, 50 µg/kg C^{14}-BPA) with BPS (s.c. injections, 1, 3, 6, and 9 mg) results in elevated circulating levels of total BPA in mice at the 9 mg BPS dose (Pollock et

al., 2018) and hypothesized that the BPA kinetics may be altered due to competition for metabolic enzymes, such as sulfotransferase (SULT) or UDP-glucuronosyltransferase (UGT) (Pollock et al., 2018).

Fetal toxicokinetics of bisphenols

We have demonstrated that, like BPA (Corbel et al., 2013; Patterson et al., 2013), both BPS and BPF cross the placenta reaching the fetal compartment and entering the amnion. Maternal *vs*. fetal toxicokinetic parameters were different with lower concentrations detected in the fetal compartment for BPS and BPF. This finding points to an incomplete maternal to fetal transfer, supportive of an active transport, rather than passive diffusion, across the placental barrier as the transfer mechanism (Pacifici and Nottoli, 1995; Yoshikawa et al., 2002). Similar to the maternal compartment, the non-compartmental analysis also revealed marked toxicokinetic differences among all three bisphenols within the fetal compartment.

Despite BPS being the bisphenol with the highest concentration in maternal circulation, BPA had the highest fetal $C_{max\text{-}fet}$ compared to BPS and BPF. This suggests that active placental transport is more permissive for BPA compared to BPS and BPF, which may relate to differences in lipophilicity, solubility, plasma protein binding, and/or molecular weight (Griffiths and Campbell, 2015). In this study, we observed that only BPA had an F/M ratio > 1. Because an F/M ratio > 1 reflects that the chemical has a higher affinity to bioaccumulate in the fetus compared to the mother, we would predict that only BPA would accumulate in the fetal compartment. In fact, the higher $logK_{o/w}$ for BPA and BPF (3.32 and 2.91) points to a higher risk of bioaccumulation in tissues compared to BPS (1.65). However, BPS's long $t_{1/2\text{-}fet}$ (402.2 ± 102.6 h) demonstrates that despite a lower placental transfer, the clearance from the fetal compartment occurs at a much slower rate to that of BPA ($t_{1/2\text{-}fet}$: 52.0 ± 20.3 h), resulting in BPS's long persistence in the fetal compartment. Previous studies have also demonstrated that conjugated BPA can

remain trapped in the fetal compartment (Nishikawa et al., 2010; Corbel et al., 2013). This phenomenon has been hypothesized to occur due to the inability of water-soluble conjugated BPA to cross the placenta, and is therefore excreted in fetal urine and trapped in the amniotic fluid (Corbel et al., 2013). In this study, we have not evaluated the conjugated forms of bisphenols and thus cannot confirm this for BPS. It has also been hypothesized that once present in the amniotic fluid, BPA may be swallowed and recirculated into the fetus (Corbel et al., 2013). The accumulation of BPA in amniotic fluid has been previously reported in rhesus monkeys (Patterson et al., 2013) and rats (Doerge et al., 2011). In support of the idea that the amniotic fluid may serve as a reservoir that helps recirculate bisphenols into the fetal compartments, we demonstrate that BPS and BPF can also accumulate in the amniotic fluid up to 72 h after administration (Table 1.4). Given the predicted $t_{1/2\text{-fet}}$, BPS may be detected in the fetal compartment up to a month after administration.

Chemical accumulation in the fetal compartment is not unique to bisphenols and has been reported with other chemicals (Mitro et al., 2015). Previous studies have shown that a decrease in the efflux transporter placental-glycoprotein (P-gp), also known as MDR1 or ATP-binding cassette transporter B1 (ABCB1), is associated with an accumulation of P-gp substrates (Bloise et al., 2017), such as BPA (Yoshikawa et al., 2002), in the fetal compartment. It is possible that the accumulation of both BPA and BPS, and the comparatively high levels of BPA reached in fetal circulation could potentially be the result of competition between bisphenols for efflux through transporters, such as P-gp. Additional complexity arises from the fact that BPA can also modify ABC transporters in placental cells (Jin and Audus, 2005).

Our previous work has shown that gestational BPS, but not BPA, leads to a placental disruption (Gingrich et al., 2018). Despite a longer BPS half-life in the fetal compartment, the fact that both chemicals are detectable in the amniotic fluid and fetal compartment after 72 h suggests that the placenta was

exposed to either bisphenol to a similar degree. Whether effects on the placenta may relate to different placental bioaccumulation of BPA and BPS or a different exposure in bioactive unconjugated bisphenols, remains unknown. It is also important to note that the current results are specific to mid- to late gestation. Numerous studies have demonstrated how gestational age modifies toxicokinetics, which largely depend on changes in placental transport receptor, cytochrome P450 enzymes expression in liver and placenta, or fetal to maternal albumin ratio (Ewing et al., 2015). These factors should be considered when extrapolating current results to other gestational ages.

In addition to amniotic fluid, this study is the first to detect bisphenols in fetal urine after maternal exposure. In addition to previous studies reporting BPA excretion through meconium (Arbuckle et al., 2015), our study demonstrates that the fetus, like the mother, is also able to excrete bisphenols through urine. Urinary BPA concentrations are similar in the mother compared to the fetus after 72 h of exposure. A similar relationship has been reported for other chemicals, such as triclosan (Arbuckle et al., 2015). Although it has been demonstrated *in vitro* that there are differences in glucuronidation efficiency between bisphenols (Karrer et al., 2018), these glucuronidation rates were determined in adult human liver and intestinal microsomes, whose metabolic profiles are developmental stage dependent (Chiba et al., 1997). However, because we did not evaluate conjugated forms of bisphenols it is not possible to address such conjugation rates in our fetal urine samples. On the other hand, we detected BPS above the limit of quantification in both amniotic fluid and fetal samples prior to exposure (t_0) in over half of the samples analyzed. As observed for BPF, and because animals were housed outdoors until a week prior to surgery, we hypothesize that these higher baseline BPS concentrations in the fetal compartment are the combination of the longer half-life for BPS in the fetal compartment (402.2 ± 102.6 h or ~16.5 days) and potential environmental exposures (i.e. water source (Wan et al., 2018b)) prior to the surgical intervention.

1.5. Conclusion

To our knowledge, this is the first report on the toxicokinetics of either BPS or BPF in a pregnancy model. This study has not only demonstrated differences in toxicokinetic parameters among three of the most prevalent bisphenols, but also between differences in maternal and fetal toxicokinetics. Toxicokinetic differences among bisphenols call for a more careful approach when extrapolating kinetic information from one bisphenol chemical to another.

Chapter 1b: Pregnancy Physiologically-Based Toxicokinetic Modeling of Bisphenol A and Bisphenol S

The high quality maternal and fetal pair-matched toxicokinetic data set generated in Chapter 1, can further be used to development pregnancy-physiologically based toxicokinetic (P-PBTK) models for bisphenols. P-PBTK models are a series of mathematical formulae which incorporate *in vivo* and *in vitro* toxicokinetic data and physiological parameters like blood flow, chemical placental transfer and tissue partitioning, to make exposure predictions. Here, we have successfully developed such a model. Using a previously published P-PBTK model for BPA in mice (Kawamoto et al. 2007), we developed comprehensive preliminary P-PBTK models for BPA and BPS more relevant to human exposure, which include fetal liver deconjugation, placental transport, and a parsimonious maternal compartment. The models were calibrated to three rich toxicokinetic data sets for bisphenols in pregnant sheep; one derived from aim one (Gingrich et al. 2019), and two collected from other publications (Grandin et al. 2018, Corbel et al. 2013). These data are presented as an accepted abstract to the 59th Annual Society of Toxicology Annual Meeting.

Physiologically-based pregnancy toxicokinetic models for bisphenol A and bisphenol S

Jeremy Gingrich*, David Filipovic*, Yong Pu, Sudin Bhattacharya, Almudena Veiga-Lopez

Michigan State University, East Lansing, Michigan 48824, USA

*authors contributed equally to this work

Abstract

The predictive power of physiologically based toxicokinetic (PBTK) models can help inform human health risk assessment for potentially toxic chemicals in the environment. Bisphenol S (BPS) is the second most

abundant bisphenol, after bisphenol A (BPA), detected in humans in the United States. We have recently demonstrated that BPS can cross the placental barrier and disrupt placental function. Differences in physicochemical properties, toxicokinetics, and exposure outcomes between BPA and BPS prevent direct extrapolation of existing BPA PBTK models to other bisphenols. The current study aimed to develop pregnancy-specific PBTK (P-PBTK) models for BPA and BPS. Novel paired maternal and fetal pregnancy data sets for total, unconjugated, and conjugated BPA and BPS plasma concentrations from three independent studies in sheep were used for model calibration. The two developed nine-compartment (maternal blood, liver, kidney, fat, and rest of body, placenta, and fetal liver, blood and rest of body) models simulate maternal and fetal experimental data for both BPA and BPS within one standard deviation of the experimental data points, highlighting the robustness of the model. Simulations were run to examine fetal exposure following daily maternal exposure to BPA or BPS at the tolerably daily intake dose for these chemicals over a two-week period, showing fetal accumulation of 0.28 and 0.09 ng/ml BPA and BPS, respectively. These models advance our understanding of bisphenolic compound toxicokinetics during pregnancy and may be used as a quantitative comparison tool in future p-PBTK models for related chemicals.

Chapter 2: Effects of Chronic Maternal Exposure to Bisphenol A and Bisphenol S on the Placenta

Data from aim one (Gingrich et al. 2019), and others (Grandin et al. 2018) demonstrate the accumulation of both BPA and BPS in the fetus during gestation. As perinatal exposure to bisphenolic chemicals occurs in >90% of pregnancies, and can result in metabolic, reproductive, and neurologic disorders in the progeny, the second aim of this dissertation was to determine if BPA or BPS, given chronically during gestation, at an environmentally relevant exposure dose, was able to impair placental function. The following study (Gingrich et al. 2018) uses singleton pregnant sheep randomly distributed into three treatment groups (n=7-8 per treatment), each receiving daily subcutaneous injections of either corn oil (control), BPA or BPS (0.5 mg/kg/day) from gestational day (GD) 30-100, to answer this question. Throughout, maternal blood was collected to assess circulating factors pertinent to placental health, like progesterone and pregnancy associated glycoproteins. After allowing for a 20-day chemical washout period, pregnancies were terminated (GD 120), and placentas were collected and subjected to gene expression, western blotting, and immunohistochemical and histopathological analyses. This is the first study to demonstrate that gestational exposure to BPS, but not BPA, results in a placental endocrine defect suggestive of a dysfunction in the trophoblast fusogenic signaling pathway. This adds to the body of literature in support of EDC-induced placental pathologies.

Gestational bisphenol S impairs placental endocrine function and the fusogenic trophoblast signaling pathway

Jeremy Gingrich[1], Yong Pu[1], Jennifer Roberts[2], Rajendiran Karthikraj[3], Kurunthachalam Kannan[3], Richard Ehrhardt[1, 2], Almudena Veiga-Lopez[1]

[1]Department of Animal Science, College of Agriculture and Natural Resources, Michigan State University, East Lansing, Michigan 48824, USA

[2]Department of Large Animal Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824, USA

[3]Wadsworth Center, New York State Department of Health, and Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, Empire State Plaza, P.O. Box 509, Albany, New York 12201-0509, USA

Funding sources: Research reported in this publication was supported the National Institute of Environmental Health Sciences of the National Institute of Health (1K22ES026208 to A.V-L.), Michigan State University (MSU), MSU AgBioResearch, and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (Hatch MICL02383). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

2.1. Introduction

Worldwide production of plastics has reached 300 million tons annually, many of which are considered to contain endocrine disrupting chemicals (EDCs). The burden of EDCs exposures has risen in the past decades (Skakkebaek et al. 2011), and so has the concern that these chemicals may pose a risk to human

health (Heindel et al. 2015). Bisphenols are among the most prevalent chemicals worldwide (Vandenberg et al. 2010) used in the production of plastics and manufacturing of consumer products. Regulations on use of bisphenol A (BPA) in certain consumer products has increased the demand for other substitutes, such as bis (4-hydroxyphenyl) sulfone, commonly known as bisphenol S (BPS) (Chen et al. 2016). BPS can be found in personal care products, food, and paper products (Liao and Kannan 2013; Liao and Kannan 2014; Liao et al. 2012). Notably, BPS is detected in >80% of human urine samples (Asimakopoulos et al. 2016; Liao et al. 2012b; Ye et al. 2015) and fetal cord blood (Liu et al. 2017), and the exposure range is lower, but in the same order of magnitude to that reported for BPA (Liao et al. 2012; Ye et al. 2015). At the current rate, BPS may become as widespread as BPA (Liao et al. 2012). In fact, it has already surpassed BPA in certain geographic areas (Asimakopoulos et al. 2016; Liao et al. 2012).

Exposure to EDCs is of concern especially during vulnerable periods of development, such as fetal life (Schneider et al. 2014; Stel and Legler 2015). During pregnancy, the placenta partly serves as the lungs, kidneys, liver, endocrine, and immune systems to the developing fetus and plays a critical role in ensuring normal fetal growth and development by acting as the immediate interface between maternal and fetal blood circulation (Benirschke et al. 2012). Many factors can impair this feto-placental-maternal balance, including maternal diet, stress, lifestyle modifications and/or exposure to EDCs (Fowden et al. 2015; Reynolds et al. 2013; Tait et al. 2015; Vaughan et al. 2011). Because most steroid receptors are expressed in the placenta (Fowden et al. 2015), it is an especially vulnerable target to xenosteroids.

As it relates to bisphenolic compounds, placental studies thus far have been restricted to BPA. To date, one *in vivo* study reported that BPA (50 mg/kg) reduces the placental layer size (Tait et al. 2015), while *in vitro* studies showed effects on placental invasion (Lan et al. 2017; Spagnoletti et al. 2015),

hydroxysteroid dehydrogenase (HSD) activity (Rajakumar et al. 2015), expression of transporters (Sieppi et al. 2016), and miRNA expression (Avissar-Whiting et al. 2010). Receptor affinity differences among bisphenols (Rosenmai et al. 2014) and the emerging use of other bisphenols (BPB, BPE, BPF, and BPS), warrant their study as it relates to their impact on placental function. Among all BPA analogues, BPS is the most distinct compared to BPA with the weakest estrogenic and anti-androgenic activity, but the highest progestogenic activity (Grignard et al. 2012; Molina-Molina et al. 2013; Rosenmai et al. 2014). These marked functional differences call for toxicological risk assessment of BPA analogues. Thus far, toxicological studies of BPS during prenatal life have been restricted to water flea (Chen et al. 2002), zebrafish (Ji et al. 2013; Kinch et al. 2015; Naderi et al. 2014), rats (Castro et al. 2015) and mice (Catanese and Vandenberg 2017; LaPlante et al. 2017), but there is virtually no information on the effects of BPS on placental function.

The unique structure of the placenta allows for the transfer of gases, water, inorganic and organic molecules, including hormones. The placenta's endocrine capacity is responsible for the majority of maternal and fetal circulating progesterone, which holds a key role in pregnancy maintenance for most mammalian species (Wooding et al. 2008a). In humans, progesterone is synthesized by the placental syncytiotrophoblast, which forms the outer most layer of the chorionic villi where feto-maternal exchange occurs. The syncytiotrophoblast forms by fusion of villous cytotrophoblasts, which are located basal to the syncytiotrophoblast layer. E-cadherin expressing cytotrophoblasts in contact with neighboring cytotrophoblasts can form adherens junctions. These junctions allow for cell-to-cell transfer of pro-fusogenic transcription factors, such as glial cell missing factor 1 (GCM1); which in turn regulate the activation of the protein syncytin-1, encoded by endogenous retrovirus envelope genes (*ERVW*) (Black et al. 2010; Huang et al. 2014). Syncytin-1, then, promotes cytotrophoblast homokaryonic fusion events, forming the multinucleated syncytiotrophoblast layer.

Because of the complexity of the placenta, EDC studies on placental function to date are limited, and often rely on placental cell studies that utilize either human primary cultures or cell lines (Gorrochategui et al. 2014; Huuskonen et al. 2015; Meruvu et al. 2016; Vitku et al. 2016; Zhang et al. 2015; Zhao et al. 2014). Although *in vitro* approaches inform about specific pathways involved, animal *in vivo* studies provide a more holistic understanding of exposure effects during pregnancy. In this study, we have used a comparative *in vivo* approach using sheep as an animal model to understand the effects of BPA and BPS on placental function. Sheep, like humans, can be monovulatory, which reduces potential confounding factors seen in litter-bearing species, such as the intrauterine fetal position phenomenon (vom Saal et al. 1999). Despite inherent differences in placentation between primates and ruminants (Fowden et al. 2015; Wooding et al. 2008a), sheep are also considered excellent models to study placental function (Fowden et al. 2015; Mourier et al. 2017), allow for temporal monitoring of placental endocrine changes (Roberts et al. 2017), and offer advantages critical for research that focus on the feto-maternal transfer of EDCs (Corbel et al. 2015). Similar to the human syncytiotrophoblast placental layer, the sheep placenta also has multinucleate cells (referred to as binucleate cells) in the placental trophoblast layer (Igwebuike 2006; Wooding et al. 2008b). The formation of these multinucleated cells has been proposed to occur through a similar fusogenic process to that of humans involving *enJSRV*, another endogenous retrovirus envelope gene (Black et al. 2010; Huang et al. 2014; Koshi et al. 2012). Additionally, these binucleate cells are responsible for the endocrine capacity of the sheep placenta; producing progesterone and pregnancy-specific glycoproteins, both of which are involved in pregnancy maintenance.

The objectives of this study were to assess if gestational exposure the bisphenolic compounds, particularly, BPA and BPS, would result in placental disruption. Specifically, we investigated their effect

on placental endocrine changes, including progesterone and pregnancy-associated glycoproteins (PAGs). In addition, we have investigated their effect on placental morphology, binucleate cell number, and expression of proteins and genes involved in trophoblast cell fusion.

2.2. Methods

Animals

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Michigan State University (MSU), are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals, and meet the ARRIVE guidelines for reporting animal research (Kilkenny et al. 2010). The study was conducted at the MSU Sheep Research Facility (East Lansing, MI; 42.7° N, 84.4° W) using an in-house flock of a cross between Polypay x Dorset breeds. Female sheep (n = 23) at first parity were bred using a time mated pregnancy strategy using three vasectomized rams as previously described (Pu et al. 2017). After estrus detection by vasectomized rams, females were moved with a fertile ram; only one fertile purebred Polypay ram was used in this experiment to reduce paternal variability. Fertility of the ram had been previously tested using a breeding soundness exam. Once mated, the females were randomly assigned to the different treatment groups, blocking for body condition and weight.

Starting 3 weeks before and continuing until 30 days after breeding, breeder ewes were fed a total mixed ration to allow unlimited consumption of a diet providing an energy concentration of 2.9 Mcal/kg of digestible energy and crude protein concentration of 12%. This diet was designed to provide a level of energy and protein intake that would optimize conditions for embryo survival. From days 30 to 100 of gestation, energy concentration was reduced to 2.6 Mcal/kg of digestible energy. Beginning 7 weeks before lambing (100 days of gestation), ewes were fed a total mixed ration designed to maximize dry

matter intake (40% and 70% neutral detergent fiber digestibility). This diet was enriched in energy (3.0 Mcal/kg digestible energy) and crude protein (14%) to exceed the requirements of fetal growth and minimize loss of maternal energy and protein reserves as previously described (Pu et al. 2017).

Gestational treatments

Gestational BPA and BPS treatment consisted of daily subcutaneous injections of BPA (0.5 mg/kg/day; purity ≥99%, Cat# 239658; Lot# MKBQ5209V; Aldrich Chemical Co., Milwaukee, WI, USA) or BPS (4,4'-sulfonyl diphenol, Cat# 146915000, Lot# A0337011, Acros Organics, Geel, Belgium) in corn oil from days 30 through 100 of gestation (term: ~ 147 days). Control (C) mothers received corn oil vehicle. The use of a large animal model limited the study to a single dose per chemical. The internal dose of BPA achieved in umbilical arterial samples using the 0.5 mg/kg/day dose has already been published (Veiga-Lopez et al. 2013) and is targeted to produce maternal blood levels of BPA to that of the median level of BPA measured in maternal circulation of US women (Veiga-Lopez et al. 2015). The same dose of BPS was used to match the BPA exposure dose used in the study. Although main exposure route or bisphenol exposures is oral (Liao and Kannan 2013), the use of sheep as a model, a ruminant species, requires the use of an alternative exposure route to avoid confounding factors from ruminal metabolism. The choice of the s.c. route relates to previous work that has demonstrated that both oral and s.c. administration of BPA exhibit nearly identical phenotypes in a neonatal rat model for prostate health (Prins et al. 2011). The window of exposure starting at GD30 was aimed to avoid confounding effects during early embryonic development and implantation. In addition, the discontinuation of exposure at GD100 allowed for a washout period to avoid confounding effects from the continuous exposure until collection of placental tissue. Number of breeders used were 8, 8, and 7, in C, BPA, and BPS groups, respectively. Number of singleton:twin pregnancies was 6:2, 5:3, 6:1, in C, BPA, and BPS groups, respectively. Number

of female:male fetuses were 6:4, 6:5, 4:4, in C, BPA, and BPS groups, respectively. The mother was considered the experimental unit.

Sample collection and placental characterization

Maternal serum samples were collected in all pregnant females (n = 23) every 2 weeks from gestational day (GD) 30 to 120 for hormonal, protein, and biochemical analyses, and frozen until assayed. To assess placental endocrine function, pregnancy associated glycoprotein 1 (PAG1) and pregnancy specific protein B (PSPB) were measured in all maternal samples. In addition, progesterone was assayed at GD45, GD75, and GD105 of pregnancy. To determine effects of bisphenols on maternal physiological status, a serum biochemistry panel was carried out in GD75 samples. Details of these assays are described below.

Placental samples were collected on gestational day 120 ± 1 (term: ~147 days). All mothers were euthanized with a barbiturate overdose (sodium pentobarbital, i.v.; Fatal Plus; Henry Schein, Melville, NY, USA). A midline incision was performed, the uterus exposed, and the placenta harvested until further processing. All placentas were weighed, number of placentomes counted and classified as types A, B, C, or D, as previously described (Beckett et al. 2014). A type A placentome next to the umbilicus was collected and the maternal and fetal portions of the placentome separated and frozen immediately. The placenta was then perfused with Carnoy's fixative. A placentome near the umbilicus was placed in Carnoy's fixative for 24 hours and processed for paraffin embedding. All placentomes (one per pregnancy) were sectioned (5 µm) with a microtome and center cross-sections of the placentome used for histological (placental stereology and binucleate cell counts) and immunohistochemistry (e-cadherin and GCM1) studies. Frozen placentomes were used to assess gene expression.

Hormonal, biochemical, and protein circulating concentrations

Two commercially available assays were used to measure PAGs in this study; the enzyme-linked immunosorbent assay (ELISA) BioPRYN assay (BioTracking Inc., Moscow, ID, USA) and the Bovine Pregnancy Test Kit (IDEXX Laboratories, Westbrook, ME, USA). Ruminant species have over 20 variants of PAGs and each of these ELISA kits detects a different subset of PAG variants.

The presence of pregnancy-specific protein B (PSPB) in serum was determined using the commercially available quantitative ELISA BioPRYN assay as previously described (Roberts et al. 2017). In brief, serum samples (150 µL) were incubated in a 96-well plate coated with PSPB antibodies overnight. After washing and dry blotting, each well was incubated with the detector solution (secondary labeled antibody) for 1 hour, washed and incubated with the enhancer solution (anti-IgG horseradish peroxidase conjugate) for 1 hour. After washing, each well was incubated with 3,3,5,5'-tetramethylbenzidine for 15 min and the reaction stopped with 1N HCl. Within 30 min, the plate was read at 630 nm (SpectraMax 384 PLUS, Molecular Devices, Sunnyvale, CA, USA). All procedures were done at room temperature. Standard line was accepted with a fit $R^2 > 0.989$. Mean intra-assay coefficient of variation (CV) based on two quality control pools were < 1 and 2%. The inter-assay CV for the same quality control were 4.7 and 14.2%, respectively.

The Bovine Pregnancy Test Kit (IDEXX Laboratories) was used to semi-quantitatively determine PAG1 and has been validated for ovine species as previously described (Roberts et al. 2017). This kit detects the variants PAG-4, PAG-6, PAG-9, PAG-16, and PAG-19. Here, we use the term PAG1 to refer to the group of modern PAGs detected by this assay and secreted by the binucleate cells of the placenta (Sousa et al. 2006). In brief, maternal serum (100 µL) and assay controls (positive and negative) were pipetted into 96-well, anti-PAG antibody coated plates along with sample diluent, sealed, and incubated for 60

min at 37 ºC in a forced air incubator (Mini-Hybridization Oven, Hybaid, Franklin, MA, USA). Between assay steps, plates were washed 4 times with wash solution (405 LS Washer, BioTek, Winooski, VT, USA). The detector solution (anti-PAG antibody) was added to each well, covered, and incubated for 30 min at room temperature. The plates were incubated for 30 min at room temperature with conjugate solution (anti-IgG-horseradish peroxidase) followed by an incubation with substrate solution (tetramethylbenzidine) for 15 minutes at room temperature. Finally, stop solution was added and absorbance (450 nm) determined on a microtiter plate spectrophotometer (Elx808, BioTek, Winooski, VT, USA). Sample values were reported as serum sample minus negative controls after subtracting the mean absorbance value of the negative controls from the absorbance of each sample value. The intra-plate and inter-plate CVs for the positive controls were 2.7 and 6.3%, respectively.

Progesterone concentrations in serum were determined using a commercially available direct, competitive ELISA assay (Ridgeway Science, Gloucestershire, United Kingdom) following manufacturer instructions. Briefly, standards (0.6 to 15.0 ng/mL), serum samples and a plate control (10 ng/mL) were diluted 1:20 with progesterone-enzyme conjugate and 200 µL pipetted into anti-progesterone-coated 96-well plates in duplicate. Plates were sealed with adhesive plate seals and incubated at 37 °C for 2 hours on a rotating shaker at 250 rpm/min. After incubation, plates were washed five times, allowing buffer to sit in plates two minutes between each wash. After washing, 200 µL of the substrate was added and plates incubated for 30 min at room temperature in the dark. Absorbance (550 nm) was read using a microtiter plate spectrophotometer (Elx808, BioTek, Winooski, VT, USA). Progesterone concentrations for each sample and plate control were calculated from the standard curve generated with a 4-parameter logistic regression model (Gen5 2.06.10, BioTek, Winooski, VT, USA). Intra- and inter-plate CV for the plate control were 3.6 and 8.1%, respectively.

Full biochemistry analyses (total protein, albumin, globulin, serum ions (Na^+, K^+, Cl^-, P, Mg^{2+}, Ca^{2+}, and I^-), total bilirubin, anion gap, osmolarity, cholesterol, creatinine, urea nitrogen, alkaline phosphatase, aspartate aminotransferase, creatine kinase, and γ-glutamyltransferase) were performed at the American Association of Veterinary Laboratory Diagnosticians (AAVLD) accredited Diagnostic Center for Population and Animal Health (DCPAH) at Michigan State University.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from placental tissue was extracted from the maternal portion of a type A placentome (most common type in all pregnancies) using a RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The fetal portion of the placentome is highly vascularized and was not used for mRNA extraction. A Nanodrop (Thermo Fisher Science, Wilmington, NC, USA) was used to assess RNA quality and concentration. RNA (1 µg; A260/A280: 2.0 ± 0.05; RNA concentration: 300 ± 50 ng/µL) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Promega, Madison, WI, USA). Quantitative real time PCR (ABI-Quant Studio 7 Flex Real-Time PCR System, ThermoFisher, Carlsbad, CA, USA) was performed to examine the mRNA expression of the genes related to trophoblast fusion: envelop Jaagsiekte sheep retrovirus (*enJSRV*) and hyaluronoglucosaminidase 2 (*HYAL2*). Primer sequences are shown in Table 2.S1 and designed using the *Ovis aries* genome. Fast start SYBR green master mix (Invitrogen, Warrington, UK) was used for qPCR. The amplification reaction included template denaturation and polymerase activation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Melt curve analyses were performed for all genes, and the specificity, as well as the integrity of the PCR products was confirmed by the presence of a single peak. For all genes, an agarose gel was run to assess single product amplification. The levels of mRNA encoding the indicated genes were normalized against housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L27

(*RPL27*) to calculate relative fold change to that of the control using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Results using both housekeeping genes provided with the same results, but only *GAPDH* data are shown.

Histology and immunohistochemistry

Staining was performed on 5 μm serial cross-sections of paraffin embedded placentomes. One section from each placentome was stained with hematoxylin and eosin for histological examination. In brief, sections were deparaffinized in xylene, rehydrated, incubated in Mayer's hematoxylin for 3 min and eosin for 30 sec, dehydrated in ethanol and mounted with acrytol mounting medium. For immunohistochemistry, sections were deparaffinized in xylene, rehydrated through an ethanol series, treated with 3% hydrogen peroxide, and antigen retrieved using an EMS 2100 Retriever (Hatfield, PA, USA) with sodium citrate buffer. Sections were then subjected to a streptavidin and biotin block (SP-2002, Vector Laboratories, Burlingame, CA, USA), followed by 4% normal goat serum in phosphate buffered saline (PBS) block. Details of antibody information are listed in Table 2.S2. Mouse anti-e-cadherin and mouse anti-GCM1 primary antibodies were incubated at 4 °C overnight, and biotinylated *Wisteria floribunda* lectin was incubated at room temperature for 30 min. Biotinylated goat anti-mouse IgG secondary antibody was applied to e-cadherin and GCM1 sections. All sections were subjected to Vectastain ABC peroxidase kit (#PK-6100, Vector Laboratories, Burlingame, CA, USA) and visualized with a 2% 3,3'-diaminobenzidine (DAB) substrate solution. DAB development time was equal across all treatment groups. All sections for the same antibody were incubated at the same time so that staining intensity could be compared among samples of different treatment groups. Slides were then rehydrated through ethanol and xylene washes and mounted with acrytol mounting medium (#13518, EMS, Hatfield, PA, USA).

Image analysis

All images were captured using an Olympus BX41 microscope with an Olympus DP71 camera or a Leica DMLB microscope with a Leica DFC480 camera on a 20X or 40X objective lens. All imaging and analyses were conducted blind to treatment group. Unless otherwise noted, all images were taken within the feto-maternal interface of the placentome. The selection of the interface for image acquisition was done to avoid skewed contributions from the maternal or fetal compartments. Systematic random images (10 per placentome section/animal) of hematoxylin and eosin stained placentome sections were obtained across the placentome for stereological examination. Using a 256-square grid overlay with a tissue area of 140.6 mm^2 the contribution of intervillous space area and area of maternal and fetal compartments was assessed. At each grid intersection, the tissue was classified as fetal, maternal, or intervillous space. Results for all 256 intersections were summed for each individual, averaged to treatment group, and presented as a percent relative to the total area at the feto-maternal interface. Systematic random images (10 per placentome section/animal) were captured for e-cadherin, GCM1, and lectin (stain for binucleate cells). Image analyses for e-cadherin and GCM1 were performed by selection and identification of DAB-positive stain area using the Fiji software immunohistochemistry analysis plug-in "*IHC toolbox*" (Schindelin et al. 2012). Binucleate cells were manually identified and counted as such when lectin stain was present in uni- or binucleate cells or absent in binucleate cells.

Maternal-to-fetal transfer of BPS

A pilot study to assess maternal-to-fetal placental transfer of BPS was conducted. At gestational day 117-118, two pregnant sheep (both carrying a singleton male fetus) underwent surgery to place catheters in the descending aorta and inferior vena cava as previously described (Ehrhardt et al. 2002). In brief, fasted animals were sedated using xylazine (0.2 mg/kg) and anesthetically induced with ketamine (8 mg/kg). Anesthesia was maintained using inhaled isoflurane (2% vaporized). After sterile

preparation of surgical site, a midline laparotomy was performed for uterine access. Through a small hysterotomy in the uterine horn, the fetal hind limb was isolated by external uterine palpation, and exposed past the tibio-tarsal joint. The cranial tibial vein was isolated with fine forceps, and catheterized with a polyvinyl catheter (Saint-Gobain Performance Plastics, Beaverton, MI, USA; inner diameter: 0.86 mm, outer diameter: 1.37 mm). The catheter was threaded through the cranial tibial vein into the inferior vena cava (20 cm) and secured laterally to the fetal hind limb using a non-absorbable suture (silk, USP 3-0). The uterine incision was closed with an absorbable monofilament suture (polyglycolic acid, USP 0) using an inverted pattern finished with a single Cushing suture to avoid peri-uterine adhesion. Catheters were exteriorized through a small incision (1 cm) in the flank of the animal nearest to the hysterotomy. Muscle and peritoneum were closed using absorbable suture material (polyglycolic, USP 1) in an interrupted pattern. Midline skin incision was closed using non-absorbable suture (silk, USP 0) in an interrupted mattress pattern. Catheter patency was maintained through daily flushing with sterile saline, followed by heparinized saline. Fetuses all received injections (i.m.) and amniotic infusions of penicillin G (300,000 U) at the time of surgery, and every 2 days after surgery. Additionally, all ewes received penicillin G (6,000 U/kg BW) and gentamycin sulfate (2 mg/kg BW) 24 hours before surgery, at the time of the surgery, and every 12 hours for 3 days after surgery. One week after surgery, a single injection of BPS (0.5 mg/kg BW, s.c.) was administered to the pregnant female and fetal arterial blood samples were collected 1 hour after the BPS administration. Fetal arterial blood samples collected prior to maternal BPS administration were used as their baseline control. In addition, blank samples (double distilled water) were collected to assess potential BPS contamination from sampling collection materials. All samples were processed in glass vials with glass Pasteur pipets, stored in glass vials, and kept frozen until assayed.

Bisphenol S analysis

BPS (analytical standard) and the internal standard $^{13}C_{12}$-BPS were purchased from Sigma Aldrich (St. Louis, MO, USA) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively. An API 4500™ electrospray QTRAP mass spectrometer (ESI-MS/MS; Applied Biosystems, AB Sciex, Framingham, MA, USA) interfaced with an Agilent 1260 HPLC (Agilent Technologies Inc., Santa Clara, CA) was used for the analysis. A Zorbax SB-Aq (150 mm x 2.1 mm, 3.5 µm; Agilent Technologies Inc., Santa Clara, CA) column serially connected to a Javelin guard column (Betasil® C18, 20 × 2.1 mm, 5 µm) was used for the chromatographic separation of BPS using methanol and water as mobile phases. An isotopic dilution method was used for the quantification of BPS. BPS was extracted from sheep serum and other fluids by a liquid-liquid extraction method (Liao et al. 2012) with slight modifications. Briefly, 250 µL of serum was transferred into a 15-mL polypropylene tube and spiked with 20 ng of the internal standard (IS). Five hundred microliters of 1.0 M ammonium acetate buffer (pH = 5.5) that contained 22 units of *β*-glucuronidase was added and digested at 37° C overnight (~15 h) in an incubator shaker. The samples were extracted twice with 10 mL (2 × 5 mL) of ethyl acetate by shaking in a mechanical shaker for 2 h. The extracts were combined and washed with 1 mL of ultra-pure water and centrifuged at 4500 x g for 10 min. The ethyl acetate layer was transferred into another 15-mL tube and concentrated to near-dryness under a gentle nitrogen stream. Then, 250 µL of methanol was added, vortex mixed, and transferred into a vial for HPLC–MS/MS analysis. For every 15 samples, a procedural blank, a matrix blank (human sera, Sigma Aldrich, St. Louis, MO, USA) and a matrix spike experiment were performed. The matrix spike recoveries ranged between 85.0 and 98.9% and the mean absolute recovery for the assay of IS was 90%. The instrumental LOD and LOQ were 0.02 ng/mL and 0.07 ng/mL, respectively. Trace level (0.005 ng/mL) of BPS was found in procedural blanks (n = 4) and that value was subtracted from all samples.

Statistical analysis

All data are presented as mean ± SEM. Appropriate transformations were applied, as needed, to account for normality of data. Comparisons among the three treatment groups were analyzed by a mixed model or ANOVA with Tukey posthoc tests with number of fetuses as a covariate using PASW Statistics for Windows release 18.0.1. Differences were considered significant at $P<0.05$.

2.3. Results

Gestational BPS disrupts placental endocrine function

Pregnant sheep exposed to BPS daily from GD30 to GD100 had reduced maternal serum concentrations of trophoblastic proteins PAG1 and PSPB (Figure 2.1) reflective of impaired placental endocrine function. This effect was significant from GD75 to GD90 for PAG1 (Figure 2.1A; $P<0.05$) and from GD60 to GD90 for PSPB (Figure 2.1B; $P<0.05$). Gestational BPA exposure did not have an effect on maternal PAG or PSPB plasma concentrations. Discontinuation of the BPS exposure at GD100 resulted in a partial recovery of both PAG1 and PSBP maternal serum concentrations. During late gestation, serum progesterone concentrations increase when progesterone secretion shifts from corpus luteum to placental origin. This normal physiological increase in serum progesterone levels tended to be lower in the BPS group in comparison to that of the control group (Figure 2.1C). Progesterone concentrations were unaffected by BPA exposure. Serum biochemistry analyses at GD75 did not find any significant differences in total protein, albumin, and globulin (Figure 2.1D). BPA- and BPS-exposed females had lower phosphorous compared to that of the control and creatinine was higher in BPS-exposed females compared to control and BPA-exposed females, although both phosphorous and creatinine were within normal reference values (Table 2.S3). All other biochemistry parameters studied were not significantly different among groups.

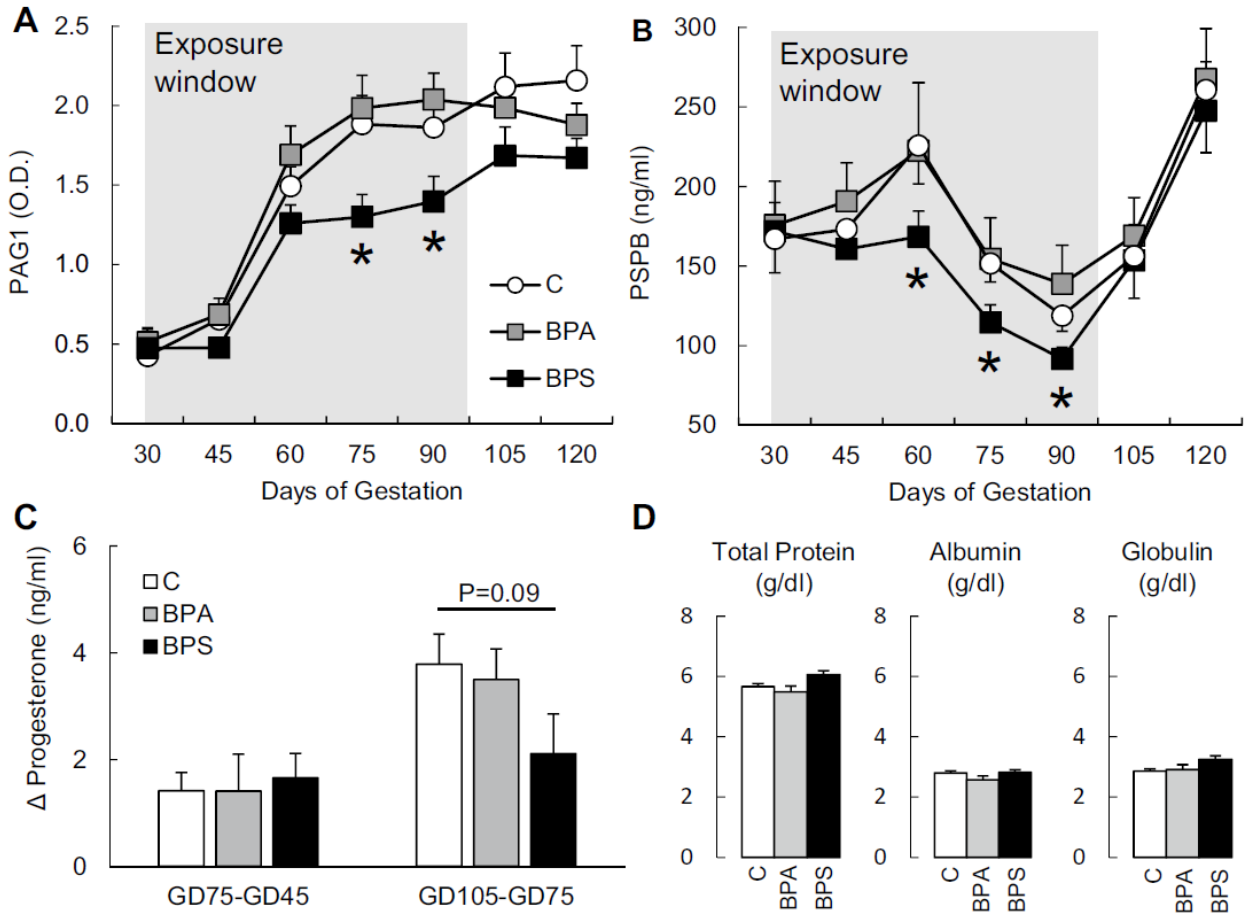

Figure 2.1: Maternal serum pregnancy-associated glycoprotein 1 and pregnancy-specific protein B in control, BPA, and BPS exposed females. Maternal serum (mean ± SE) pregnancy associated glycoprotein 1 (PAG1; (A)) and pregnancy specific protein B (PSPB (B)) in control (*open circles*), BPA- (*gray squares*) and BPS- (*closed squares*) exposed females. Change in maternal serum progesterone between GD45 and GD75 and between GD75 and GD105 (C) in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females. Maternal serum (mean ± SE) total protein, albumin, and globulin at GD75 (D) in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females. N=6-7/group. Asterisks denote statistical differences between control and BPS-exposed group at P<0.05.

Maternal-to-fetal transfer of BPS

One hour after a single injection of vehicle (corn oil) or BPS (0.5 mg/kg BW, s.c.) to the pregnant animal, BPS concentrations in fetal arterial blood samples were 0.02 ng/mL (LOD) in both control fetuses and 4.9 and 10.6 ng/mL in the two BPS fetuses. Mean BPS concentration in blank samples that had not been in contact with collection materials was 0.020 ± 0 ng/mL. Mean BPS concentration in blank samples that had been in contact with collection materials (fetal catheter and collection syringe) was 0.038 ± 0.017 ng/mL.

Gestational BPS does not affect placental gross morphology and histology

Histology of placentomes and placental membranes did not reveal pathological signs of fibrosis, picnosis, necrosis, or active inflammation (Figure 2.2; top right). Gestational exposure to BPA or BPS did not alter placental weight when compared to that of the control group, but tended to be higher in BPS *vs*. BPA group (P=0.07; Figure 2.2C). Placentome count assessed at the time of collection showed no significant difference in the number of placentomes among treatment groups (Figure 2.2D). All placentomes were of types A for all placentas with the exception of one BPS-exposed placenta that had ~20% type C or D placentomes (data not shown). There was no difference in fetal or maternal tissue contribution or intervillous space at the feto-maternal interface among treatment groups (Figure 2.2E). We have previously reported that gestational BPS exposure resulted in lower biparietal diameter in male fetuses, without fetal body weight differences (Pu et al. 2017).

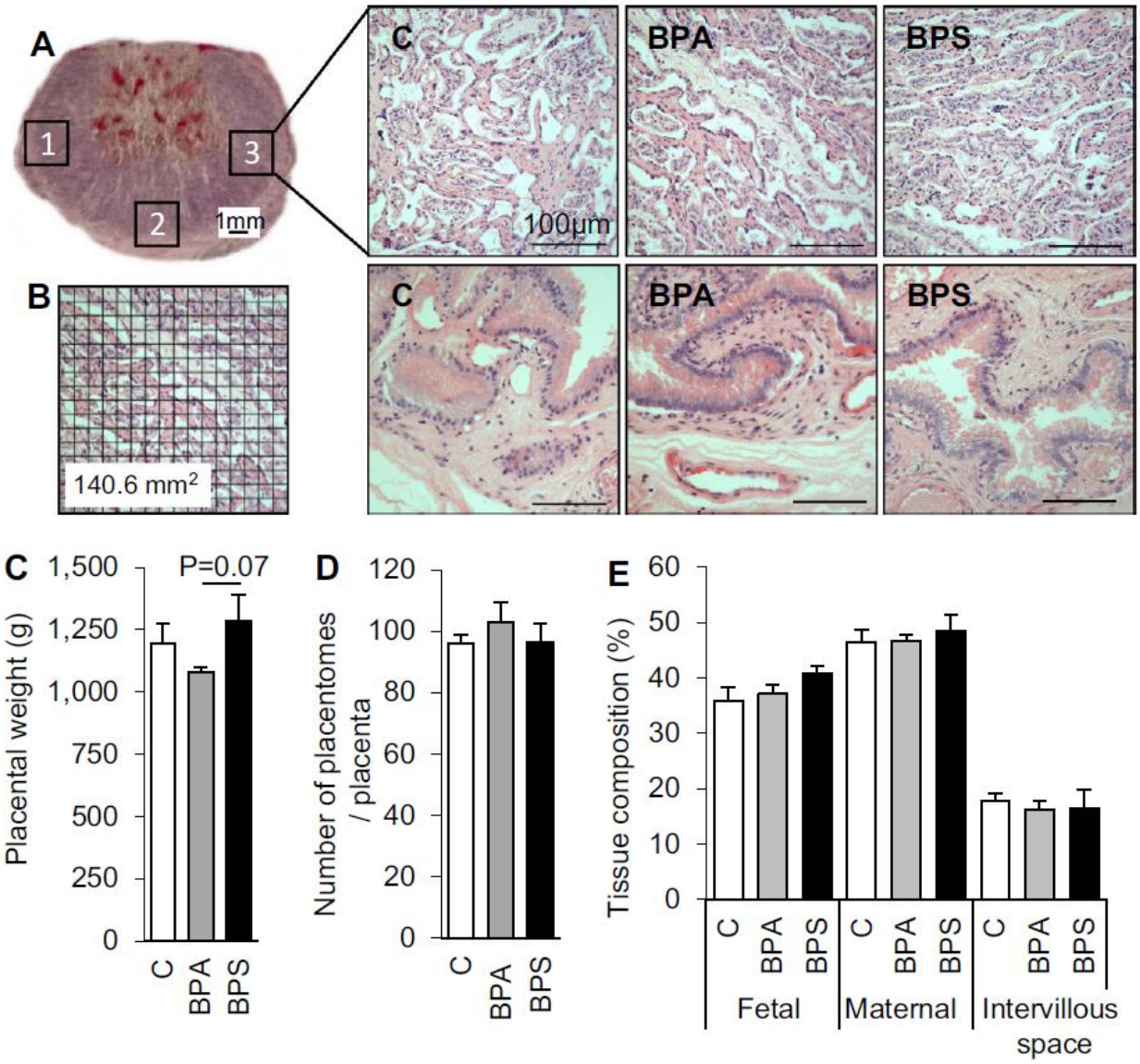

Figure 2.2: Placentome cross section stained with hematoxylin and eosin and stereology. (A) Placentome cross-section stained with hematoxylin and eosin. Numbered squares (1, 2, and 3) denote approximate areas where images were taken for analysis of placental stereology and immunohistochemistry of e-cadherin, GCM1, and lectin (Figures 2.3, 2.4, and 2.5). Representative images of placentome cross-sections (*top*) and intercotyledonary membranes (*bottom*) in control (*left*), BPA- (*middle*) and BPS- (*right*) exposed females at gestational day (GD) 120 (20 days after discontinuation of exposure). (B) Example of placentome image with grid overlay used for placental

Figure 2.2 (cont'd): stereology (Figure 2.2E). (C) Mean (± SE) placental weight, in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females. (D) Number of placentomes per placenta. All placentome types were included (see text for details). (E) Placental tissue distribution, depicting percent intervillous space and fetal and maternal tissue contributions in control (*open bars*), BPA (*gray bars*), and BPS (*black bars*) exposed placentas. N=6-7/group. Four to five images studied per placentome.

Gestational BPS impairs the syncytialization signaling pathway

To examine if the reduction in maternal placental function reflected a reduced trophoblastic function *in vivo*, the syncytialization signaling pathway was explored in GD120 placentomes (20 days after chemical exposure had ended). Primarily localized in the fetal trophoblastic region of the placentome, e-cadherin immunostain was significantly reduced in the BPS group (Figure 2.3A) compared to the control and BPA-exposed groups. mRNA expression of genes involved in trophoblast cell fusion (*enJSRV* ($P=0.06$) and *HYAL2* ($P<0.05$)) were also downregulated in BPS-exposed placentas compared to that of the control group (Figure 2.3B). GCM1 immunostain was significantly increased in prenatal BPS exposure when compared to the control and BPA-exposed groups (Figure 2.4).

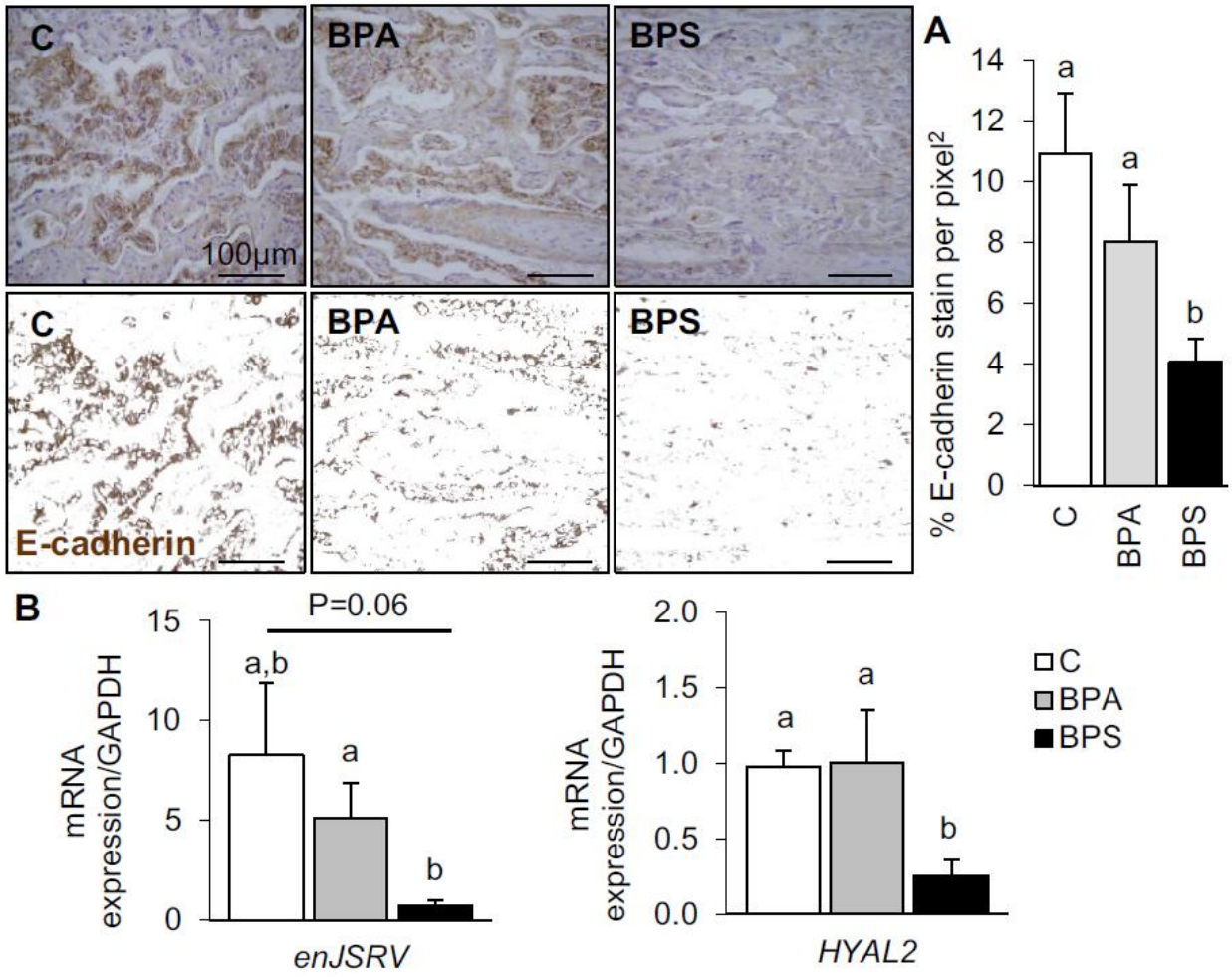

Figure 2.3: Representative images and quantification of placentome cross sections immunostained against e-cadherin. Representative images of placentome cross-sections immunostained against e-cadherin (*top*) and respective processed images for imaging analyses (*bottom*) in control (*left*), BPA- (*middle*) and BPS- (*right*) exposed females at gestational day (GD) 120 (20 days after discontinuation of exposure). **(A)** E-cadherin protein (mean ± SE) quantification in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females by immunohistochemistry (*histogram*). N=6-7/group. Ten images studied per placentome. Different letters denote statistical differences among treatment groups at P<0.05. **(B)** mRNA expression (mean ± SE) of envelop Jaagsiekte sheep retrovirus (*enJSRV*) and hyaluronoglucosaminidase 2 (*HYAL2*) in control (*open bars*), BPA- (*gray bars*), and BPS- (*closed bars*)

Figure 2.3 (cont'd): exposed females. N=6-7/group. Different letters denote statistical differences among treatment groups at P<0.05.

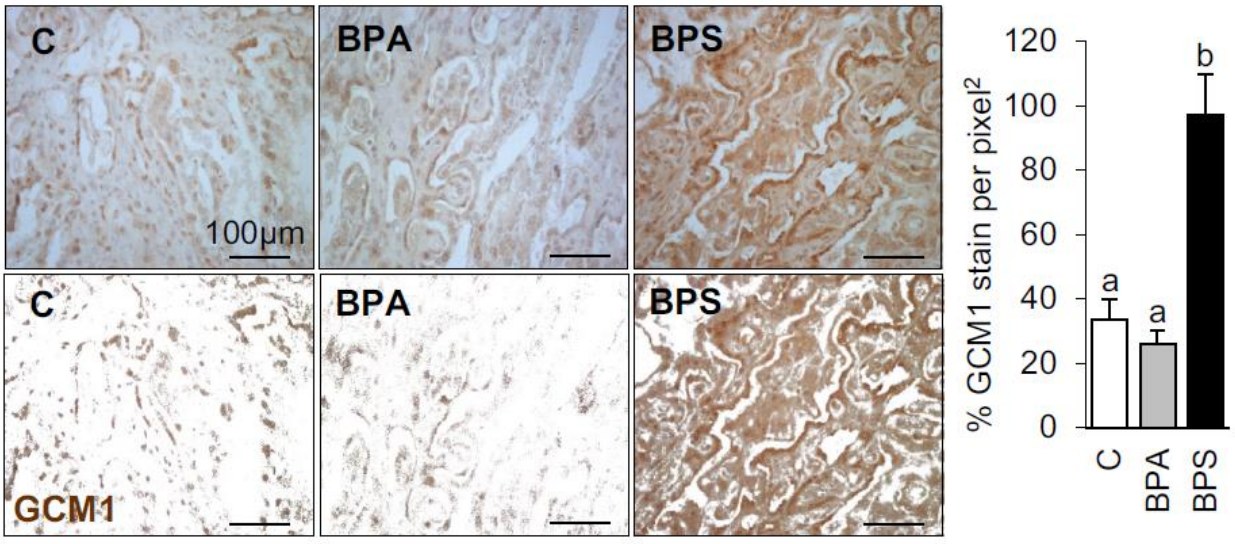

Figure 2.4: Representative images and quantification of placentome cross sections immunostained against GCM1. Representative images of placentome cross-sections immunostained against GCM1 (*top*) and respective processed images for imaging analyses (*bottom*) in control (*left*), BPA- (*middle*) and BPS- (*right*) exposed females at gestational day (GD) 120 (20 days after discontinuation of exposure). GCM1 protein (mean ± SE) quantification in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females by immunohistochemistry. N=6-7/group. Ten images studied per placentome. Different letters denote statistical differences among treatment groups at P<0.05.

Gestational BPS reduces binucleate cell population

To assess the role that changes observed in cell adhesion and fusogenic proteins expression had on steroidogenic placental cell populations, binucleate cells were identified with lectin stain (Figure 2.5A) and quantified in GD120 placentomes. A significant reduction (22.8%; P<0.02) in trophoblast-derived

binucleate cell number was observed in BPS-exposed placentas when compared to the control group (Figure 2.5B). No significant change was observed in the BPA-exposed group.

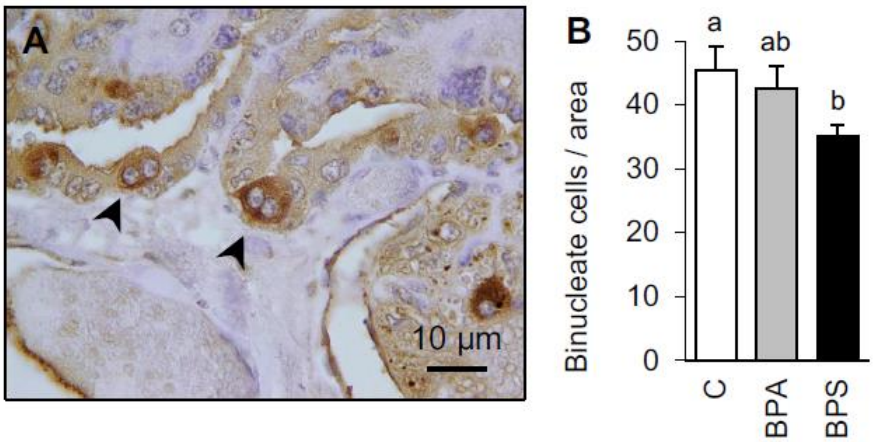

Figure 2.5: Lectin immunostained binucleate cell quantification. Arrows denote binucleate cells. (B) Binucleate cell number (mean ± SE) quantification in control (*open bars*), BPA- (*gray bars*) and BPS- (*closed bars*) exposed females. N=6-7/group. Binucleate cell number count ranged from 25-73 cells per image. Ten images studied per placentome. Different letters denote statistical differences among treatment groups at $P<0.05$.

2.4. Discussion

The effects of endocrine disrupting chemicals on placental function remain poorly understood. In this study, we evaluated placental endocrine and trophoblast fusogenic function in sheep exposed to BPA or BPS during pregnancy. Our comparative experimental design demonstrates for the first time the deleterious effects that BPS can exert on placental function. We have demonstrated that mid-gestation exposure to BPS results in impaired endocrine placental function, reduction of binucleate cells, downregulation of the cell to cell adhesion protein e-cadherin, genes involved in trophoblast fusion (*enJSRV* and *HYAL2*) and compensatory upregulation of the fusogenic transcription factor GCM1 (Figure

2.6). Importantly, at the same exposure dose, gestational exposure to BPA did not result in an abnormal placental phenotype. We also demonstrated that BPS can cross the placental barrier and reach the developing fetus. Overall, this study proves that the placenta is susceptible to BPS, highlights the intrinsic differences among bisphenolic chemicals, and the need to further investigate the safety of BPA analogue exposures on placental function in humans.

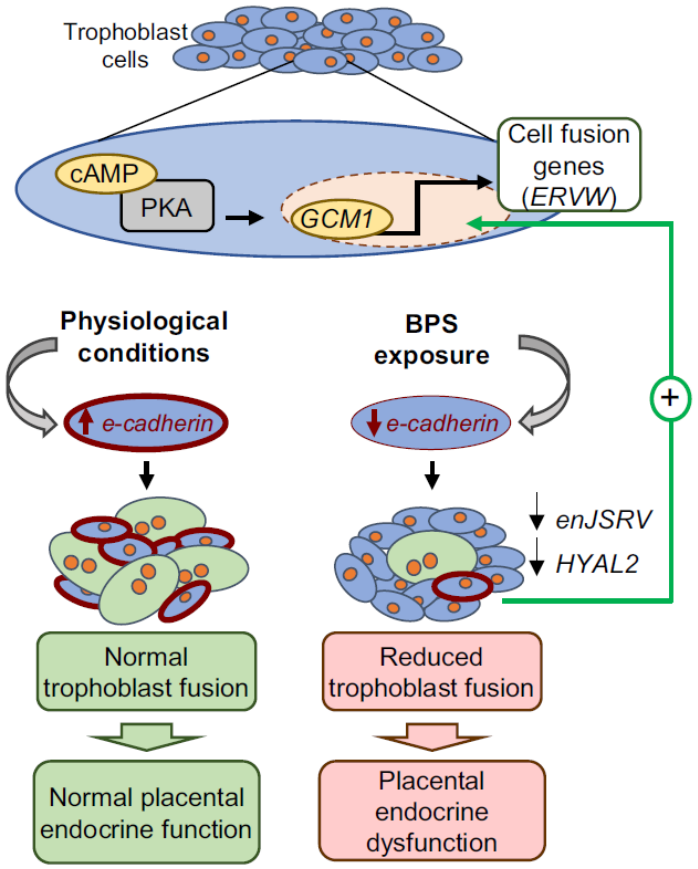

Figure 2.6: BPS-induced placental dysfunction working model. Trophoblast cell fusion is a cAMP-activated of the protein kinase A (PKA) pathway which triggers the expression of the transcription factor glial cell missing factor 1 (GCM1). GCM1 in turn stimulates transcription activity of fusogenic genes, such as endogenous retrovirus envelope genes (*ERVW*). ERVW genes, such as *enJSRV* in sheep, can promote cell fusion, resulting in binucleate cell formation. However, before cell fusion can occur, cell to cell

Figure 2.6 (cont'd): communication between two adjacent cells has to take place. E-cadherin, a transmembrane protein, facilitates cell to cell communication by the formation of adherens junctions in trophoblast cells (*red cellular membranes*). Under physiological conditions (*left side*), e-cadherin expression facilitates cell-to-cell communication enabling trophoblast fusion to occur and binucleate cells to form, resulting in normal placental endocrine function. Our findings demonstrate that gestational BPS exposure (*right side*) results in lower placental e-cadherin expression, preventing the formation of adherens junctions required for cell fusion and thus reducing the number of binucleate cells and expression of fusogenic genes (*enJSRV* and *HYAL2*). Altogether, this results in placental endocrine dysfunction. We hypothesize that the upregulation of GCM1 expression is a compensatory mechanism to overcome reduced trophoblast fusion rate.

Gestational BPS impairs placental endocrine function

Gestational exposure to BPS reduced maternal circulating levels of pregnancy associated glycoproteins (PAG1 and PSPB). PAGs are proteins from the aspartic proteinase family that are secreted by the trophoblast layer of the ruminant placenta (Sousa et al. 2006). Although PAGs biological significance remains elusive, these proteins are required for pregnancy maintenance in ruminants (Wallace et al. 2015). Because factors such as heat stress can reduce circulating PAGs (Thompson et al. 2013) and reduction in circulating PAGs have been associated with early and late pregnancy loss in cattle (Pohler et al. 2016), PAGs have been proposed as biomarkers for pregnancy health in ruminant species (Wallace et al. 2015). Our results further support this premise given that mothers were otherwise healthy (no alterations in the biochemical profile). The recent discovery of the presence of an aspartic proteinase in the human placenta, homologous to PAGs in other domestic species (Majewska et al. 2017), opens the opportunity to use PAGs as biomarkers in the context of human placental pathology.

The effect of BPS on circulating progesterone further supports a compromise in placental endocrine function. However, this effect was time-dependent. BPS did not affect plasma progesterone concentration during early mid-gestation, but rather during late mid-gestation. The lack of change in the early mid-gestation phase (GD45-GD75) is likely due to a relatively stable secretion of progesterone until GD75 in sheep (Bedford et al. 1972) of mainly ovarian origin (Harrison and Heap 1978). Under physiological conditions, progesterone continues to increase through the late mid-gestation phase reflecting a physiological shift from ovarian to placental production of progesterone at mid-gestation (Harrison and Heap 1978) and continues to increase until birth (Bedford et al. 1972). BPS exposure halted progesterone's physiological increase through the late mid-gestation phase (GD75 to GD105) supportive of an impaired placental endocrine function. Such an effect in progesterone concentrations during pregnancy has only been reported in a few EDCs *in vivo*. For instance, triclosan reduces progesterone leading to abortion, but only at high exposure doses (600mg/kg/day) (Feng et al. 2016). In humans and sheep, low maternal serum progesterone is linked to pregnancy complications of unknown etiologies, such as preterm birth and spontaneous abortions (Arck et al. 2007; Fylling et al. 1973; Schindler 2005; Van Calster et al. 2016). Additional studies are necessary to elicit the involvement of EDCs in the development of these pregnancy complications. This study could not address if uninterrupted gestational exposure to BPS through pregnancy would have resulted in preterm delivery. To note is that progesterone supplementation is used in humans for preterm birth prevention in at-risk pregnancies (Newnham et al. 2014; Rundell and Panchal 2017), which highlights the significance of placental progesterone homeostasis on pregnancy maintenance.

The longitudinal experimental design allowed us to track progression of the placental defect. Although the reduction in PAGs was not significantly different until GD60 (30 days after the start of the exposure),

PAG1 concentrations began to be numerically lower two weeks (GD45) after the exposure begun. This indicates that a minimum exposure of two weeks for BPS to elicit a detectable change in placental endocrine function. Importantly, the discontinuation of the exposure (GD100) before tissue harvest (GD120), resulted in partial and full recovery in PAG1 and PSPB circulating concentrations, respectively. This is supportive of the reversibility of the observed effect. Since the number of binucleate cells were lower in BPS-exposed placentas at GD120, we hypothesize that the placenta has the ability to buffer a significant loss (~20%) of endocrine cells while continuing to partially maintain endocrine function and pregnancy. This is similar to other organs such as the liver and kidney that can continue to compensate homeostatic balance despite significant cell loss (Barai et al. 2010; Shoup et al. 2003). More information is required to understand the limitations of this buffering ability in the placenta and the threshold to successfully overcome a significant loss of placental endocrine cells.

We have demonstrated that BPS can cross the ovine placental barrier and reach fetal circulation. In this study, BPS fetal concentrations reached ~7 ng/mL (one hour after maternal administration), which is 100-fold higher compared to human fetal circulation (0.03-0.12 ng/mL) (Liu et al. 2017). These findings support human transplacental transfer of BPS reaching fetal circulation (Liu et al. 2017) and are similar to those observed in BPA-exposed fetuses (Veiga-Lopez et al. 2013). We have previously reported that fetal biparietal diameter tended to be lower in BPS-exposed males, but not females (Pu et al. 2017) despite a discontinuation of the exposure for 20 days prior to pregnancy termination. This suggests that fetal development may be directly affected by BPS (BPS can cross the placental barrier) or indirectly via placental endocrine dysfunction. Additional work is required to determine if BPS-induced placental defects result in long-term consequences for the progeny. Interestingly, we noticed that BPS had a stronger effect in placentas from male fetuses for many of the outcomes studied (binucleate cell

number, GCM1, e-cadherin) than in placentae from female fetuses. However, due to the number of animals per group (n=7-8) results have been presented for all pregnancies combined.

Gestational BPS disrupts trophoblast fusogenic function

In sheep, placental endocrine function at the cellular level is dependent on trophectoderm derived uni- and bi-nucleate trophoblast cells. During placentation, ovine trophoblast cells undergo homokaryonic fusion to form binucleate trophoblast cells through a similar event to that of human syncytiotrophoblast formation (Huang et al. 2014; Koshi et al. 2012). One of the first events in cell fusion is the formation of adherens junctions between adjacent cells. This process is facilitated by the transmembrane protein e-cadherin, whose expression is reduced upon gestational exposure to BPS. We have not investigated the mechanism by which BPS results in e-cadherin downregulation. To date, underlying mechanisms by which bisphenols can modulate e-cadherin expression in the placenta remain unclear (Borman et al. 2017; Borman et al. 2015; Wang et al. 2015), although inhibition experiments have pointed out to the estrogen receptor 1 (ESR1) as the mechanism by which BPA modulates e-cadherin in hemangioma cells (Zhai et al. 2016).

Loss of e-cadherin naturally occurs in human pregnancies between the first and second trimester (Zhou et al. 1997). Lower than normal expression has also been reported in placenta accreta (Duzyj et al. 2015), placenta percreta (Incebiyik et al. 2016) and placentas of somatic cell nuclear transfer derived embryos (Kohan-Ghadr et al. 2011). Additionally, a down regulation of genes encoding cadherin-associated protein (CTNNAL) have also been reported in preeclamptic placentas complicated with hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome (Buimer et al. 2008). The loss of e-cadherin hampers the fusogenic ability of the placenta resulting in aberrant cell adhesion, initiation of an invasive phenotype, and ultimately may result in trophoblast cells undergoing epithelial to

mesenchymal transition (Kokkinos et al. 2010). This is a common phenomenon observed in carcinoma invasiveness (Peinado et al. 2007; Schmalhofer et al. 2009). In this work, we have not further explored the tumorigenic nature of the exposure to BPS. However, other EDCs such as phthalates and BPA have been reported to trigger epithelial to mesenchymal transition in colorectal cancer cells (Chen et al. 2015) and ovarian cells (Kim et al. 2015). Although e-cadherin expression is required prior to cell fusion, its expression is reduced during cell fusion (Coutifaris et al. 1991). Given the reduced number of binucleate cells observed upon gestational BPS exposure, the lower e-cadherin expression is unlikely to be reflective of higher cell fusion events.

The observed reduction in e-cadherin protein and mRNA expression was accompanied by an upregulation of the transcription factor GCM1 in BPS-exposed placentas. Trophoblast cell fusion is controlled by a cAMP-mediated process which activates the transcription factor GCM1. GCM1, in turn, stimulates transcription activity of fusogenic genes, such as *syncytin-1*, encoded by endogenous retrovirus envelope genes (ERVW). The ovine homolog for *syncytin-1* is *enJSRV* (Black et al. 2010), which tended to be downregulated by BPS. *HYAL2*, a cellular receptor for *enJSRV* (Rai et al. 2001) and exclusively expressed in binucleate cells (Dunlap et al. 2005) was also downregulated. The cell-to-cell fusion cascade is triggered by ERVW genes in both humans and ruminants (Black et al. 2010; Huang et al. 2014). Blocking the expression of ERVW in sheep results in a slower trophectoderm growth and inhibition of binucleate cell differentiation during the preimplantation period (Dunlap et al. 2006). GCM1 is a critical factor for normal placentation across mammalian species, as homozygous GCM1 knockout mice are embryonic lethal, present with an absent or amorphous placental labyrinth, and do not form a syncytiotrophoblast layer (Anson-Cartwright et al. 2000). Additionally, expression of GCM1 is repressed during the trophoblast fusion process (syncytialization) in humans (Kashif et al. 2011), which is supportive of our findings and proposed compensatory mechanism (lower binucleate cell number and

higher GCM1 expression; Figure 2.6). Effects of *GCM1* overexpression vary depending on the placental cell types (Hughes et al. 2004); it arrests trophoblast stem cells proliferation, blocks differentiation of trophoblast giant cells, but is not sufficient to induce formation of the syncytiotrophoblast (Hughes et al. 2004). Additional research is required to predict the phenotype that BPS-induced GCM1 overexpression may have on the human placenta.

Because binucleate trophoblast cells are responsible for the production of hormones such as progesterone, and pregnancy associated glycoproteins in ruminant species (Wallace et al. 2015), BPS-induced low binucleate cell number is likely the direct cause of the observed reduction in PAG1, PSPB, and progesterone. The direct effect of BPS on placental endocrine function is further supported by the recovery observed in circulating PAG1 and PSPB after the discontinuation of the BPS treatment. Importantly, this cellular loss was not associated with placental weight reduction, changes in placental gross morphology or histopathology; reflective of a specific endocrine disrupting effect. Loss of syncytiotrophoblasts, the human homolog for the ovine binucleate cells, occurs in reactive oxygen species states and has been observed in preeclampsia and placentas of IUGR pregnancies (Wu et al. 2015). Additionally, apoptosis of syncytiotrophoblasts can be induced in hypoxic or reperfusion states (Wu et al. 2015). Macroscopic and histologic evaluation of BPS-exposed placentas and placentomes, did not reveal any evidence of hypoxia or necrosis. Further studies are required to evaluate the direct cause that lead to the loss of binucleate cells observed upon BPS exposure.

Gestational BPA does not affect placental endocrine function

In this study, BPA did not affect any of the endocrine or placental aspects investigated. The comparative experimental design and similar dose exposure levels used for both bisphenols help support previously reported differences in the mechanism of action and receptor affinities of both of these bisphenolic

compounds (Grignard et al. 2012; Molina-Molina et al. 2013; Rosenmai et al. 2014). However, this needs to be further investigated in the context of placental cells. The lack of effects on the placenta differs from previous work where mice exposed to BPA (50 mg/kg/day) during early pregnancy resulted in reduction of the spongiotrophoblast layer of the placenta (Tait et al. 2015). The difference between studies is likely due to the 100-fold difference in dose exposure, but could also relate to the species or route of exposure. Other aspects of placental function reported to be affected upon BPA exposure *in vitro* (Avissar-Whiting et al. 2010; Lan et al. 2017; Rajakumar et al. 2015; Spagnoletti et al. 2015) need further validation in an *in vivo* model.

2.5. Conclusion

This study demonstrates, for the first time, detrimental effects of gestational BPS, but not BPA, exposure on placental development and endocrine function. This work adds to prior work demonstrating that BPS can affect endocrine organs (LaPlante et al. 2017) and highlights intrinsic differences between the two bisphenolic compounds, BPA and BPS. Further research is required to evaluate the safety of BPA analogues.

Chapter 2b: Bisphenol S Disrupts *In Vitro* Placental Trophoblast Cell Fusion

Similarities between human and sheep placental development allows for robust interspecies translatability. The novel data from aim two suggests that gestational exposure to BPS in humans could impair syncytialization, the cytotrophoblast fusion process in human placentogenesis. Due to the inherent ethical concerns of exposing humans to toxic chemicals during pregnancy, an *in vitro* approach of placental syncytialization was employed. Here, primary isolated human placental cytotrophoblasts (n=6) were subjected to a 96-hour syncytialization protocol in the presence or absence of BPS. In support of aim two, BPS exposure inhibited epidermal growth factor-mediated syncytialization. This suggests a conserved mechanism of placental disruption by BPS in two species. Preliminary data are presented below in an abstract accepted for presentation at the 59th Annual Society of Toxicology Annual Meeting.

Bisphenol S Impairs Human Cytotrophoblast Syncytialization Through Competitive Epidermal Growth Factor Receptor Binding

Elvis Ticiani, Jeremy Gingrich, Yong Pu, and Almudena Veiga-Lopez

Michigan State University, East Lansing, Michigan 48824, USA

Abstract

Bisphenol S (BPS) is an endocrine disrupting chemical and the second most abundant bisphenol detected in humans. Recently, we have demonstrated that *in utero* exposure to BPS reduces the endocrine capacity of the ovine placenta by reducing binucleate cell number. During placentation, ovine trophoblast cells undergo homokaryotic fusion to form binucleate trophoblast cells, a process similar to

that of human syncytiotrophoblast formation. Syncytiotrophoblasts are essential for hormone production and nutrient exchange. Given that BPS reduces binucleate cell number in the ovine epitheliochorial placenta, we hypothesized that BPS will also impair trophoblast syncytialization in the human hemochorial placenta. To test this hypothesis, we used human cytotrophoblast cells (hCTBs) that were isolated from healthy placentas (n=6; IRB#15-484) derived from elective C-section pregnancies at term. hCTBs were cultured for 96 h with: 1) Vehicle (0.1% DMSO), 2) BPS (200 ng/ml), 3) human epidermal growth factor (hEGF, 10ng/ml), or 4) BPS+hEGF. hEGF was used as a syncytialization-induction signal and served as a positive control. Exposure to BPS reduced hEGF-mediated syncytialization rate (Vehicle: 11.58±1.17%, BPS: 11.23±1.17%, EGF: 21.71±1.17%, BPS+EGF: 11.97±1.17%; $P<0.001$). We additionally tested whether BPS could inhibit the EGF response by blocking EGFR phosphorylation in MDA-MD-231 breast cancer cells due to their high expression of EGFR. Cells were exposed to the same aforementioned treatments and phosphorylation of EGFR (pEGFR) and AKT (pAKT), a downstream signal to EGFR, were assessed by western blotting. As expected, hEGF upregulated pEGFR and pAKT (15.2 and 10.1-fold, respectively, compared to the vehicle), but hEGF+BPS reduced pEGFR and pAKT (5.1 and 1.2-fold, respectively,compared to the hEGF group). To test if BPS directly competes with hEGF for the EGFR binding site we used an EGF/EGFR AlphaLISA assay, and demonstrated that BPS blocked EGF binding in a dose dependent fashion, with a reduction of up to 58%. This is the first study to demonstrate that BPS can 1) prevent EGF-mediated trophoblast syncytialization, 2) block EGFR phosphorylation, and 3) compete with EGF for receptor binding. Given the role of EGFR in placental development, including trophoblast proliferation and differentiation, BPS interference may result in placenta dysfunction.

Supported by NIEHS R01 ES027863 to A.V-L.

Chapter 3: Effect of BPS on Gap Junction Intercellular Communication

Gap junction intercellular communication (GJIC) is necessary for placental syncytialization. Data from aim two suggest that BPS may repress GJIC in the placenta. Unfortunately, primary isolated human placental trophoblast cells are not suitable for functional assays that assess this outcome. Presented in the peer-reviewed publication that follows (Gingrich et al. 2020b, *under review at Chemosphere*), GJIC was assessed in steroidogenic ovarian theca cells, which have a similar expression of gap junction proteins as trophoblast cells, in the presence of BPS or one of four other EDCs (BPA, BPF, triphenyltin chloride (TPT), and perfluorooctanesulfonic acid (PFOS)) for validation. GJIC in the ovary is necessary for ovarian function and is temporospatial regulated during follicular development and ovulation. Theca cells are located on the outermost layer of the follicle, where they provide with structural, vascular, and steroidogenic support. To assess GJIC, primary isolated sheep theca cells (n=8) were grown in monoculture, acutely exposed to EDCs at non-apoptotic environmentally relevant exposure concentrations and were subjected to a scrape loading and dye transfer (SL/DT) assay. Interestingly, BPS, but no other EDC tested, was able to enhance theca cell GJIC in a dose- and time-dependent manner. A signal-protein inhibitor approach was then used to evaluate the mechanism(s) through which BPS may be eliciting its effect; implicating both phospholipase C and mitogen-activated protein kinase (MAPK), inhibitors of which were able to attenuate the BPS effect. Primary isolated human theca cells (n=3) were then subjected to the same BPS exposure conditions in order to evaluate the translational relevance of these findings. Here, we also demonstrate that BPS enhances GJIC in human theca cells, an effect that could be fully attenuated with a MAPK pathway inhibitor, suggestive of a conserved mechanism. Since upregulation of GJIC could result in hyperplasia of the theca cell layer or prevent ovulation by holding the oocyte in meiotic arrest, further studies will be necessary to understand the *in vitro* to *in vivo* translatability, and the implications of these findings on reproductive outcomes.

Bisphenol S enhances gap junction intercellular communication in ovarian theca cells

Jeremy Gingrich[1,2], Yong Pu[1], Brad L. Upham[3], Madeline Hulse[4], Sarah Pearl[4], Denny Martin[4], Anita Avery[4,5], Almudena Veiga-Lopez[1]

[1]Department of Animal Science, Michigan State University, East Lansing, MI 48824, USA

[2]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824, USA

[3]Department of Ped. and Human Development, Michigan State University, East Lansing, MI, 48824, USA

[4]Department of Obstetrics and Gynecology, Sparrow Health System, Lansing, MI, 48912, USA

[5]Department of Obst., Gynec. and Repro. Bio., Michigan State University, East Lansing, MI, 48824, USA

Funding sources: This work was supported by Michigan State University General Funds, AgBioResearch and the United States Department of Agriculture (USDA) National Institute of Food and Agriculture, and Hatch project. Research reported in this publication was partially supported by the National Institute of Environmental Health Sciences (NIEHS) of the National Institute of Health under Award Number R01ES027863. J.G. was supported in part by the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health (NICHD) under award number T32HD087166. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

3.1. Introduction

Nearly one out of every six couples suffer from infertility; a quarter of which remain etiologically unexplained, and a third are of female origin (Practice Committee of the American Society for Reproductive 2015; Thoma et al. 2013). The basis for female infertility often relates to ovarian

dysfunction and includes diseases that have steroid hormone imbalances such as polycystic ovarian syndrome, premature ovarian insufficiency, and luteal dysfunction (CDC 2017). There is growing evidence that exposure to environmental contaminants is a contributing factor to the rising infertility trend over the past four decades (Palioura and Diamanti-Kandarakis 2015; Rutkowska and Diamanti-Kandarakis 2016).

The ovarian antral follicle is comprised of two distinct cell types that sustain oocyte development and maturation; granulosa and theca. Theca cells are steroidogenic cells at the outer layer of the follicle that provide structural and vascular support, and androgen and progesterone synthesis (Young and McNeilly 2010). Prior to ovulation, theca cells synthesize testosterone that is transferred extracellularly into neighboring granulosa cells for estrogen synthesis. Intercellular communication within the follicle and transport of testosterone across follicular layers is thus essential for follicular growth and oocyte maturation (Orisaka et al. 2009). Regulated through gonadotropin-mediated events (El-Hayek and Clarke 2015; Norris et al. 2008; Sommersberg et al. 2000), gap junction intercellular communication (GJIC) within the follicle is also critical for oocyte meiotic arrest and resumption. Granulosa cells allow intercellular transfer of cGMP to the oocyte which indirectly increases the local concentration of cAMP, holding the cell in a state of meiotic arrest via protein kinase A (PKA)-induced cell signaling (Shuhaibar et al. 2015). Reduction in cGMP, driven in part through a mitogen-activated protein kinase (MAPK)-dependent mechanism (Norris et al. 2008), allows phosphodiesterase 3A (PDE3a)-controlled cAMP hydrolysis resulting in meiotic resumption and ovulation (Mehlmann 2005; Richards and Ascoli 2018). Theca cells aid in control of the ovulatory process through a steroid feedback loop (Young and McNeilly 2010) and by stimulation of proliferation and anti-apoptotic responses in granulosa cells (Orisaka et al. 2009). Upon ovulation, granulosa and theca cells undergo luteinization, a cellular differentiation process that includes an estrogen-to-progestogen shift in follicular steroidogenesis. This shift towards

progesterone synthesis is essential for fertilization, embryo implantation, and pregnancy maintenance in mammalian species. To note, connexin 37 (Cx37) gap junctions have been implicated in the transfer of important, yet unidentified signals, from the oocyte to the granulosa cells to prevent luteinization prior to ovulation (Winterhager and Kidder 2015). Theca cells functions are modulated by steroid hormones, growth factors, and bone morphogenetic proteins (BMP) (Young and McNeilly 2010). Additionally, they are highly sensitive to a variety of stimuli, including nutrition (Williams et al. 2001), stress (Zhu et al. 2016), heat stress (Nteeba et al. 2015), and bacterial insult (Magata et al. 2014). Increasing evidence also suggests that endocrine disrupting chemicals (EDCs) can disrupt ovarian function (Craig and Ziv-Gal 2018; Patel et al. 2015), specifically theca cell steroidogenesis (Gregoraszczuk et al. 1999; Grochowalski et al. 2001; Kendall et al. 2006; Lee et al. 2013).

Gap junctions are intercellular communication channels that are formed by apposition of connexin proteins organized into hexameric connexons. Once connexons from two adjacent cells dock end-to-end, intercellular channels are formed to allow diffusion of molecules <1 kDa in mass or <1.6 nm in diameter, a process known as gap junction intercellular communication. Assembly and activation of connexon channels can occur through multiple established pathways including protein kinase C (PKC) (Long et al. 2007), PKA (Pidoux et al. 2014), phosphatidyl choline-phospholipase C (PC-PLC) (Machala et al. 2003a; Upham et al. 2008) and MAPK (Warn-Cramer et al. 1996). Connexins are expressed in the ovary of mammalian species (Gershon et al. 2008), including humans (Furger et al. 1996; Wang et al. 2009). There are over twenty connexin genes in vertebrates, which are expressed in a tissue and a cell-specific manner (Sohl and Willecke 2004b; Srinivas et al. 2018). Connexin 43 (Cx43) is the most highly expressed connexin in the ovary and it is essential for ovarian follicle formation in mice (Ackert et al. 2001; Juneja et al. 1999). However, Cx43 knockout mice are embryonic lethal, making reproduction in the absence of Cx43 difficult to study (Nishii et al. 2014) as no Cx43 ovarian conditional knockout mice

have been developed to date. However, when compared to ovaries from $Cx43^{+/+}$ wildtype mice, ovaries from $Cx43^{-/-}$ knockout mice grafted into kidney capsules of ovariectomized mice resulted in folliculogenesis arrest at the secondary follicle stage (Ackert et al. 2001). In the ovary, formation of gap junctions allows the development of a metabolic syncytium between the oocyte and its supporting cells (Kidder and Vanderhyden 2010), but can also enable theca-to-granulosa paracrine signaling via ATP release (Tong et al. 2007). Connexin expression in the antral follicle is a dynamic process. Specifically, Cx43 has reduced expression during the peri-ovulatory period (Borowczyk et al. 2006; Granot and Dekel 2002; Okuma et al. 1996; Sela-Abramovich et al. 2005) enabling meiosis resumption (Norris et al. 2008) and the initiation of the ovulatory process (Borowczyk et al. 2006). In mice, theca cell gap junctions are primarily composed of Cx32 and Cx26 (Wright et al. 2001) and have significantly lower levels of GJIC than granulosa cells in the bovine ovary (Johnson et al. 2002). Theca-derived BMP4 and BMP7 contribute to the downregulation of Cx43 and gap junction intercellular communication in granulosa cells (Chang et al. 2013; Chang et al. 2014) through SMAD-dependent signaling (Chang et al. 2013; Rossi et al. 2016) in both mice and humans. Despite this, the role of gap junctions in theca cells is not yet well established.

GJIC plays a central role in coordinating intracellular signal transduction initiated by extracellular signals (endocrine, paracrine, and autocrine); in turn, controlling gene expression of neighboring cells via signaling molecules like cAMP, cGMP, and ATP (Mese et al. 2007; Trosko 2011; Zong et al. 2016). This process can be modulated by EDCs with steroid hormone binding activity via non-genomic actions (Vinken et al. 2009). Despite the significant role that intercellular communication plays in the ovarian follicle, scant information is available regarding the impact that EDCs have on ovarian GJIC (Dominguez et al. 2016). The purpose of this study was to investigate the effects of known EDCs, specifically bisphenols A (BPA), bisphenol S (BPS), bisphenol F (BPF), triphenyltin chloride (TPT), and

perfluorooctanesulfonic acid (PFOS) on GJIC in ovarian theca cells. BPA is a synthetic organic chemical, and the most widely used bisphenol in the manufacturing of epoxy resins and industrial and consumer plastics (Liao and Kannan 2013; Liao et al. 2012). The recent ban of BPA from certain consumer products (Metz 2016) has raised concerns about the safe use of other bisphenolic compounds like BPS and BPF. Additionally, we have included two non-bisphenolic EDCs of concern, TPT and PFOS in this study. We hypothesized that all five chemicals would reduce GJIC in theca cells. To test this hypothesis, we used primary theca cells from two monovulatory species: sheep and human. We further investigated the molecular pathways by which these chemicals alter GJIC in ovarian theca cells by using a signal-protein inhibitor approach.

3.2. Materials and Methods

Chemicals

All chemicals used in the study are listed in Table 3.1 and were dissolved in DMSO. All groups were administered the same solvent volume in media (0.1% DMSO). The doses of chemicals used were chosen for their environmental exposure relevance (ranges of human exposure for BPA: 0.14 - 792 ng/ml in urine, BPS: 0.07 - 211.9 ng/ml in urine, BPF: 0.14 - 298.7 ng/ml in urine, PFOS: 4.5-5.52 ng/ml in urine, and TPT: not detectable - 155 ng/ml in blood) (CDC 2016; Pu et al. 2019; Worley et al. 2017).

Table 3.1: Chemicals used.

Chemical (*target pathway*)	Catalog #	Purity	Source	Concentration	Exposure
Bisphenol A (BPA)	239658	99%	Sigma	1 - 10,000 ng/ml	24 h
Bisphenol S (BPS)	146915000	99.7%	Acros	1 - 10,000 ng/ml	24 h
Bisphenol F (BPF)	B47006	> 98%	Sigma	1 - 10,000 ng/ml	24 h
Perfluorooctanesulfonic acid (PFOS)	77282	98%	Sigma	50 ng/ml	24 h
Triphenyltin chloride (TPT)	45492	NR	Sigma	10 ng/ml	24 h
GF109203X (*panPKC inhibitor*)	NC9686383	98%	Enzo	1 µM	30 min
SB202190 (*p38 MAPK inhibitor*)	S7067	> 98%	Sigma	1 µM	30 min
D609 (*PC-PLC inhibitor*)	1437	> 98%	Tocris	50 µM	30 min
ET-18-OCH_3 (*PI-PLC inhibitor*)	3022	NR	Tocris	30 µM	15 min
H89 (*PKA inhibitor*)	B1427	> 98%	Sigma	40 µM	30 min
U0126 (*MEK1/2 inhibitor*)	1144	99%	Tocris	70 µM	30 min
CW008 (*PKA activator*)	5495	> 98%	Tocris	0.25 µM	24 h
Phorbol 12-myristate 13-acetate (TPA)	1201	> 99%	Tocris	5 nM	15 min
Lucifer yellow	PK-CA707-80015	> 99%	Promocell	0.5 mg/ml	5 min
Rhodamine-dextran	R8881	NR	Sigma	0.5 mg/ml	5 min

NR: Purity not reported.

Generation of primary sheep ovarian theca cells, purity, and luteinization

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Michigan State University and are consistent with the National Research Council's Guide for the Care and Use of Laboratory Animals and the current Animal Welfare Act. Ovine theca cells were used in this study as they represent an excellent *in vitro* model to study GJIC in the context of human chemical exposures and ovarian function as sheep and human are both monovulatory, and have similar ovarian sizes, steroid feedback regulatory mechanisms (Kasa-Vubu et al. 1992; Nippoldt et al. 1989), and connexin protein expression to humans (Borowczyk et al. 2006; Winterhager and Kidder 2015). Isolation of theca cells was performed on eight multiparous Polypay x Dorsett breed sheep at gestational day 120 of pregnancy as previously described (Pu et al. 2019). In brief, the theca interna cell layer of antral follicles was isolated by microdissection. Then, theca cells were dispersed using collagenase I (1 mg/ml) supplemented with 10 µg/ml deoxyribonuclease (DNAse I) in Ca^{2+}/Mg^{2+}-free buffer. The cell suspension was filtered, then fractioned on a discontinuous Percoll gradient (44% and 35% Percoll). Plated cells were maintained in basic medium consisting of DMEM/F12 media supplemented with 1% heat inactivated fetal bovine serum (FBS, Cat#: 35- 010-CV, Corning Inc., Corning, NY, USA), 2 mM L-glutamine, 10 mM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin in 5% CO_2 at 39°C. Theca cell purity was determined using theca cell markers fibulin 5 (Hatzirodos et al. 2015) and vimentin (Wang et al. 2018) by immunocytochemistry. Purity of all isolated primary cell cultures was > 95% (Pu et al. 2019). Theca cells were kept frozen at -80 ºC until used for GJIC assays. Theca cells luteinization was performed as previously described (Pu et al. 2019). In brief, theca cells were grown in DMEM/F12 medium supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine, 1% heat inactivated FBS, 250 ng/ml ovine luteinizing hormone (LH; U.S. National Hormone and Peptide Program), and 50 ng/ml insulin-like growth factor-1 (IGF-1), and cultured for 72 h.

Ethics, exclusion criteria, and generation of human ovarian theca cells

Human ovarian tissue was obtained after written informed consent following Institutional Review Board (IRB: 17-1066 M) approval from Sparrow Health System and Michigan State University and is consistent with relevant guidelines and regulations. Ovaries were collected from healthy non-pregnant women (n = 3, aged 31 - 46 years) undergoing surgical uni- or bilateral oophorectomy. Exclusion criteria included polycystic ovarian syndrome, ovarian cancer, menopause, drug addiction, HIV, and/or hepatitis B or C diagnoses. Only follicles with a healthy appearance, and ≥ 3 mm in size were chosen to pool for the generation of a theca cell line per individual. All ovaries were processed within 1 h of surgical removal and kept at 4°C. The theca interna cell layer of human antral follicles was isolated as previously described (Pu et al. 2019) and enzyme-digested as described for sheep. Changes to the enzyme digestion for human theca cells included more collagenase I (3 mg/ml) and DNase I (300 IU/ml). Theca cells were kept frozen at -80 ºC until used for GJIC assays.

Cell viability assay

Cell viability was determined using an MTT assay as previously described (Palaniappan et al. 2013). In brief, theca cells (n = 8 primary cultures, with at least 3 replicates per experiment) were seeded into 96-well plates. Cells were treated with increasing concentrations of BPA, BPS, or BPF (0, 1, 10, 20, 50, 100, 200, 500, 1,000, and 10,000 ng/ml) for 72 h, or pathway inhibitors at concentrations and times (15 or 30 min) listed in Table 3.1. All chemicals tested were dissolved in DMSO to a final concentration of 0.1%. Vehicle group received 0.1% DMSO. Medium was then replaced with MTT working solution (50 µg/ml) and incubated for 4 h. Wells were rinsed, and DMSO added to each. Cell viability was determined by absorbance quantification at 570 nm using a microplate reader (SpectraMax M5e, Molecular Devices, LLC, Sunnyvale, CA, USA).

Experimental design

To test if three bisphenols (BPA, BPS, and BPF), PFOS, and/or TPT alter GJIC, ovine pre-luteinized theca cells were subjected to a GJIC assay as outlined below. Once plated cells reached 90% confluency, media was replaced for exposure media consisting of basic medium supplemented with DMSO (0.1%, vehicle), BPA, BPS, BPF, TPT, or PFOS for 24 h, followed by a 2-h serum starving step, and subsequent gap junction communication assays using a scrape loading dye transfer assay (see below).

Bisphenols (BPA, BPS, and BPF) exposure doses (1, 100, and 1,000 ng/ml) were chosen to cover a range of exposures from environmentally relevant to supraphysiological. Given that BPS altered GJIC, BPS was also tested at these additional doses: 10, 200, and 500 ng/ml. We then investigated if the observed GJIC BPS effect occurred in a theca cells independent of their stage (pre-luteinized, luteinizing, and post-luteinized). Luteinizing cells chemical exposure occurred concomitantly with LH during the last 24 h of the 72-h differentiation period, and luteinized cells were exposed after the 72 h luteinizing period in the absence of LH. The effect of BPS (at 200 ng/ml) exposure duration on GJIC was also assessed in pre-luteinized theca cells for 3, 6, 12, and 24 h.

To assess signal transduction mechanisms mediating changes in GJIC, specific gap junction pathway inhibitors (GF109203X, SB202190, D609, ET-18-OCH_3, H89, and U0126) and a PKA activator (CW008) were used. Pathways in which these inhibitors function and concentrations and time of exposure at which all test compounds were used as outlined in Table 3.1. Primary ovine theca cell cultures (n = 8) were used in the aforementioned experiments, with a minimum of 3 replicates per treatment group per experiment. Human theca cell GJIC experiments (n = 3 primary theca cell cultures per treatment group) followed the same method to that described for ovine cells, at a fixed BPS exposure dose (200 ng/ml) and using a MAPK inhibitor (1 μM SB202190) for GJIC pathway analysis.

Gap junction intercellular communication assay

Theca cells were plated at 50 x 10^4 cells/dish on a 60 mm-diameter tissue culture dishes (Corning Inc., Corning, NY, USA) and cultured in basic medium supplemented with 10% FBS. After 24 h, media was replaced with normal basic medium (containing only 1% FBS). Confluent cells were passed and seeded at 25 x 10^4 cells/dish in 35 mm-diameter tissue culture dishes and cultured in basic medium supplemented with 10% FBS. After 24 h, media was replaced with basic medium (1% FBS). Once cells reached 90% confluency, media was replaced for exposure media consisting of basic medium supplemented with DMSO (0.1%, vehicle), BPA, BPS, BPF, TPT, or PFOS for 24 h. To stimulate intercellular communication, cells were serum starved 2-h prior to the GJIC assay (Lin et al. 2003). For the gap junction pathway inhibitor experiments, inhibitors were added in the last 15 to 30 min (Table 3.1) of the serum starvation step. GJIC was assessed using the scrape loading/dye transfer technique as previously described (Upham 2011). In brief, cells were washed with Ca^{2+}-Mg^{2+}-PBS and then lucifer yellow and rhodamine-dextran were added to each plate. With gentle pressure, a surgical scalpel blade was rolled through a cell monolayer. Passive diffusion of the lucifer yellow from the loaded cells into the adjacent cells was allowed for 5 min. Due to its high molecular weight and inability to pass through gap junction channels, the rhodamine-dextran was used to visualize the dye loaded cells (*not shown*). Cells were then washed with Ca^{2+}-Mg^{2+}-PBS, fixed with 4% neutral buffered formalin and stored at 4°C until imaging.

Image analyses

To quantify theca cell GJIC, 6 to 10 images per primary cultured cell line per treatment group were captured using an Eclipse TE2000-U inverted microscope (Nikon, Toyko, Japan) with a CoolSNAP-ProCF camera (Media Cybernetics, Rockville, MD, USA). Images were quantified using Fiji image analysis software (Schindelin et al. 2012). The freehand selection tool was used to manually determine the area of co-joined lucifer yellow positive cells, and then averaged to determine the area of dye diffusion. A

vehicle group was included in each run, and the average area of dye diffusion was normalized to the vehicle group.

Statistical analysis

All data are presented as a mean ± SEM. Appropriate transformations were applied, as needed, to account for normality of data. Comparisons among the treatment groups were analyzed by mixed model ANOVA with Tukey posthoc tests using experiment run as a covariate. Differences were considered significant at $P < 0.05$.

3.3. Results

Effects of bisphenols (BPA, BPS, and BPF) on cell viability

Cell viability was unaltered by any bisphenol (range: 1 to 10,000 ng/ml; Figure 3.1). Additionally, TPT, PFOS, or inhibitors used at the concentration tested (Table 3.1) did not affect cell viability (*data not shown*). Cell morphology was similar across treatment groups and was not affected by exposure to bisphenols, TPT, PFOS, or inhibitors at the concentrations tested.

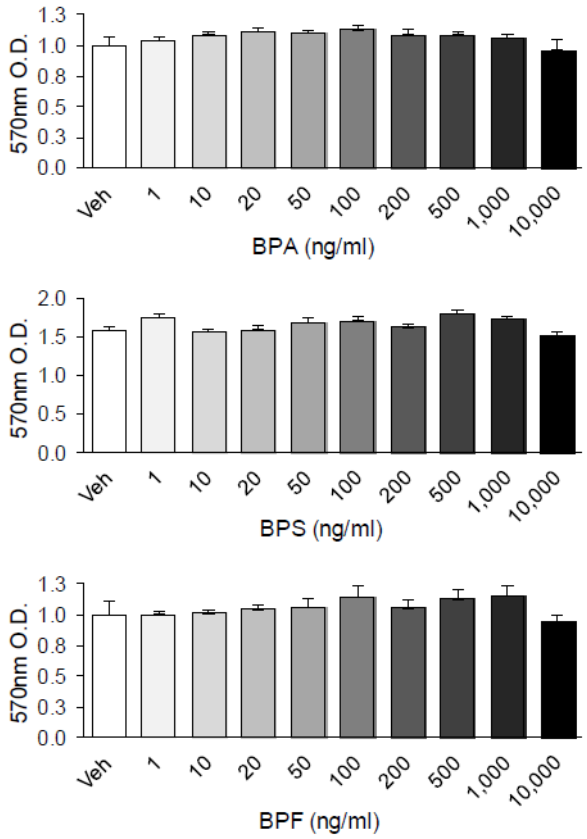

Figure 3.1: Cytotoxicity assay for bisphenol A, bisphenol S, and bisphenol F in pre-luteinized ovine theca cells after 72 h of exposure. Cytotoxicity assay for bisphenol A (BPA), bisphenol S (BPS), and bisphenol (BPF) in pre-luteinized ovine theca cells after 72 h of exposure (range: 1 to 10,000 ng/ml). Data are expressed as mean ± SEM. N = 3 primary theca cell cultures. No significant differences between exposure concentrations were detected. Veh: DMSO vehicle control.

BPS enhances gap junction communication in a cell stage-, time-, and dose-dependent manner

BPA and BPF, at all doses tested (ranging: 1 to 1,000 ng/ml), had no effect on GJIC in pre-luteinized theca cells (Figure 3.2, *left and center panels*) following a 24 h exposure, and thus no further experiments were pursued with these two chemicals. PFOS and TPT, additionally, had no effect on GJIC (Figure 3.2, *right panel*). However, pre-luteinized theca cells exposed for 24 h to BPS increased GJIC in a dose-dependent

fashion (Figure 3.3). GJIC increased starting at an exposure dose as low as 10 ng/ml, peaked at 200 ng/ml, and plateaued at higher doses.

To assess if exposure time affects the BPS-induced increase in GJIC in pre-luteinized theca cells, we used the BPS dose that resulted in a ~2-fold increase in GJIC (200 ng/ml), for exposure times of 3, 6, 12, and 24 h. The increase in GJIC was time-dependent with 12 h and 24 h exposures resulting in the highest GJIC response (Figure 3.3C).

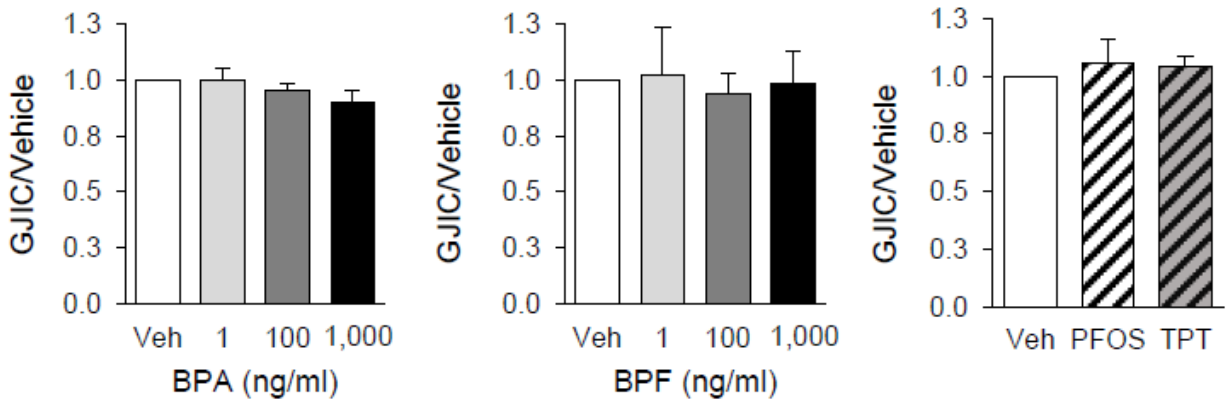

Figure 3.2: Effects of exposure to common endocrine disrupting chemicals on gap junction intercellular communication in ovine primary theca cells. GJIC was assessed using the scrape loading dye transfer assay in pre-luteinized ovine theca cells exposed to bisphenol A (BPA), bisphenol F (BPF), perfluorooctanesulfonic acid (PFOS; 50 ng/ml), or triphenyltin chloride (TPT; 10 ng/ml). Data are represented as mean ± SEM. The effects were normalized to the vehicle group GJIC. Eight different primary theca cell cultures were used in at least 3 independent experiments. Veh: DMSO vehicle control.

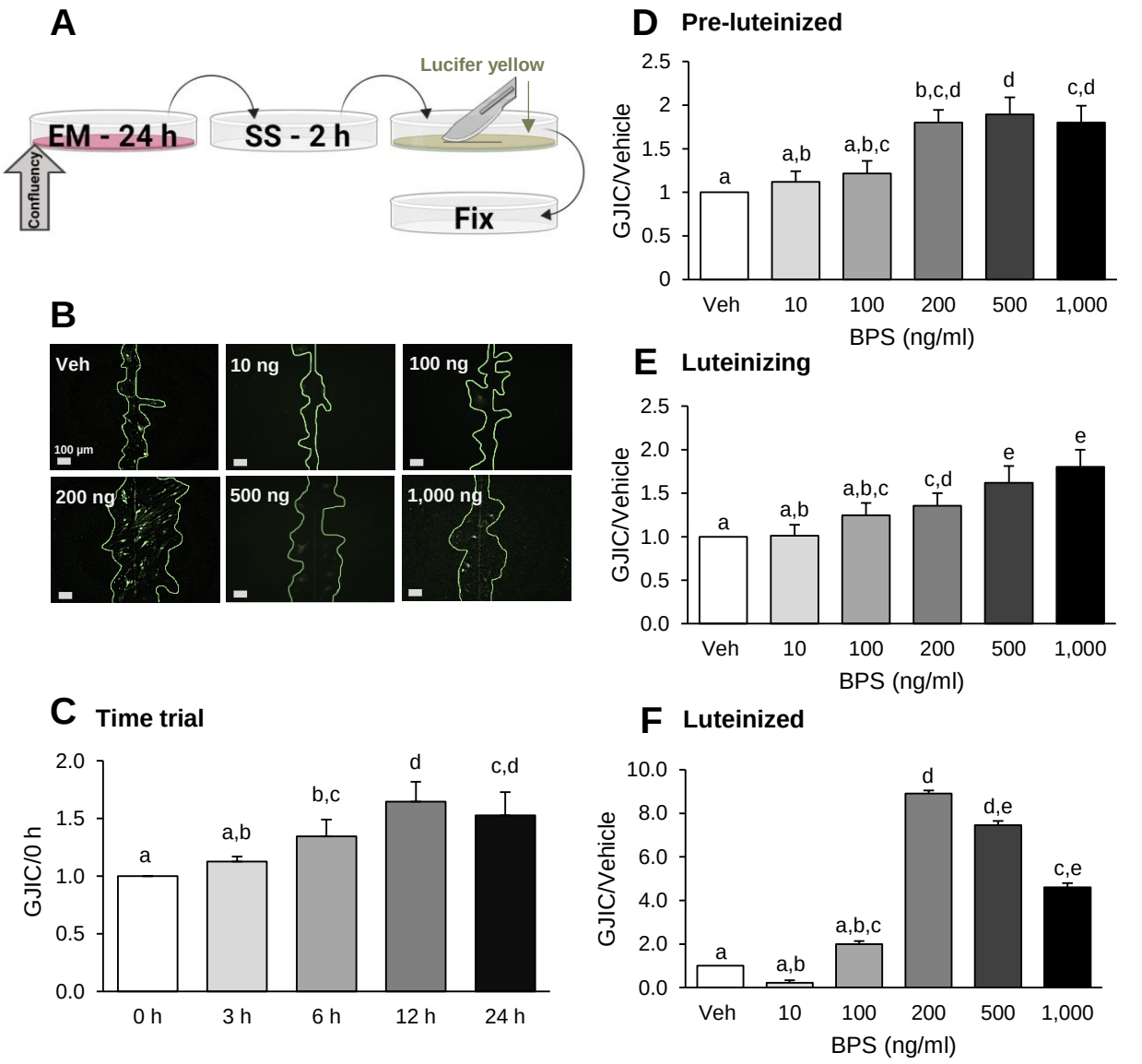

Figure 3.3: Dose- and time-dependent effects of bisphenol S exposure on gap junction intercellular communication in ovine primary theca cells. Scheme of exposure (A): At ~80% confluency, cells are exposed to exposure medium (EM) containing BPS followed by a 2 h serum starvation (SS) period. Cells were then subjected to the scrape loading dye/transfer assay, using lucifer yellow dye (*arrow*) and a scalpel blade, before fixation (see text for additional details). Representative images from scrape loading dye transfer assay (B) used to assess GJIC in pre-luteinized ovine theca cells. White lines were drawn to facilitate visualization of the lucifer yellow intercellular transfer. BPS effect on GJIC (LS means ± SEM) in

Figure 3.3 (cont'd): a time-course in pre-luteinized theca cells (C) and in a dose-response in pre-luteinized (D), luteinizing (E), and luteinized (F) theca cells. The effect of BPS was normalized with the dye transfer in the vehicle control. Eight different primary theca cell cultures were used for all experiments, utilizing at least 3 different theca cell cultures each. Different letters denote statistical differences among treatment groups at $P < 0.05$. Veh: DMSO vehicle control.

To test whether BPS differentially modulates GJIC depending on the luteal stage of the theca cells, we used luteinizing and luteinized theca cells. In luteinizing theca cells, BPS also induced an increase in GJIC. Although less pronounced, BPS's effect was first observed at 200 ng/ml and displayed a dose-dependent increase up to the maximum dose tested (1,000 ng/ml) (Figure 3.3E). In luteinized theca cells, the BPS effect followed a similar trend to the pre-luteinized theca cells but the effect was less robust (Figure 3.3F).

Signal-protein inhibitor/activator evaluation of BPS-enhanced gap junction communication

A signal-protein inhibitor/activator approach was used to evaluate the molecular pathways potentially involved in BPS-induced upregulation of GJIC (Figure 3.4A). First, PKA activator CW008 was used alone and in combination with BPS exposure as a positive control to enhance GJIC. CW008 exposure enhanced GJIC in pre-luteinized theca cells both alone and in combination with BPS, although the combined effect was comparable to that of BPS alone (Figure 3.4B). Additionally, TPA was used as a non-specific negative control due to its ability to internalize gap junction channels (Leithe and Rivedal 2004). Co-exposure of BPS and TPA resulted in GJIC like that observed in the vehicle group (Figure 3.4C).

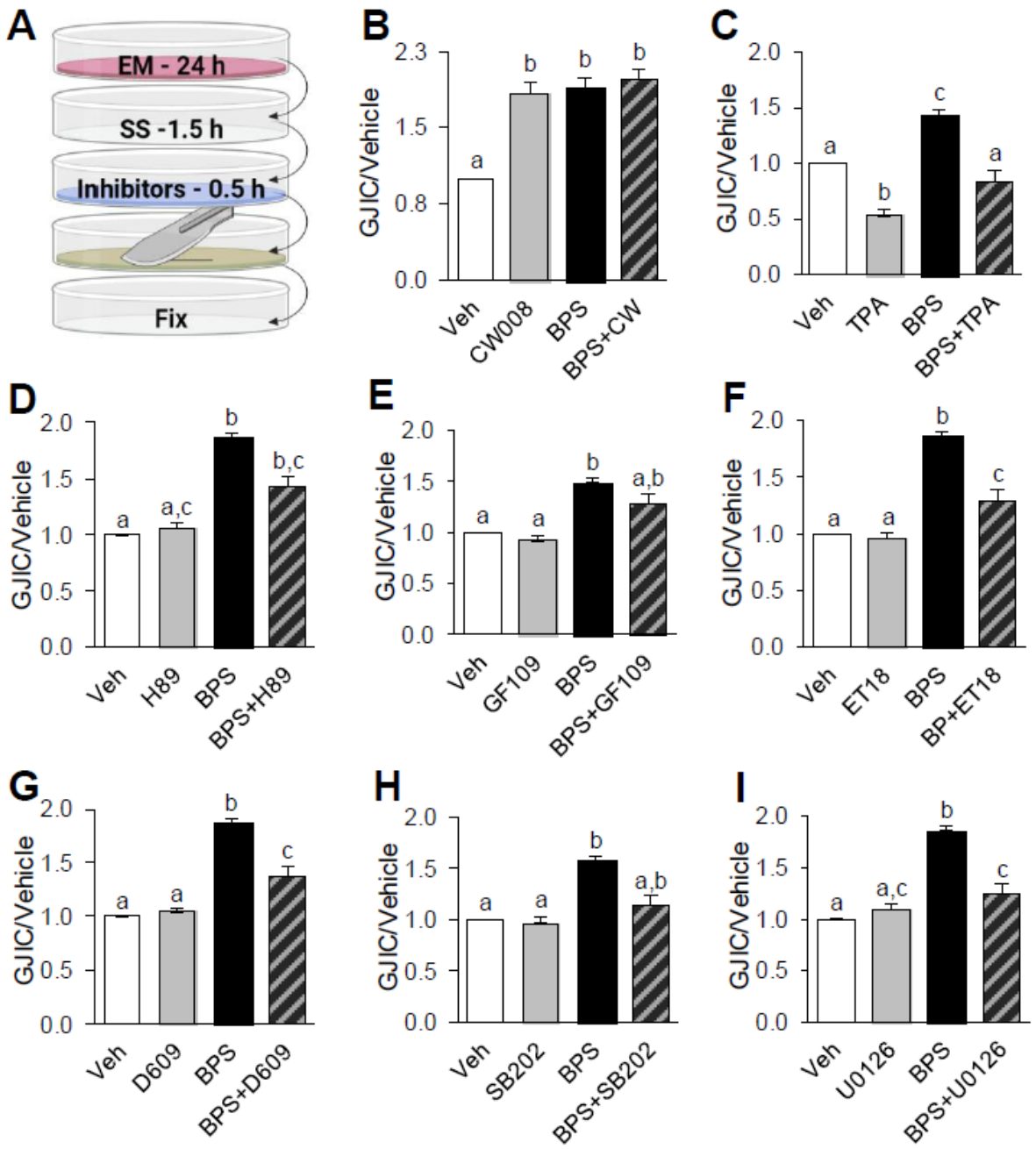

Figure 3.4: Pathway identification of bisphenol S-induced enhanced GJIC in pre-luteinized ovine primary theca cells using chemical modulators. Scheme of exposure to BPS and GJIC inhibitors (A): At ~90% confluency, cells are exposed to exposure medium (EM) containing BPS followed by a 2 h serum starvation (SS) period, where inhibitors were added in the last 15 - 30 min. Cells were then subjected to the scrape loading dye transfer assay, which was run using lucifer yellow (*green plate*) and a scalpel

Figure 3.4 (cont'd): blade, prior to fixation (see text for additional details). GJIC (mean ± SEM) in pre-luteinized ovine theca cells exposed to BPS with and without: positive control CW008 (PKA activator) (B), negative control phorbol 12-myristate 13-acetate (TPA) (C), or GJIC pathway inhibitors H89 (PKA inhibitor) (D), GF109203X (panPKC inhibitor) (E), ET-18-OCH_3 (PI-PLC inhibitor) (F), D609 (PC-PLC inhibitor) (G), SB202190 (p38 MAPK inhibitor) (H), or U0126 (MEK1/2 inhibitor) (I). See Table 3.1 for inhibitor exposure times. GJIC is expressed relative to the vehicle group. Eight different primary theca cell cultures were used in at least 3 independent experiments. Different letters denote statistical differences among treatment groups at $P < 0.05$. Veh: DMSO vehicle control.

To evaluate the involvement of the PKA pathway, H89 was used as an inhibitor of PKA-induced connexin phosphorylation. H89 partially reduced (by ~23%) the BPS-induced enhanced GJIC (Figure 3.4D). PKC pathway activation was tested using GF109203X as a panPKC inhibitor (Figure 3.4E), ET-18-OCH_3 as a phosphatidylinositol-phospholipase C (PI-PLC)-dependent PKC inhibitor (Figure 3.4F), and D609 as a phosphatidylcholine-phospholipase C (PC-PLC)-dependent PKC inhibitor (Figure 3.4G). Both PLC-dependent PKC pathway inhibitors were able to significantly attenuate the BPS-induced enhanced GJIC by ~29 and ~31%, respectively. Finally, to understand the role of MAPK in BPS-induced upregulation of GJIC, a p38 MAPK inhibitor (SB202190) (Figure 3.4H), and a MEK1/2 inhibitor (U0126) (Figure 3.4I) were used. Both SB202190 and U0126 were able to partially prevent the BPS-induced enhanced GJIC to that of the vehicle control group by ~39 and ~33%, respectively. No pathway inhibitors tested individually affected GJIC compared to the vehicle control.

BPS enhances gap junction communication in human theca cells

GJIC in primary isolated human theca cells upon BPS exposure was also tested. At the time and dose optimized in ovine primary theca cell lines (24 h exposure to 200 ng/ml BPS), BPS was able to induce

approximately a ~1.5-fold increase in GJIC, which was significantly attenuated with a MAPK inhibitor (Figure 3.5).

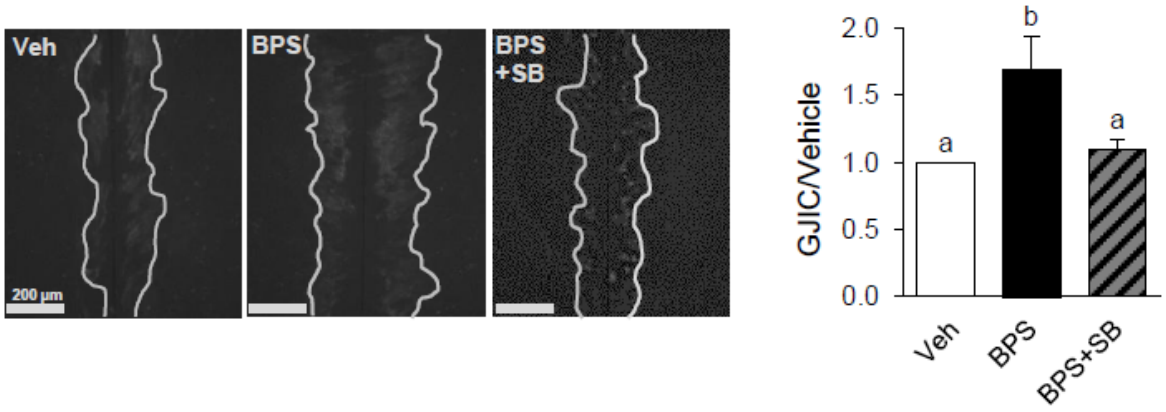

Figure 3.5: Effect of bisphenol S exposure on GJIC in human theca cells. Effect of bisphenol S (BPS) exposure on GJIC in human theca cells. Representative images from scrape loading dye transfer assay used to assess GJIC in pre-luteinized human theca cells. White lines were drawn to facilitate visualization of the lucifer yellow intercellular transfer. GJIC was assessed following exposure to vehicle control (Veh, 0.1% DMSO), BPS, or BPS in combination with MAPK inhibitor SB2020190 (SB, 1 µM). Data are represented as mean ± SEM and were normalized to dye transfer in the vehicle control. Different letters denote statistical differences among treatment groups at $P < 0.05$. Three different primary human theca cell cultures were used.

3.4. Discussion

We demonstrated that BPS, but not four other common EDCs (BPA, BPF, TPT, and PFOS), enhanced GJIC in both a dose- and time-dependent manner in ovine primary ovarian theca cells. BPS-induced enhancement of GJIC was also evident in primary human theca cells. The lowest observed adverse effect level (LOAEL) for enhanced GJIC was 10 and 100 ng/ml BPS in pre-luteinized, and luteinizing and luteinized cells, respectively. Importantly, both LOAEL doses fall within environmentally relevant human

exposures (Qiu et al. 2018; Zhao et al. 2018). Thus far, chemotherapeutic agents have been shown to enhance GJIC (Babica et al. 2016; Liu et al. 2013; Sovadinova et al. 2015; Vinken et al. 2006; Wu et al. 2016; Xiao et al. 2013; Yang et al. 2014), while most EDCs (dichlorodiphenyltrichloroethane (Masten et al. 2001), polychlorinated biphenyls (Kang et al. 1996; Machala et al. 2003a), perfluorinated compounds (Hu et al. 2002; Upham et al. 1998; Upham et al. 2009), methoxychlor,vinclozolin (Babica et al. 2016b)), lipid by-products (octadecatetraenoate (Hasler et al. 1991)), cigarette smoke relevant polycyclic aromatic hydrocarbons (Osgood et al. 2017; Osgood et al. 2014; Siegrist et al. 2019; Tai et al. 2007; Upham et al. 2008; Velmurugan et al. 2015), cannabinoids (Δ(9)-tetrahydrocannabinol and cannabinol (Upham et al. 2003)), plant derivatives (licorice root; (Davidson and Baumgarten 1988; Tanaka et al. 1999)), and pharmaceuticals (phenobarbital; (Klaunig et al. 1990; Ren and Ruch 1996)) result in a dose- and time-dependent reduction of GJIC.

Reduced GJIC, *in vivo,* is recognized as a hallmark in the early stages of cancer, while late stage or metastatic cancers present with both down- and up-regulated GJIC, particularly during the vascularization of tumors (Aasen et al. 2017), including in the ovary (Hanna et al. 1999). Inhibition of GJIC has been associated with a chemicals' higher carcinogenic potential (Rosenkranz et al. 2000). On the contrary, substances that enhance GJIC, such as green tea micronutrients genistein and epicatechin (Ale-Agha et al. 2002; Sai et al. 2000) and other plant antioxidants (Babica et al. 2016; Nakamura et al. 2005; Nakamura et al. 2005b; Sovadinova et al. 2015; Upham et al. 2007), are hypothesized to have cancer preventive properties, and drugs enhancing GJIC have been viewed as novel chemotherapeutics (Wu et al. 2016; Yang et al. 2014). Despite this, in the context of the ovarian reproductive cycle where GJIC is temporospatially regulated (Kidder and Mhawi 2002), enhanced GJIC could result in pathological outcomes like hyperplasia of the theca cell layer, a pathology seen in hyperandrogenism (Czyzyk et al. 2017). Importantly, a BPS-induced increase in GJIC, if also occurring intergranulosal or granulosa-to-

oocyte, could impair follicle development and oocyte maturation. We are not aware of disease etiologies associated with altered GJIC in the ovary. However, cellular arrest before antral follicle development in Cx43-inhibited human granulosa cells (Winterhager and Kidder 2015). Importantly, enhanced GJIC has been associated with the development of diabetic cardiomyopathy (Wang et al. 2017) and in the brain is associated with epileptic hypersynchronous neuronal activity (Xie et al. 2015). Upregulation of connexin mRNA expression and protein abundance is implicated in a model for Parkinson's disease (Xie et al. 2015) and also occurs following prion disease on-set (Lee et al. 2016) highlighting the role of GJIC imbalances during in disease states.

During ovulation, just prior to luteinization, there is a BMP4- and BMP7-mediated gradual loss in GJIC within the preovulatory antral follicle for oocyte maturation to occur (Borowczyk et al. 2006; Granot and Dekel 2002; Norris et al. 2008; Okuma et al. 1996; Sela-Abramovich et al. 2005; Winterhager and Kidder 2015). Therefore, BPS-induced enhanced GJIC occurring during the latest stages of preovulatory follicle growth, especially if GJIC is also altered in granulosa cells, could alter transfer of signaling molecules, like BMPs, preventing final oocyte maturation and progression into metaphase II. This may ultimately result in lower oocyte fertilization rates and reduced fertility. BPS also retained the ability to enhance GJIC during and after theca cell luteinization, a time when theca cells shift steroid synthesis from androgens to progesterone.

Estradiol and the xenoestrogen BPA have been previously reported to reduce GJIC via a down regulation in connexin mRNA expression in the ovarian cumulus-oocyte cell complex (Acuna-Hernandez et al. 2018; Zhang et al. 2019; Zhang et al. 2019b), an observation we did not observed in theca cells at environmentally relevant doses. BPS can also act as a xenoestrogen (Pelch et al. 2019), and the observed BPS-induced enhancement in GJIC could thus potentially be due to upregulation of connexin expression.

However, connexin mRNA expression does not necessarily translate to a change in GJIC (Genetos et al. 2012), particularly in the context of EDC exposures (Zhang et al. 2019b), and therefore functional outcomes such as intercellular dye transfer should be assessed when studying changes in GJIC. Since enhanced GJIC occurs following exposure to BPS, the interplay between BPS, connexin expression, and the changing hormonal milieu throughout folliculogenesis and ovulation should be studied further.

To understand the mechanism by which BPS increases GJIC, we co-incubated theca cells with pharmacological inhibitors directed to specific signal-protein targets (Figure 3.6). Our results demonstrate that BPS can act through multiple phosphorylation pathways. These pathways occur in parallel or upstream of those commonly implicated in GJIC regulation (Kurtenbach et al. 2014). PKA, PKC, and PLC pathways have redundant roles in the phosphorylation of connexins, or can indirectly regulate other pathways implicated in GJIC (Cesen-Cummings et al. 1998; Hossain et al. 1998; Kanemitsu and Lau 1993; Machala et al. 2003b; Ruch et al. 2001; Upham et al. 2008). These redundant roles are likely why only a partial, albeit significant, attenuation of BPS-induced enhanced GJIC was achieved by any of the signal-protein inhibitors, both in sheep and human theca cells. To note, these phosphorylation checkpoints are implicated in other cellular processes such as invasion, differentiation, Ca^{2+} ion signaling and influx, and neuronal responsiveness, cancer metastasis, and the development of diabetes (Morrison 2012; Putney and Tomita 2012; Sassone-Corsi 2012; Sotogaku et al. 2007; Tarafdar and Michie 2014). Additionally, there are 21 known connexins expressed in humans (Sohl and Willecke 2004a), at least 6 of which (Cx26, Cx32, Cx37, Cx40, Cx43, and Cx45) are present in the normal human ovary, and at least 11 (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx40, Cx43, Cx46, and Cx50) have been reported in ovarian cancers (Gershon et al. 2008). Although the function of each connexin is not fully understood, expression of different connexins within the ovary can result in essential pathway redundancy needed to provide compensatory communication mechanisms upon specific pathway

malfunction (Sinyuk et al. 2018). This makes the evaluation and identification of specific biochemical pathways altered in BPS-enhanced GJIC challenging.

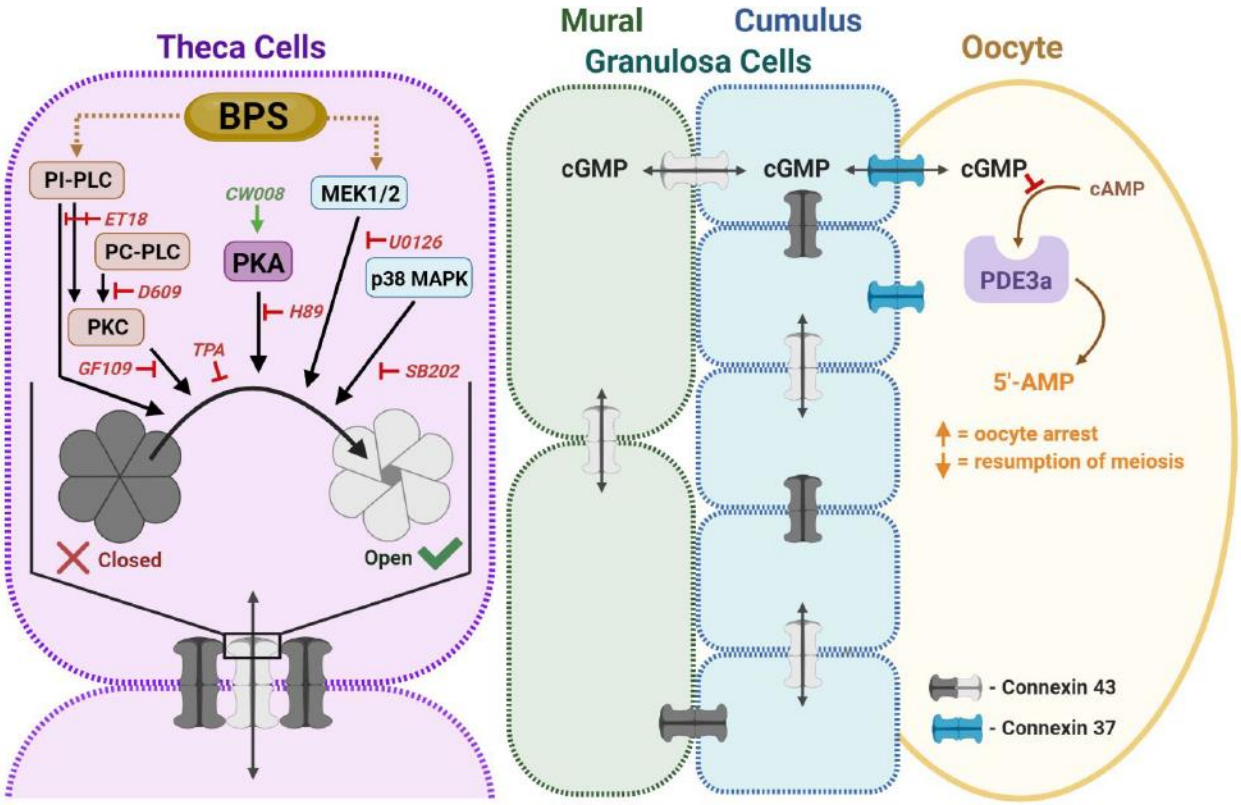

Figure 3.6: Working model for BPS' modulation of theca cell GJIC. The antral follicle is formed by theca cells (*purple*), mural granulosa cells (*green*), cumulus granulosa cells (*blue*), and the oocyte (*yellow*). Connexin 37 and 43 form gap junction channels that connect interthecal, intergranulosal and granulosa-to-oocyte cell communication, while theca-to-granulosa cell communication primarily occurs through paracrine signaling. GJIC in the ovary is temporospatially regulated, in part, by phosphodiesterase 3a (PDE3a), and needed for cellular arrest and meiosis resumption in the oocyte. Connexin phosphorylation signals opening, function, and recycling of gap junction channels. Pathways involved in GJIC regulation include protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK) signaling. In theca cells, BPS can modulate GJIC through phosphatidylinositol-specific phospholipase C

Figure 3.6 (cont'd): (PI-PLC) independent of PKC activation, as well as through MAPK/ERK kinase (MEK1/2). ET18: ET-18-OCH_3, GF109: GF109203X, PC-PLC: phosphatidyl choline-specific phospholipase C, SB202: SB202190, and TPA: phorbol 12-myrisate 13-acetate.

Bisphenols and perfluorinated chemicals, both common classes of EDCs, have recently been reported to reduce GJIC in the ovary both, functionally or through a reduction in connexin expression (Acuna-Hernandez et al. 2018; Dominguez et al. 2019; Lopez-Arellano et al. 2019). To our knowledge, BPS is the first bisphenol congener and EDC reported to enhance GJIC. Importantly, gestational exposure to BPS in sheep reduces the population of progesterone-producing cells in the placenta (Gingrich et al. 2018). This was hypothesized to be due to an imbalance in cellular fusion at the commitment stage, which requires gap junction communication after cell apposition (Gingrich et al. 2018). Additional reproductive outcomes such as delayed onset of puberty (Shi et al. 2019), increased testosterone production (Shi et al. 2019), decreased ovary weight (Nevoral et al. 2018), and decreased number of antral and pre-antral follicles (Nevoral et al. 2018) are reported outcomes in female mice exposed to BPS *in vivo*. To our knowledge, neither GJIC or connexin expression was assessed or implicated in any of these BPS-related outcomes.

3.5. Concluding remarks

This study provides a novel model to evaluate the role of GJIC in theca cells. Using this model, we have demonstrated that BPS enhances GJIC in ovine ovarian theca cells. This effect is reproducible in primary human theca cells, highlighting the translational relevance of these findings. The inhibitor approach used demonstrates the promiscuity of BPS in altering multiple signaling pathways regulating GJIC in theca cells (Figure 3.6). Importantly, since GJIC alterations can disrupt folliculogenesis, the role of BPS in mediating ovarian-mediated sub- or infertility remains to be evaluated.

Chapter 3b: A Modified Parachute Assay

The parachute assay is a method of assessing GJIC by measuring dye transfer from two cell populations, usually of the same type (homocellular GJIC), denoted as donors and acceptors. Donor cells are loaded with a gap junction permissible dye, like calcein, and seeded onto a confluent monolayer of unlabeled acceptor cells. Donors and acceptors remain in co-culture for a set period, are washed off, and GJIC is quantified as the number of cells positive for calcein. However, this method assumes that donor cells do not remain attached to the acceptors, potentially leading to error-prone calculations. Since the scrape loading and dye transfer assay used in aim three was not suitable for testing placental cell GJIC, a modified version of the parachute assay was developed to assess GJIC in the non-cancer derived, commercially available, human trophoblast cell line HTR8/SVneo. Here, we use a lentiviral transfection method to distinguish donors from acceptors. This improved method shows that washing does not remove all donor cells and demonstrates an improvement to the current method. Preliminary data are presented below in an abstract accepted for platform presentation at the 59th Annual Society of Toxicology Annual Meeting. This model represents a novel tool, adaptable to high throughput screening technology, to further assess the effects of EDC exposures on GJIC in the placenta.

A modified parachute assay for accurate assessment of gap junction intercellular communication in placental trophoblast cells

Jeremy Gingrich[1,2], Yong Pu[1], Almudena Veiga-Lopez[1]

[1]Department of Animal Science, Michigan State University, East Lansing, MI 48824, USA

[2]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824, USA

Abstract

Gap junction intercellular communication (GJIC) can be assessed with a parachute assay, where fluorescent dye-loaded donor cells are seeded onto acceptors and dye diffuses to adjacent cells with active gap junctions. GJIC is expressed as the percent dye^+ cells. Since cells remain in co-culture for up to 4 h, donor cells can attach, but the assay does not permit distinction between donors and acceptors, which is a major limitation. Updates to this method use DiI dye, but DiI can be effluxed by donors over time resulting in false positives. We aimed to develop a parachute assay with distinguishable populations and hypothesized that it will assess GJIC comparable to current methods such as the scrape load/dye transfer assay (SL/DT) and is more accurate than methods lacking donor detection. Since intercellular communication is required during placental development, we used the trophoblast cell line HTR8-SVneo as the acceptor and HTR8-SVneo expressing red-fluorescent protein (RFP) as the donor. The assay was optimized under positive-control conditions using PKA activator, CW008. We have previously shown that gestational exposure to bisphenol S (BPS) reduces binucleate trophoblast cell number in sheep by disrupting fusogenic cell signaling. Since GJIC is one of the first steps in cell fusion, we also tested if BPS alters trophoblast cell GJIC. Donors and acceptors were exposed for 24 h to DMSO (0.1%), CW008 (1 µM), BPS (200 ng/mL), 12-O-tetradecanoylphorbol 13-acetate (TPA; 10 nM; neg. control), or combinations of the three. Donors loaded with calcein AM (10 µM) were then added at 10,000 cells/well onto acceptors. Cells remained in co-culture for 3 h then fixed, DAPI stained, and imaged. Number of transfer events was calculated as: $\Sigma calcein^+$ cells minus ΣRFP^+ cells over total cells, normalized to the control. BPS enhanced GJIC in HTR8-SVneo cells, like CW008. TPA significantly attenuated both chemical-induced enhanced GJIC. As predicted, exclusion of donors during quantification resulted false positives for both control treatments. This is the first report of a parachute assay method for measuring GJIC in placental cells. Like the SL/DT assay, this novel parachute method reliably detects both enhanced and suppressed GJIC. The ease and accuracy of quantification over current methods make this new

assay optimal for automation and is a useful tool for *in vitro* toxicological placental testing. Supported by NIEHS R01ES027863 to AVL. JG was supported by NICHD T32HD087166.

Chapter 4: Closing Remarks

Humans are exposed to a myriad of compounds classified as endocrine disrupting chemicals (EDCs) due to their ability to interfere with normal endocrine function. One class of EDCs are bisphenols, which have been studied in this dissertation to advance our understanding of EDC exposures on the development and function of the placenta. Bisphenols are used predominantly in the production of plastics and ubiquitously present in the environment (Konieczna et al. 2015, Eckardt and Simat 2017). Sources of human exposure to bisphenols include consumer products, food, water, and indoor dust (Liao and Kannan 2013; Liao et al. 2012). Bisphenol A (BPA) is the most widely detected in human circulation, closely followed by emerging EDCs bisphenol S (BPS) and bisphenol F (BPF). Importantly, bisphenols can cross from the maternal circulation – through the placenta – to reach the developing fetus. Investigating exposure outcomes during vulnerable windows of development, such as pregnancy and fetal life, is needed to better understand exposure risk and chemical safety. Being a transient, yet complex fetal endocrine organ, the placenta is particularly susceptible to EDC exposures (Gingrich et al. 2020). Despite this, literature on EDC exposures and placental outcomes, particularly for bisphenols and emerging EDCs, is lacking. This dissertation provides new and comprehensive information regarding the effect of bisphenolic EDCs on the development and function of the placenta. This chapter will address some of the strengths and shortcomings from the aims in this dissertation and propose future studies that could further expand our knowledge into the effects of EDCs on placental function and pregnancy outcomes.

Aim 1 investigated the pregnancy toxicokinetics of the three most common bisphenols (BPA, BPS, and BPF), using sheep. One major strength of this work, which focuses predominantly on the comparative evaluation of emerging bisphenols like BPS and BPF, was the use of an animal model that allowed the collection of high quality, pair-matched maternal and fetal blood samples. The uniqueness of this study has unveiled the potential for BPS, whose fetal elimination half-life is approximately 402

hours, to accumulate and persist in fetal circulation to a much larger extent than BPA (half-life ~52 hours). One limitation of this study was that bisphenols measurements were reported as total bisphenol (unconjugated + conjugated). Because of this, we were unable to evaluate the toxicokinetics of the unconjugated and conjugated bisphenol forms. Future studies should incorporate these measurements to better evaluate the bioactive parent compound. However, the quality and reliability of this dataset permitted its use in the development of physiologically-based toxicokinetic models (PBTK) for BPA and BPS. To our knowledge, this will be the first available pregnancy-specific PBTK model for BPS. Pregnancy-specific PBTK models allow for *in silico* risk predictions in humans and can be used to estimate fetal chemical exposures from maternal biomonitoring samples. We have also used this model to gain insight into fetal bisphenol steady state concentrations following a two-week exposure regimen relevant to human exposures. Due to the inherent ethical concerns of purposeful exposure of pregnant humans to bisphenols, pregnancy-specific PBTK models are incredibly useful tools in understanding gestational chemical exposures. This work adds to the growing number of *in silico* models available to understand chemical exposure risk.

EDC exposures do not just occur as a single chemical alone but in mixture, and gestational exposures are no exception (Woodruff et al. 2011). Aim 1 has allowed us to improve our understanding of the potential toxicokinetic interactions among bisphenols when exposure occurs in a mixture. By comparing non-compartmental toxicokinetics between a mixture of bisphenols (BPA, BPS, and BPF) to BPS alone, we demonstrated that during pregnancy there is no change in BPS toxicokinetics in the mother or fetus whether BPS is given alone or in mixture (with BPA and BPF). This suggests a lack of toxicokinetic interactions between bisphenols in a pregnancy model. However, the pervasiveness and complexity of chemical mixture exposures during pregnancy warrant future studies which include such exposure regimens.

Placenta-specific outcomes following chronic gestational exposure to BPA and BPS were evaluated in Aim 2 using sheep as an animal model. We demonstrated that BPS exposure results in a placental defect related to the fusogenic machinery of trophoblast cells. This is a previously unrecognized effect of BPS. Importantly, fusion of trophoblast cells is a key regulatory event for the development and function of the placenta (Huang et al. 2014; Koshi et al. 2012). One strength of this study relates to the comparative approach used in the experimental scheme, where results from BPA exposure could be directly compared with those from BPS. Notably, BPA exposure did not result in a similar phenotype to BPS, highlighting key differences in placental outcomes between bisphenols. In this aim we used a classic animal model for the study of placental chemical transfer, the sheep. Sheep are a non-litter baring, precocial, large mammal, which as a model species allow for the longitudinal evaluation of chemical exposures and hormonal profiling. The use of sheep also avoids known confounding factors from altricial species (rodents) such as uterine implantation site and fetal position phenomena, which can lead to non-uniform chemical exposures (vom Saal et al. 1999). However, sheep and humans have both different macro and micro placental structures (Wooding et al. 2008). While the use of non-human primates in these studies would have provided the most interspecies translatable results, ethical and monetary constraints make such studies extremely challenging. An underused placenta model species is the guinea pig, which is a precocial rodent with more comparable placentation to humans than mice or rats and are more cost effective than sheep. To help strengthen the translatability of placenta-specific toxicological findings, future studies should consider incorporating more amenable animal models like sheep and guinea pig.

The doses of BPA and BPS used in Aim 2a were chosen to achieve an environmentally relevant exposure concentration to the fetus but can be deemed supraphysiologic to the mother. Since the primary aim of the study was to be able to examine fetal outcomes upon BPS exposure, the doses were chosen to consider mainly the fetal compartment. Future studies investigating placental outcomes

should incorporate lower maternal doses relevant to world-wide human exposures. Importantly, the fusogenic defect discovered in Aim 2a after *in vivo* exposure to BPS in our sheep model was recapitulated in primary isolated human placental cells *in vitro* for Aim 2b using a physiologically relevant exposure regimen. This highlights the translatability of the sheep model and has enabled us to begin to learn about the mechanistic insights into this BPS-induced placental defect. While we could not account for a sex-specific effect due to original IRB restrictions which did not permit collect this clinical data (the placenta has the sex of the fetus), current studies in the laboratory are including sex as a biological variable. Given the sex disparity in pregnancy complications, such as preterm birth (McGregor et al. 1992), this should be a future area of research. Additionally, classification of trophoblast cell populations is still an active area of research (Lee et al. 2019), making the development of *in vitro* protocols evaluating placenta-specific toxicological screening challenging. However, Aim 2b uses a broadly used method for isolation of trophoblast cells (Petroff et al. 2006). While *in vivo* studies allow for a more holistic and integrative picture of the chemical disruption, *in vitro* models allow for higher throughput and are needed to understand mechanisms of action. The placenta has a complex, cellularly heterogenous, 3-dimensional (3D) structure that is difficult to recapitulate with classic 2-dimensional (2D) *in vitro* culture models. Future studies should incorporate the use of 3D culture systems, with human cell lines, that display cell-cell interactions and shear stress to better mimic the *in vivo* environment. Such studies can aid in bridging this gap in *in vivo* to *in vitro* translational relevance.

Findings from Aim 2 demonstrated that a defect in placental trophoblast cell fusogenic machinery was induced upon exposure to BPS both *in vivo* and *in vitro*. Given that gap junction intercellular communication (GJIC) is a key regulatory process during trophoblast fusion (Pidoux et al. 2014), we investigated whether BPS could alter GJIC in Aim 3. The widely accepted scrape loading/dye transfer assay (SL/DT) used to assess GJIC requires a confluent monolayer of cells. Since primary isolated human cytotrophoblasts do not proliferate in culture, we used another steroidogenic cell that produces

progesterone, the ovarian theca cell (Young and McNeilly 2010) as a cell model. We have demonstrated for the first time that BPS can alter GJIC in sheep theca cells, which was done in a comparative exposure scheme to four other common EDCs (BPA, BPF, PFOS, and TPT). This result was time- and dose-dependent and occurred regardless of cell stage (pre- or post-luteinized). Altogether, these findings represent a comprehensive approach into the evaluation of how bisphenols can differentially affect GJIC. Although this outcome still needs to be confirmed in placental cells, we now have provided with a proof of concept that BPS enhances GJIC in steroidogenic cells. Moreover, we have shown this to be translationally relevant, recapitulating this effect in human primary theca cells. Like the fusogenic defect induced by BPS, enhanced GJIC is also a conserved mechanism across species. To note, only one approach, the SL/DT assay, was used in Aim 3a. To validate these results using a different approach, I develop a modified parachute assay for the assessment of GJIC in the placenta (Aim 3b). Using this modified technique, we have been able to resolve one of the main shortcomings of the original parachute assay, which is the distinction between donor and acceptor cells, using a lentiviral transfection method. By using this modified parachute technique, we can reduce the error from counting attached donor cells by approximately 25%. This assay places us in a great position to begin developing future studies capable of exploring GJIC specific to placental toxicology.

When proceeding with future studies evaluating the effects of EDC exposures on the placenta, the development of systematic analyses and standardized exposure protocols will help increase interstudy reproducibility and overall data confidence. There are currently no available US Environmental Protection Agency guideline studies for placenta-specific outcomes. This creates not only a barrier for research groups which may identify only placenta-specific outcomes from being used in regulatory decision processes but may also prevent reproducibility among studies. Because of this, we need to make sure that a conscious effort is made to establish a guideline for studies that evaluate

placenta-specific outcomes. It is only then that placenta-specific outcomes can be effectively incorporated into chemical risk assessment.

APPENDICES

APPENDICES

APPENDIX A: Supplementary Tables

Table 2.S1: Primers for quantitative real time PCR.

Gene	Primers	Length (bp)	Accession
enJSRV – Forward LOC105613207	TTAGAGGTGTAGCCAAAG	120	XM_015094295
enJSRV – Reverse LOC105613207	CAGTTGCTATAAGGGTGA		
HYAL2 - Forward	CGTGGACTCACAGGGCTTAG	204	NM_001009754
HYAL2 - Reverse	GGTCTCGTTGCTGGTGGTA		

Note: Accession number from NCBI gene database.

Table 2.S2: Antibodies and dilutions used for immunocytochemical analyses.

Type	**Antibody**	**Company**	**Catalog**	**Dilution**	**Source**
Primary	e-cadherin	BD Biosciences, Franklin Lakes, NJ, USA	610181	1:500	Rabbit
Primary	GCM1	Abcam, Cambridge, UK	ab88748	1:40	Mouse
Primary	*Wisteria floribunda* lectin	Vector Laboratories, Burlingame, CA, USA	B-1355	1:100 (10µg/ml)	*Wisteria floribunda*
Secondary	Biotinylate anti-mouse IgG	Abcam, Cambridge, UK	ab64255	Ready-to-use	Goat

Table 2.S3: Maternal serum biochemistry.

Metabolite	Units	Control	BPA	BPS	ANOVA	Range (Mean ± SE)[1]
Na^+	(mmol/L)	144.88 ± 0.91	142.25 ± 1.49	144.14 ± 1.24	*0.310*	139 - 152
K^+	(mmol/L)	5.69 ± 0.23	5.55 ± 0.19	5.56 ± 0.17	*0.860*	3.9 -5.4
Na-K ratio		25.75 ± 1.15	25.88 ± 0.72	26.00 ± 0.76	*0.982*	na
Cl^-	(mmol/L)	107.5 ± 0.89	107.13 ± 1.43	107.29 ± 0.75	*0.969*	95 -103
Ca^{2+}	(mg/dL)	10.26 ± 0.16	9.96 ± 0.25	10.39 ± 0.11	*0.290*	11.5 - 12.8 (12.16 ± 0.28)
Mg^{2+}	(mg/dL)	2.26 ± 0.08	2.31 ± 0.05	2.40 ± 0.10	*0.444*	2.2 - 2.8 (2.5 ± 0.3)
P	(mg/dL)	6.50 ± 0.31^a	5.35 ± 0.31^b	5.63 ± 0.29^b	*0.035*	5.0 - 7.3 (6.4 ± 0.2)
I^-	(µg/dL)	127.75 ± 15.65	140.63 ± 12.99	140.29 ± 13.72	*0.765*	na
Total CO_2	(mmol/L)	23.75 ± 0.56	23.00 ± 0.6	23.57 ± 0.90	*0.714*	21 - 28
Anion Gap	(mmol/L)	19.38 ± 0.38	17.63 ± 0.71	18.57 ± 0.78	*0.162*	na
Osmolarity	(mmol/L)	298.25 ± 1.92	293.38 ± 3.23	295.86 ± 2.09	*0.394*	na
Urea Nitrogen	(mg/dL)	13.75 ± 0.94	15.87 ± 1.23	11.14 ± 2.09	*0.096*	8 - 20
Total Bilirubin	(mg/dL)	0.1 ± 0.00	0.11 ± 0.01	0.13 ± 0.02	*0.288*	0.1-0.5 (0.23 ± 0.1)
Cholesterol	(mg/dL)	55.87 ± 2.61	53.63 ± 2.67	61.29 ± 5.06	*0.313*	52 - 56 (64 ± 12)
Alkaline Phosphatase	(U/L)	191.25 ± 19.51	144.87 ± 15.03	156.86 ± 20.21	*0.191*	68 - 387 (178 ± 102)
Aspartate Aminotransferase	(U/L)	74.50 ± 3.93	71.25 ± 3.01	81.86 ± 6.57	*0.278*	60 - 280 (307 ± 43)
Creatinine	(mg/dL)	0.91 ± 0.04^a	0.95 ± 0.05^b	1.1 ± 0.03^c	*0.008*	0.8 - 1.3
Creatine Kinase	(U/L)	148.00 ± 32.57	284.38 ± 106.06	350.86 ± 133.51	*0.342*	100 - 547
γ-Glutamyltransferase	(U/L)	50.25 ± 4.01	42.12 ± 3.02	43.00 ± 2.06	*0.165*	20 - 52 (33.5 ± 4.3)

[1]Range and Mean values obtained from references listed below.
Different letters denote statistical differences among treatment groups at $P<0.05$.
na: not available

APPENDIX B: Permissions for Reprint

Gingrich, J., E. Ticiani and A. Veiga-Lopez (2020). "Placenta disrupted: endocrine disrupting chemicals and pregnancy." Trends Endocrinol Metab doi: 10.1016/j.tem.2020.03.003. [Epub ahead of print].

Elsevier authors retain the right to include whole articles in a thesis or dissertation. Permission is not required. http://www.elsevier.com/about/our-business/policies/copyright#Author-rights

Gingrich, J., Y. Pu, R. Ehrhardt, R. Karthikraj, K. Kannan and A. Veiga-Lopez (2019). "Toxicokinetics of bisphenol A, bisphenol S, and bisphenol F in a pregnancy sheep model." Chemosphere **220**: 185-194.

Order license ID: 1025062-1 for reprint in a thesis/dissertation

https://marketplace.copyright.com/rs-ui-web/mp/license/d2a5fd97-b1a8-4241-bd82-7c30ac1eb55a/22a0baae-61e1-44aa-80f6-c5261574e25c

Gingrich, J., Y. Pu, J. Roberts, R. Karthikraj, K. Kannan, R. Ehrhardt and A. Veiga-Lopez (2018). "Gestational bisphenol S impairs placental endocrine function and the fusogenic trophoblast signaling pathway." Arch Toxicol **92**(5): 1861-1876.

Order license ID# 4792010941573

License Number	4792010941573
License date	Mar 18, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Archives of Toxicology
Licensed Content Title	Gestational bisphenol S impairs placental endocrine function and the fusogenic trophoblast signaling pathway
Licensed Content Author	Jeremy Gingrich et al
Licensed Content Date	Mar 17, 2018
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	electronic
Portion	full article/chapter
Will you be translating?	no
Circulation/distribution	1 - 29
Author of this Springer Nature content	yes
Title	Effects of Endocrine Disrupting Chemicals on Placental Development and Function
Institution name	Michigan State University
Expected presentation date	Jun 2020
Requestor Location	Jeremy Gingrich 2597 Martina Dr. Apt. 25 HOLT, MI 48842 United States Attn: Jeremy Gingrich

Gingrich, J., Y. Pu, B.L. Upham, M. Hulse, S. Pearl, D. Martin, A. Avery and A. Veiga-Lopez (2020b). "Bisphenol S enhances gap junction intercellular communication in ovarian theca cells." Chemosphere [under *review*].

Elsevier authors retain the right to include whole articles in a thesis or dissertation. Permission is not required. http://www.elsevier.com/about/our-business/policies/copyright#Author-rights

BIBLIOGRAPHY

BIBLIOGRAPHY

Abu-Qare, A. W., A. Abdel-Rahman, C. Brownie, A. M. Kishk and M. B. Abou-Donia (2001). "Inhibition of cholinesterase enzymes following a single dermal dose of chlorpyrifos and methyl parathion, alone and in combination, in pregnant rats." J Toxicol Environ Health A **63**(3): 173-189.

Aasen, T., M. Mesnil, C. C. Naus, P. D. Lampe and D. W. Laird (2017). "Gap junctions and cancer: communicating for 50 years." Nat Rev Cancer **17**(1): 74.

Accustandard. (2017). "Flame Retardant Standards Guide." Retrieved 11/12/2019, from https://www.accustandard.com/assets/Flame_Retardant_Guide.pdf.

Ackert, C. L., J. E. Gittens, M. J. O'Brien, J. J. Eppig and G. M. Kidder (2001). "Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse." Dev Biol **233**(2): 258-270.

Acuna-Hernandez, D. G., L. Arreola-Mendoza, R. Santacruz-Marquez, S. P. Garcia-Zepeda, L. Y. Parra-Forero, J. A. Olivares-Reyes and I. Hernandez-Ochoa (2018). "Bisphenol A alters oocyte maturation by prematurely closing gap junctions in the cumulus cell-oocyte complex." Toxicol Appl Pharmacol **344**: 13-22.

Adibi, J. J., R. M. Whyatt, R. Hauser, H. K. Bhat, B. J. Davis, A. M. Calafat, L. A. Hoepner, F. P. Perera, D. Tang and P. L. Williams (2010). "Transcriptional biomarkers of steroidogenesis and trophoblast differentiation in the placenta in relation to prenatal phthalate exposure." Environ Health Perspect **118**(2): 291-296.

Adibi, J. J., Y. Zhao, L. V. Zhan, M. Kapidzic, N. Larocque, H. Koistinen, I. T. Huhtaniemi and U. H. Stenman (2017). "An Investigation of the Single and Combined Phthalate Metabolite Effects on Human Chorionic Gonadotropin Expression in Placental Cells." Environ Health Perspect **125**(10): 107010.

Ahbab, M. A., C. Guven, E. A. Kockaya and N. Barlas (2017). "Comparative developmental toxicity evaluation of di- n-hexyl phthalate and dicyclohexyl phthalate in rats." Toxicol Ind Health **33**(9): 696-716.

Ahmed, R. G., A. W. El-Gareib and H. M. Shaker (2018). "Gestational 3,3',4,4',5-pentachlorobiphenyl (PCB 126) exposure disrupts fetoplacental unit: Fetal thyroid-cytokines dysfunction." Life Sci **192**: 213-220.

Ale-Agha, N., W. Stahl and H. Sies (2002). "(-)-Epicatechin effects in rat liver epithelial cells: stimulation of gap junctional communication and counteraction of its loss due to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate." Biochem Pharmacol **63**(12): 2145-2149.

Al-Saleh, I., I. Al-Doush, A. Alsabbaheen, D. Mohamed Gel and A. Rabbah (2012). "Levels of DDT and its metabolites in placenta, maternal and cord blood and their potential influence on neonatal anthropometric measures." Sci Total Environ **416**: 62-74.

Ando, M. (1978). "Transfer of 2,4,5,2',4',5'-hexachlorobiphenyl and 2,2-bis-(p-chlorophenyl),1,1,1-trichloroethane (p,p'-DDT) from maternal to newborn and suckling rats." Arch Toxicol **41**(3): 179-186.

Andra, S. S., C. Austin, J. Yang, D. Patel and M. Arora (2016). "Recent advances in simultaneous analysis of bisphenol A and its conjugates in human matrices: Exposure biomarker perspectives." Science of the Total Environment **572**: 770-781.

Anson-Cartwright, L., K. Dawson, D. Holmyard, S. J. Fisher, R. A. Lazzarini and J. C. Cross (2000). "The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta." Nat Genet **25**(3): 311-314.

Arbuckle, T. E., L. Weiss, M. Fisher, R. Hauser, P. Dumas, R. Berube, A. Neisa, A. LeBlanc, C. Lang, P. Ayotte, M. Walker, M. Feeley, D. Koniecki and G. Tawagi (2015). "Maternal and infant exposure to environmental phenols as measured in multiple biological matrices." Science of the Total Environment **508**: 575-584.

Arck, P., P. J. Hansen, B. Mulac Jericevic, M. P. Piccinni and J. Szekeres-Bartho (2007). "Progesterone during pregnancy: endocrine-immune cross talk in mammalian species and the role of stress." Am J Reprod Immunol **58**(3): 268-279.

Arita, Y., M. Kirk, N. Gupta, R. Menon, D. Getahun and M. R. Peltier (2018). "Effects of tributyltin on placental cytokine production." J Perinat Med **46**(8): 867-875.

Asimakopoulos, A. G., J. Xue, B. P. De Carvalho, A. Iyer, K. O. Abualnaja, S. S. Yaghmoor, T. A. Kumosani and K. Kannan (2016). "Urinary biomarkers of exposure to 57 xenobiotics and its association with oxidative stress in a population in Jeddah, Saudi Arabia." Environ Res **150**: 573-581.

Avissar-Whiting, M., K. R. Veiga, K. M. Uhl, M. A. Maccani, L. A. Gagne, E. L. Moen and C. J. Marsit (2010). "Bisphenol A exposure leads to specific microRNA alterations in placental cells." Reprod Toxicol **29**(4): 401-406.

Babica, P., L. Ctverackova, Z. Lencesova, J. E. Trosko and B. L. Upham (2016). "Chemopreventive Agents Attenuate Rapid Inhibition of Gap Junctional Intercellular Communication Induced by Environmental Toxicants." Nutrition and Cancer-an International Journal **68**(5): 827-837.

Babica, P., R. Zurabian, E. R. Kumar, R. Chopra, M. J. Mianecki, J. S. Park, L. Jasa, J. E. Trosko and B. L. Upham (2016b). "Methoxychlor and Vinclozolin Induce Rapid Changes in Intercellular and Intracellular Signaling in Liver Progenitor Cells." Toxicological Sciences **153**(1): 174-185.

Backlin, B. M., E. Persson, C. J. Jones and V. Dantzer (1998). "Polychlorinated biphenyl (PCB) exposure produces placental vascular and trophoblastic lesions in the mink (Mustela vison): a light and electron microscopic study." APMIS **106**(8): 785-799.

Baker, F. D., B. Bush, C. F. Tumasonis and F. C. Lo (1977). "Toxicity and persistence of low-level PCB in adult wistar rats, fetuses, and young." Arch Environ Contam Toxicol **5**(2): 143-156.

Baldwin, K. R., A. L. Phillips, B. Horman, S. E. Arambula, M. E. Rebuli, H. M. Stapleton and H. B. Patisaul (2017). "Sex Specific Placental Accumulation and Behavioral Effects of Developmental Firemaster 550 Exposure in Wistar Rats." Sci Rep **7**(1): 7118.

Ballabh, P., E. S. Lo, J. Kumari, T. B. Cooper, I. Zervoudakis, P. A. Auld and A. N. Krauss (2002). "Pharmacokinetics of betamethasone in twin and singleton pregnancy." Clin Pharmacol Ther **71**(1): 39-45.

Barai, S., S. Gambhir, N. Prasad, R. K. Sharma and M. Ora (2010). "Functional renal reserve capacity in different stages of chronic kidney disease." Nephrology (Carlton) **15**(3): 350-353.

Basak, S., V. Srinivas and A. K. Duttaroy (2018). "Bisphenol-A impairs cellular function and alters DNA methylation of stress pathway genes in first trimester trophoblast cells." Reprod Toxicol **82**: 72-79.

Beckett, E. M., O. Astapova, T. L. Steckler, A. Veiga-Lopez and V. Padmanabhan (2014). "Developmental programing: impact of testosterone on placental differentiation." Reproduction **148**(2): 199-209.

Bedford, C. A., F. A. Harrison and R. B. Heap (1972). "The metabolic clearance rate and production rate of progesterone and the conversion of progesterone to 20 -hydroxypregn-4-en-3-one in the sheep." J Endocrinol **55**(1): 105-118.

Benirschke, K., G. Burton and R. N. Baergen (2012). Pathology of the human placenta. Berlin ; London, Springer.

Bernier, M. R. and L. N. Vandenberg (2017). "Handling of thermal paper: Implications for dermal exposure to bisphenol A and its alternatives." PLoS One **12**(6): e0178449.

Bird, S. D. (2017). "Artificial placenta: Analysis of recent progress." Eur J Obstet Gynecol Reprod Biol **208**: 61-70.

Birks, L., M. Casas, A. M. Garcia, J. Alexander, H. Barros, A. Bergstrom, J. P. Bonde, A. Burdorf, N. Costet, A. Danileviciute, M. Eggesbo, M. F. Fernandez, M. C. Gonzalez-Galarzo, G. Regina, W. Hanke, V. Jaddoe, M. Kogevinas, I. Kull, A. Lertxundi, V. Melaki, A. N. Andersen, N. Olea, K. Polanska, F. Rusconi, L. Santa-Marina, A. C. Santos, T. Vrijkotte, D. Zugna, M. Nieuwenhuijsen, S. Cordier and M. Vrijheid (2016). "Occupational Exposure to Endocrine-Disrupting Chemicals and Birth Weight and Length of Gestation: A European Meta-Analysis." Environ Health Perspect **124**(11): 1785-1793.

Birnbaum, L. S. and E. A. Cohen Hubal (2006). "Polybrominated diphenyl ethers: a case study for using biomonitoring data to address risk assessment questions." Environ Health Perspect **114**(11): 1770-1775.

Black, S. G., F. Arnaud, M. Palmarini and T. E. Spencer (2010). "Endogenous retroviruses in trophoblast differentiation and placental development." Am J Reprod Immunol **64**(4): 255-264.

Blanc-Lapierre, A., G. Bouvier, A. Gruber, K. Leffondre, P. Lebailly, C. Fabrigoule and I. Baldi (2013). "Cognitive disorders and occupational exposure to organophosphates: results from the PHYTONER study." Am J Epidemiol **177**(10): 1086-1096.

Bloise, E., S. Petropoulos, M. Iqbal, A. Kostaki, T. M. Ortiga-Carvalho, W. Gibb and S. G. Matthews (2017). "Acute Effects of Viral Exposure on P-Glycoprotein Function in the Mouse Fetal Blood-Brain Barrier." Cell Physiol Biochem **41**(3): 1044-1050.

Blundell, C., Y. S. Yi, L. Ma, E. R. Tess, M. J. Farrell, A. Georgescu, L. M. Aleksunes and D. Huh (2018). "Placental Drug Transport-on-a-Chip: A Microengineered In Vitro Model of Transporter-Mediated Drug Efflux in the Human Placental Barrier." Adv Healthc Mater **7**(2).

Borman, E. D., W. G. Foster and D. deCatanzaro (2017). "Concurrent administration of diethylhexyl phthalate reduces the threshold dose at which bisphenol A disrupts blastocyst implantation and cadherins in mice." Environmental Toxicology and Pharmacology **49**: 105-111.

Borowczyk, E., M. L. Johnson, J. J. Bilski, P. P. Borowicz, D. A. Redmer, L. P. Reynolds and A. T. Grazul-Bilska (2006). "Expression of gap junctional connexins 26, 32, and 43 mRNA in ovarian preovulatory follicles and corpora lutea in sheep." Can J Physiol Pharmacol **84**(10): 1011-1020.

Bouwman, H., H. van den Berg and H. Kylin (2011). "DDT and malaria prevention: addressing the paradox." Environ Health Perspect **119**(6): 744-747.

Bradbury, J. (2008). "Secrets of the placental labyrinth unlocked." Development **135**(12): e1201-e1201.

Buimer, M., R. Keijser, J. M. Jebbink, D. Wehkamp, A. H. van Kampen, K. Boer, J. A. van der Post and C. Ris-Stalpers (2008). "Seven placental transcripts characterize HELLP-syndrome." Placenta **29**(5): 444-453.

Burton, G. J., A. L. Fowden and K. L. Thornburg (2016). "Placental Origins of Chronic Disease." Physiol Rev **96**(4): 1509-1565.

Cabaton, N., D. Zalko, E. Rathahao, C. Canlet, G. Delous, M. C. Chagnon, J. P. Cravedi and E. Perdu (2008). "Biotransformation of bisphenol F by human and rat liver subcellular fractions." Toxicol In Vitro **22**(7): 1697-1704.

Cabaton, N., M. C. Chagnon, J. C. Lhuguenot, J. P. Cravedi and D. Zalko (2006). "Disposition and metabolic profiling of bisphenol F in pregnant and nonpregnant rats." J Agric Food Chem **54**(26): 10307-10314.

Canton, R. F., D. E. Scholten, G. Marsh, P. C. de Jong and M. van den Berg (2008). "Inhibition of human placental aromatase activity by hydroxylated polybrominated diphenyl ethers (OH-PBDEs)." Toxicol Appl Pharmacol **227**(1): 68-75.

Cao, J., L. H. Guo, B. Wan and Y. Wei (2011). "In vitro fluorescence displacement investigation of thyroxine transport disruption by bisphenol A." J Environ Sci (China) **23**(2): 315-321.

Cao, S., L. Ye, Y. Wu, B. Mao, L. Chen, X. Wang, P. Huang, Y. Su and R. S. Ge (2017). "The Effects of Fungicides on Human 3beta-Hydroxysteroid Dehydrogenase 1 and Aromatase in Human Placental Cell Line JEG-3." Pharmacology **100**(3-4): 139-147.

Caserta, D., A. Graziano, G. Lo Monte, G. Bordi and M. Moscarini (2013). "Heavy metals and placental fetal-maternal barrier: a mini-review on the major concerns." Eur Rev Med Pharmacol Sci **17**(16): 2198-2206.

Castro, B., P. Sanchez, J. M. Torres and E. Ortega (2015). "Bisphenol A, bisphenol F and bisphenol S affect differently 5alpha-reductase expression and dopamine-serotonin systems in the prefrontal cortex of juvenile female rats." Environ Res **142**: 281-287.

Catanese, M. C. and L. N. Vandenberg (2017). "Bisphenol S (BPS) Alters Maternal Behavior and Brain in Mice Exposed During Pregnancy/Lactation and Their Daughters." Endocrinology **158**(3): 516-530.

Cavazos-Rehg, P. A., M. J. Krauss, E. L. Spitznagel, K. Bommarito, T. Madden, M. A. Olsen, H. Subramaniam, J. F. Peipert and L. J. Bierut (2015). "Maternal age and risk of labor and delivery complications." Matern Child Health J **19**(6): 1202-1211.

CDC. (2016, 9/2016). "Personal Care and Consumer Product Chemicals and Metabolites (EPHPP_H)." Retrieved March 9, 2020, from https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/EPHPP_H.htm#URXBPF.

CDC. (2017, February 16, 2017). "Infertility." Retrieved April 5, 2017, from https://www.cdc.gov/reproductivehealth/infertility/.

CDC. (2019, 2/28/2019). "Data on Selected Pregnancy Complications in the United States." Retrieved 11/6/2019, 2019, from https://www.cdc.gov/reproductivehealth/maternalinfanthealth/pregnancy-complications-data.htm.

Cesen-Cummings, K., K. A. Warner and R. J. Ruch (1998). "Role of protein kinase C in the deficient gap junctional intercellular communication of K-ras-transformed murine lung epithelial cells." Anticancer Res **18**(6A): 4343-4346.

Chang, H. M., J. C. Cheng and P. C. Leung (2013). "Theca-derived BMP4 and BMP7 down-regulate connexin43 expression and decrease gap junction intercellular communication activity in immortalized human granulosa cells." J Clin Endocrinol Metab **98**(3): E437-445.

Chang, H. M., J. C. Cheng, E. Taylor and P. C. Leung (2014). "Oocyte-derived BMP15 but not GDF9 down-regulates connexin43 expression and decreases gap junction intercellular communication activity in immortalized human granulosa cells." Mol Hum Reprod **20**(5): 373-383.

Chen, D., K. Kannan, H. Tan, Z. Zheng, Y. L. Feng, Y. Wu and M. Widelka (2016). "Bisphenol Analogues Other Than BPA: Environmental Occurrence, Human Exposure, and Toxicity-A Review." Environ Sci Technol **50**(11): 5438-5453.

Chen, F., S. Yin, B. C. Kelly and W. Liu (2017). "Chlorinated Polyfluoroalkyl Ether Sulfonic Acids in Matched Maternal, Cord, and Placenta Samples: A Study of Transplacental Transfer." Environ Sci Technol **51**(11): 6387-6394.

Chen, M. Y., M. Ike and M. Fujita (2002). "Acute toxicity, mutagenicity, and estrogenicity of bisphenol-A and other bisphenols." Environ Toxicol **17**(1): 80-86.

Chen, Z. J., X. L. Yang, H. Liu, W. Wei, K. S. Zhang, H. B. Huang, J. P. Giesy, H. L. Liu, J. Du and H. S. Wang (2015). "Bisphenol A modulates colorectal cancer protein profile and promotes the metastasis via induction of epithelial to mesenchymal transitions." Arch Toxicol **89**(8): 1371-1381.

Chiapella, G., J. Flores-Martin, M. E. Ridano, L. Reyna, G. Magnarelli de Potas, G. M. Panzetta-Dutari and S. Genti-Raimondi (2013). "The organophosphate chlorpyrifos disturbs redox balance and triggers antioxidant defense mechanisms in JEG-3 cells." Placenta **34**(9): 792-798.

Chiba, M., J. A. Nshime and J. H. Lin (1997). "In vitro metabolism of indinavir in the human fetal liver microsomes." Drug Metab Dispos **25**(10): 1219-1222.

Chu, P. W., Z. J. Yang, H. H. Huang, A. A. Chang, Y. C. Cheng, G. J. Wu and H. C. Lan (2018). "Low-dose bisphenol A activates the ERK signaling pathway and attenuates steroidogenic gene expression in human placental cells." Biol Reprod **98**(2): 250-258.

Clegg, D. J. and M. van Gemert (1999). "Determination of the reference dose for chlorpyrifos: proceedings of an expert panel." J Toxicol Environ Health B Crit Rev **2**(3): 211-255.

Colovic, M. B., D. Z. Krstic, T. D. Lazarevic-Pasti, A. M. Bondzic and V. M. Vasic (2013). "Acetylcholinesterase inhibitors: pharmacology and toxicology." Curr Neuropharmacol **11**(3): 315-335.

Corbel, T., E. Perdu, V. Gayrard, S. Puel, M. Z. Lacroix, C. Viguie, P. L. Toutain, D. Zalko and N. Picard-Hagen (2015). "Conjugation and deconjugation reactions within the fetoplacental compartment in a sheep model: a key factor determining bisphenol A fetal exposure." Drug Metab Dispos **43**(4): 467-476.

Corbel, T., V. Gayrard, C. Viguie, S. Puel, M. Z. Lacroix, P. L. Toutain and N. Picard-Hagen (2013). "Bisphenol A disposition in the sheep maternal-placental-fetal unit: mechanisms determining fetal internal exposure." Biol Reprod **89**(1): 11.

Correia Carreira, S., L. Cartwright, L. Mathiesen, L. E. Knudsen and M. Saunders (2011). "Studying placental transfer of highly purified non-dioxin-like PCBs in two models of the placental barrier." Placenta **32**(3): 283-291.

Corsini, E., R. W. Luebke, D. R. Germolec and J. C. DeWitt (2014). "Perfluorinated compounds: emerging POPs with potential immunotoxicity." Toxicol Lett **230**(2): 263-270.

Coutifaris, C., L. C. Kao, H. M. Sehdev, U. Chin, G. O. Babalola, O. W. Blaschuk and J. F. Strauss, 3rd (1991). "E-cadherin expression during the differentiation of human trophoblasts." Development **113**(3): 767-777.

Craig, Z. R. and A. Ziv-Gal (2018). "Pretty Good or Pretty Bad? The Ovary and Chemicals in Personal Care Products." Toxicol Sci **162**(2): 349-360.

Cronier, L., J. L. Frendo, N. Defamie, G. Pidoux, G. Bertin, J. Guibourdenche, G. Pointis and A. Malassine (2003). "Requirement of gap junctional intercellular communication for human villous trophoblast differentiation." Biol Reprod **69**(5): 1472-1480.

Cross, C. E., M. F. Tolba, C. M. Rondelli, M. Xu and S. Z. Abdel-Rahman (2015). "Oxidative Stress Alters miRNA and Gene Expression Profiles in Villous First Trimester Trophoblasts." Biomed Res Int **2015**: 257090.

Cross, J. C., Z. Werb and S. J. Fisher (1994). "Implantation and the placenta: key pieces of the development puzzle." Science **266**(5190): 1508-1518.

Czyzyk, A., J. Latacz, D. Filipowicz, A. Podfigurna, R. Moszynski, P. Jasinski, S. Sajdak, M. Gaca, A. R. Genazzani and B. Meczekalski (2017). "Severe hyperandrogenemia in postmenopausal woman as a presentation of ovarian hyperthecosis. Case report and mini review of the literature." Gynecol Endocrinol **33**(11): 836-839.

Davidson, J. S. and I. M. Baumgarten (1988). "Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships." J Pharmacol Exp Ther **246**(3): 1104-1107.

Derfoul, A., F. J. Lin, E. M. Awumey, T. Kolodzeski, D. J. Hall and R. S. Tuan (2003). "Estrogenic endocrine disruptive components interfere with calcium handling and differentiation of human trophoblast cells." J Cell Biochem **89**(4): 755-770.

Doerge, D. R., N. C. Twaddle, M. Vanlandingham and J. W. Fisher (2010). "Pharmacokinetics of bisphenol A in neonatal and adult Sprague-Dawley rats." Toxicol Appl Pharmacol **247**(2): 158-165.

Doerge, D. R., N. C. Twaddle, M. Vanlandingham, R. P. Brown and J. W. Fisher (2011). "Distribution of bisphenol A into tissues of adult, neonatal, and fetal Sprague-Dawley rats." Toxicol Appl Pharmacol **255**(3): 261-270.

Dominguez, A., Z. Salazar, E. Arenas, M. Betancourt, Y. Ducolomb, H. Gonzalez-Marquez, E. Casas, M. Teteltitla and E. Bonilla (2016). "Effect of perfluorooctane sulfonate on viability, maturation and gap junctional intercellular communication of porcine oocytes in vitro." Toxicol In Vitro **35**: 93-99.

Dominguez, A., Z. Salazar, M. Betancourt, Y. Ducolomb, E. Casas, F. Fernandez, I. Bahena, A. Salomon, M. Teteltitla, R. Martinez, A. Chaparro, P. Cuapio, C. Salazar-Lopez and E. Bonilla (2019). "Effect of perfluorodecanoic acid on pig oocyte viability, intracellular calcium levels and gap junction intercellular communication during oocyte maturation in vitro." Toxicol In Vitro **58**: 224-229.

Dominguez-Lopez, P., L. Diaz-Cueto, A. Olivares, A. Ulloa-Aguirre and F. Arechavaleta-Velasco (2012). "Differential effect of DDT, DDE, and DDD on COX-2 expression in the human trophoblast derived HTR-8/SVneo cells." J Biochem Mol Toxicol **26**(11): 454-460.

Dorman F, R. E. (2012). Chapter 28 - Emerging and Persistent Environmental Compound Analysis, Elsevier.

Drenth, H. J., C. A. Bouwman, W. Seinen and M. Van den Berg (1998). "Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3." Toxicol Appl Pharmacol **148**(1): 50-55.

Dunlap, K. A., M. Palmarini, D. L. Adelson and T. E. Spencer (2005). "Sheep endogenous betaretroviruses (enJSRVs) and the hyaluronidase 2 (HYAL2) receptor in the ovine uterus and conceptus." Biol Reprod **73**(2): 271-279.

Dunlap, K. A., M. Palmarini, M. Varela, R. C. Burghardt, K. Hayashi, J. L. Farmer and T. E. Spencer (2006). "Endogenous retroviruses regulate periimplantation placental growth and differentiation." Proc Natl Acad Sci U S A **103**(39): 14390-14395.

Duzyj, C. M., I. A. Buhimschi, H. Motawea, C. A. Laky, G. Cozzini, G. Zhao, E. F. Funai and C. S. Buhimschi (2015). "The invasive phenotype of placenta accreta extravillous trophoblasts associates with loss of E-cadherin." Placenta **36**(6): 645-651.

Eckardt, M. and T. J. Simat (2017). "Bisphenol A and alternatives in thermal paper receipts - a German market analysis from 2015 to 2017." Chemosphere **186**: 1016-1025.

Ehrhardt, R. A., A. W. Bell and Y. R. Boisclair (2002). "Spatial and developmental regulation of leptin in fetal sheep." Am J Physiol Regul Integr Comp Physiol **282**(6): R1628-1635.

El-Hayek, S. and H. J. Clarke (2015). "Follicle-Stimulating Hormone Increases Gap Junctional Communication Between Somatic and Germ-Line Follicular Compartments During Murine Oogenesis." Biol Reprod **93**(2): 47.

Ema, M., A. Arima, K. Fukunishi, M. Matsumoto, M. Hirata-Koizumi, A. Hirose and T. Ihara (2009). "Developmental toxicity of dibutyltin dichloride given on three consecutive days during organogenesis in cynomolgus monkeys." Drug Chem Toxicol **32**(2): 150-157.

Ema, M., T. Itami and H. Kawasaki (1992). "Susceptible period for the teratogenicity of di-n-butyltin dichloride in rats." Toxicology **73**(1): 81-92.

EPA. (2003). "Table of Polychlorinated Biphenyl (PCB) Congeners." Retrieved 12 November, 2019, from https://www.epa.gov/pcbs/table-polychlorinated-biphenyl-pcb-congeners.

EPA. (2016). "Health Effects Support Document for PFOA." from https://www.epa.gov/sites/production/files/2016-05/documents/pfoa_hesd_final_508.pdf.

EPA. (2016b). "Health Effects Support Document for PFOS." Retrieved 13 December 2019, from https://www.epa.gov/sites/production/files/2016-05/documents/pfos_hesd_final_508.pdf.

EPA. (2017). "Exposure Levels for Evaluating Polychlorinated Biphenyls (PCBs) in Indoor School Air." Polychlorinated Biphenyls (PCBs) Retrieved 11/12/2019, from https://www.epa.gov/pcbs/exposure-levels-evaluating-polychlorinated-biphenyls-pcbs-indoor-school-air.

EPA. (2019). "TSCA Chemical Substance Inventory." Retrieved 11/6/2019, 2019, from https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory.

Ewing, G., Y. Tatarchuk, D. Appleby, N. Schwartz and D. Kim (2015). "Placental transfer of antidepressant medications: implications for postnatal adaptation syndrome." Clin Pharmacokinet **54**(4): 359-370.

Farhang, L., J. M. Weintraub, M. Petreas, B. Eskenazi and R. Bhatia (2005). "Association of DDT and DDE with birth weight and length of gestation in the Child Health and Development Studies, 1959-1967." Am J Epidemiol **162**(8): 717-725.

Feng, Y., P. Zhang, Z. Zhang, J. Shi, Z. Jiao and B. Shao (2016). "Endocrine Disrupting Effects of Triclosan on the Placenta in Pregnant Rats." PLoS One **11**(5): e0154758.

Filgo, A. J., E. M. Quist, M. J. Hoenerhoff, A. E. Brix, G. E. Kissling and S. E. Fenton (2015). "Perfluorooctanoic Acid (PFOA)-induced Liver Lesions in Two Strains of Mice Following Developmental Exposures: PPARalpha Is Not Required." Toxicol Pathol **43**(4): 558-568.

Fisher, M., T. E. Arbuckle, C. L. Liang, A. LeBlanc, E. Gaudreau, W. G. Foster, D. Haines, K. Davis and W. D. Fraser (2016). "Concentrations of persistent organic pollutants in maternal and cord blood from the maternal-infant research on environmental chemicals (MIREC) cohort study." Environ Health **15**(1): 59.

Fowden, A. L., A. J. Forhead, A. N. Sferruzzi-Perri, G. J. Burton and O. R. Vaughan (2015). "Review: Endocrine regulation of placental phenotype." Placenta **36 Suppl 1**: S50-59.

Frederiksen, H., C. Taxvig, U. Hass, A. M. Vinggaard and C. Nellemann (2008). "Higher levels of ethyl paraben and butyl paraben in rat amniotic fluid than in maternal plasma after subcutaneous administration." Toxicol Sci **106**(2): 376-383.

Frederiksen, M., C. Thomsen, M. Froshaug, K. Vorkamp, M. Thomsen, G. Becher and L. E. Knudsen (2010). "Polybrominated diphenyl ethers in paired samples of maternal and umbilical cord blood plasma and associations with house dust in a Danish cohort." Int J Hyg Environ Health **213**(4): 233-242.

Frederiksen, M., K. Vorkamp, L. Mathiesen, T. Mose and L. E. Knudsen (2010b). "Placental transfer of the polybrominated diphenyl ethers BDE-47, BDE-99 and BDE-209 in a human placenta perfusion system: an experimental study." Environ Health **9**: 32.

Fry, R. C., J. Bangma, J. Szilagyi and J. E. Rager (2019). "Developing novel in vitro methods for the risk assessment of developmental and placental toxicants in the environment." Toxicol Appl Pharmacol **378**: 114635.

Furger, C., L. Cronier, C. Poirot and M. Pouchelet (1996). "Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions." Mol Hum Reprod **2**(8): 541-548.

Furukawa, S., N. Tsuji, Y. Kobayashi, Y. Yamagishi, S. Hayashi, M. Abe, Y. Kuroda, M. Kimura, C. Hayakawa and A. Sugiyama (2017). "Effect of dibutyltin on placental and fetal toxicity in rat." J Toxicol Sci **42**(6): 741-753.

Fylling, P., O. V. Sjaastad and W. Velle (1973). "Mid-Term Abortion Induced in Sheep by Synthetic Corticoids." Journal of Reproduction and Fertility **32**(2): 305-306.

Gabory, A., T. J. Roseboom, T. Moore, L. G. Moore and C. Junien (2013). "Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics." Biol Sex Differ **4**(1): 5.

Gayrard, V., M. Z. Lacroix, S. H. Collet, C. Viguie, A. Bousquet-Melou, P. L. Toutain and N. Picard-Hagen (2013). "High bioavailability of bisphenol A from sublingual exposure." Environ Health Perspect **121**(8): 951-956.

Genetos, D. C., Z. Zhou, Z. Li and H. J. Donahue (2012). "Age-related changes in gap junctional intercellular communication in osteoblastic cells." J Orthop Res **30**(12): 1979-1984.

Geng, H. X. and L. Wang (2019). "Cadmium: Toxic effects on placental and embryonic development." Environ Toxicol Pharmacol **67**: 102-107.

Gerona, R., F. S. Vom Saal and P. A. Hunt (2019). "BPA: have flawed analytical techniques compromised risk assessments?" Lancet Diabetes Endocrinol.

Gershon, E., V. Plaks and N. Dekel (2008). "Gap junctions in the ovary: expression, localization and function." Mol Cell Endocrinol **282**(1-2): 18-25.

Geyer, R., J. R. Jambeck and K. L. Law (2017). "Production, use, and fate of all plastics ever made." Science Advances **3**(7).

Gingrich, J., Y. Pu, J. Roberts, R. Karthikraj, K. Kannan, R. Ehrhardt and A. Veiga-Lopez (2018). "Gestational bisphenol S impairs placental endocrine function and the fusogenic trophoblast signaling pathway." Arch Toxicol **92**(5): 1861-1876.

Gingrich, J., Y. Pu, R. Ehrhardt, R. Karthikraj, K. Kannan and A. Veiga-Lopez (2019). "Toxicokinetics of bisphenol A, bisphenol S, and bisphenol F in a pregnancy sheep model." Chemosphere **220**: 185-194.

Gingrich, J., E. Ticiani and A. Veiga-Lopez (2020). "Placenta disrupted: endocrine disrupting chemicals and pregnancy." Trends Endocrinol Metab doi: 10.1016/j.tem.2020.03.003. [Epub ahead of print]

Gingrich, J., Y. Pu, B.L. Upham, M. Hulse, S. Pearl, D. Martin, A. Avery and A. Veiga-Lopez (2020b). "Bisphenol S enhances gap junction intercellular communication in ovarian theca cells." Chemosphere [*in review*]

Ginsberg, G. and D. C. Rice (2009). "Does Rapid Metabolism Ensure Negligible Risk from Bisphenol A?" Environmental Health Perspectives **117**(11): 1639-1643.

Gore, A. C., D. Crews, L. L. Doan, M. La Merrill, H. B. Patisaul and A. Zota (2014). Introduction to endocrine disrupting chemicals (EDCs) a guide for public interest organizations and policy-makers. Endocrine Society**:** 1-69.

Gore, A. C., V. A. Chappell, S. E. Fenton, J. A. Flaws, A. Nadal, G. S. Prins, J. Toppari and R. T. Zoeller (2015). "Executive Summary to EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals." Endocr Rev **36**(6): 593-602.

Gorrochategui, E., E. Perez-Albaladejo, J. Casas, S. Lacorte and C. Porte (2014). "Perfluorinated chemicals: differential toxicity, inhibition of aromatase activity and alteration of cellular lipids in human placental cells." Toxicol Appl Pharmacol **277**(2): 124-130.

Gorrochategui, E., J. Casas, E. Perez-Albaladejo, O. Jauregui, C. Porte and S. Lacorte (2014). "Characterization of complex lipid mixtures in contaminant exposed JEG-3 cells using liquid chromatography and high-resolution mass spectrometry." Environ Sci Pollut Res Int **21**(20): 11907-11916.

Grandin, F. C., M. Z. Lacroix, V. Gayrard, G. Gauderat, H. Mila, P. L. Toutain and N. Picard-Hagen (2018). "Bisphenol S instead of Bisphenol A: Toxicokinetic invstigations in the ovine materno-feto-placental unit." Environ Int **120**: 584-592.

Granot, I. and N. Dekel (2002). "The ovarian gap junction protein connexin43: regulation by gonadotropins." Trends Endocrinol Metab **13**(7): 310-313.

Gregoraszczuk, E., M. Slomczynska and S. Stoklosowa (1999). "Effect of genistein, tyrphostin and herbimycin on prolactin-stimulated progesterone production by porcine theca and luteal cells." J Physiol Pharmacol **50**(3): 477-484.

Griffiths, S. K. and J. P. Campbell (2015). "Placental structure, function and drug transfer." Bja Education **15**(2): 84-89.

Grignard, E., S. Lapenna and S. Bremer (2012). "Weak estrogenic transcriptional activities of Bisphenol A and Bisphenol S." Toxicol In Vitro **26**(5): 727-731.

Grigsby, P. L. (2016). "Animal Models to Study Placental Development and Function throughout Normal and Dysfunctional Human Pregnancy." Semin Reprod Med **34**(1): 11-16.

Grindler, N. M., L. Vanderlinden, R. Karthikraj, K. Kannan, S. Teal, A. J. Polotsky, T. L. Powell, I. V. Yang and T. Jansson (2018). "Exposure to Phthalate, an Endocrine Disrupting Chemical, Alters the First Trimester Placental Methylome and Transcriptome in Women." Sci Rep **8**(1): 6086.

Grochowalski, A., R. Chrzaszcz, R. Pieklo and E. L. Gregoraszczuk (2001). "Estrogenic and antiestrogenic effect of in vitro treatment of follicular cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin." Chemosphere **43**(4-7): 823-827.

Grube, A., D. Donaldson, T. Kiely and L. Wu (2011). Pesticides Industry Sales and Usage, 2006 and 2007 Market Estimates. EPA.

Grun, F. (2014). "The obesogen tributyltin." Vitam Horm **94**: 277-325.

Gu, P. Q., L. J. Gao, L. Li, Z. Liu, F. Q. Luan, Y. Z. Peng and X. R. Guo (2012). "Endocrine disruptors, polychlorinated biphenyls-induced gC1qR-dependent apoptosis in human trophoblast cell line HTR-8/SVneo." Reprod Sci **19**(2): 181-189.

Guinazu, N., V. Rena, S. Genti-Raimondi, V. Rivero and G. Magnarelli (2012). "Effects of the organophosphate insecticides phosmet and chlorpyrifos on trophoblast JEG-3 cell death, proliferation and inflammatory molecule production." Toxicol In Vitro **26**(3): 406-413.

Guller, S. (2009). "Role of the syncytium in placenta-mediated complications of preeclampsia." Thromb Res **124**(4): 389-392.

Halla, N., I. P. Fernandes, S. A. Heleno, P. Costa, Z. Boucherit-Otmani, K. Boucherit, A. E. Rodrigues, I. Ferreira and M. F. Barreiro (2018). "Cosmetics Preservation: A Review on Present Strategies." Molecules **23**(7).

Hanna, E. A., S. Umhauer, S. L. Roshong, M. P. Piechocki, M. J. Fernstrom, J. D. Fanning and R. J. Ruch (1999). "Gap junctional intercellular communication and connexin43 expression in human ovarian surface epithelial cells and ovarian carcinomas in vivo and in vitro." Carcinogenesis **20**(7): 1369-1373.

Harada, T., M. Takeda, S. Kojima and N. Tomiyama (2016). "Toxicity and Carcinogenicity of Dichlorodiphenyltrichloroethane (DDT)." Toxicol Res **32**(1): 21-33.

Harrison, F. A. and R. B. Heap (1978). "Progesterone secretion during pregnancy in sheep with an autotransplanted adrenal and an autotransplanted ovary." J Reprod Fertil **54**(1): 153-157.

Hasler, C. M., J. E. Trosko and M. R. Bennink (1991). "Incorporation of n-3 fatty acids into WB-F344 cell phospholipids inhibits gap junctional intercellular communication." Lipids **26**(10): 788-792.

Hassanzadeh, N. (2017). "Histopathological evaluation of Zebrafish (Danio rerio) testes following exposure to methyl paraben." International Journal of Aquatic Biology **5**: 71-78.

Hatzirodos, N., K. Hummitzsch, H. F. Irving-Rodgers and R. J. Rodgers (2015). "Transcriptome comparisons identify new cell markers for theca interna and granulosa cells from small and large antral ovarian follicles." PLoS One **10**(3): e0119800.

Heindel, J. J., J. Balbus, L. Birnbaum, M. N. Brune-Drisse, P. Grandjean, K. Gray, P. J. Landrigan, P. D. Sly, W. Suk, D. Cory Slechta, C. Thompson and M. Hanson (2015). "Developmental Origins of Health and Disease: Integrating Environmental Influences." Endocrinology **156**(10): 3416-3421.

Hiromori, Y., H. Yui, J. Nishikawa, H. Nagase and T. Nakanishi (2016). "Organotin compounds cause structure-dependent induction of progesterone in human choriocarcinoma Jar cells." J Steroid Biochem Mol Biol **155**(Pt B): 190-198.

Hogberg, J., A. Hanberg, M. Berglund, S. Skerfving, M. Remberger, A. M. Calafat, A. F. Filipsson, B. Jansson, N. Johansson, M. Appelgren and H. Hakansson (2008). "Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations." Environ Health Perspect **116**(3): 334-339.

Hossain, M. Z., P. Ao and A. L. Boynton (1998). "Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinase C and mitogen-activated protein kinase." J Cell Physiol **176**(2): 332-341.

Hu, W., P. D. Jones, B. L. Upham, J. E. Trosko, C. Lau and J. P. Giesy (2002). "Inhibition of gap junctional intercellular communication by perfluorinated compounds in rat liver and dolphin kidney epithelial cell lines in vitro and Sprague-Dawley rats in vivo." Toxicol Sci **68**(2): 429-436.

Huang, H. and L. K. Leung (2009). "Bisphenol A downregulates CYP19 transcription in JEG-3 cells." Toxicol Lett **189**(3): 248-252.

Huang, Q., H. Chen, J. Li, M. Oliver, X. Ma, D. Byck, Y. Gao and S. W. Jiang (2014). "Epigenetic and non-epigenetic regulation of syncytin-1 expression in human placenta and cancer tissues." Cell Signal **26**(3): 648-656.

Huang, R., Q. Chen, L. Zhang, K. Luo, L. Chen, S. Zhao, L. Feng and J. Zhang (2019). "Prenatal exposure to perfluoroalkyl and polyfluoroalkyl substances and the risk of hypertensive disorders of pregnancy." Environ Health **18**(1): 5.

Hughes, M., N. Dobric, I. C. Scott, L. Su, M. Starovic, B. St-Pierre, S. E. Egan, J. C. Kingdom and J. C. Cross (2004). "The Hand1, Stra13 and Gcm1 transcription factors override FGF signaling to promote terminal differentiation of trophoblast stem cells." Dev Biol **271**(1): 26-37.

Hurst, C. H. and D. J. Waxman (2003). "Activation of PPARalpha and PPARgamma by environmental phthalate monoesters." Toxicol Sci **74**(2): 297-308.

Huuskonen, P., S. Auriola and M. Pasanen (2015). "Zearalenone metabolism in human placental subcellular organelles, JEG-3 cells, and recombinant CYP19A1." Placenta **36**(9): 1052-1055.

Hyttel, P. (2010). Essentials of domestic animal embryology. Edinburgh ; New York, Saunders/Elsevier.

IARC (2016). "Polychlorinated Biphenyls and Polybrominated Biphenyls." IARC Monogr Eval Carcinog Risks Hum **107**: 9-500.

Igwebuike, U. M. (2006). "Trophoblast cells of ruminant placentas--A minireview." Anim Reprod Sci **93**(3-4): 185-198.

Incebiyik, A., S. Kocarslan, A. Camuzcuoglu, N. G. Hilali, H. Incebiyik and H. Camuzcuoglu (2016). "Trophoblastic E-cadherin and TGF-beta expression in placenta percreta and normal pregnancies." J Matern Fetal Neonatal Med **29**(1): 126-129.

Ivry Del Moral, L., L. Le Corre, H. Poirier, I. Niot, T. Truntzer, J. F. Merlin, P. Rouimi, P. Besnard, R. Rahmani and M. C. Chagnon (2016). "Obesogen effects after perinatal exposure of 4,4'-sulfonyldiphenol (Bisphenol S) in C57BL/6 mice." Toxicology **357-358**: 11-20.

James, J. L., P. R. Stone and L. W. Chamley (2005). "Cytotrophoblast differentiation in the first trimester of pregnancy: evidence for separate progenitors of extravillous trophoblasts and syncytiotrophoblast." Reproduction **130**(1): 95-103.

James-Todd, T., R. Stahlhut, J. D. Meeker, S. G. Powell, R. Hauser, T. Huang and J. Rich-Edwards (2012). "Urinary phthalate metabolite concentrations and diabetes among women in the National Health and Nutrition Examination Survey (NHANES) 2001-2008." Environ Health Perspect **120**(9): 1307-1313.

Ji, K., S. Hong, Y. Kho and K. Choi (2013). "Effects of bisphenol s exposure on endocrine functions and reproduction of zebrafish." Environ Sci Technol **47**(15): 8793-8800.

Jin, H. and K. L. Audus (2005). "Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line." Placenta **26 Suppl A**: S96-S103.

Johns, L. E., K. K. Ferguson, D. E. Cantonwine, T. F. McElrath, B. Mukherjee and J. D. Meeker (2017). "Urinary BPA and Phthalate Metabolite Concentrations and Plasma Vitamin D Levels in Pregnant Women: A Repeated Measures Analysis." Environ Health Perspect **125**(8): 087026.

Johnson, M. L., D. A. Redmer, L. P. Reynolds, J. J. Bilski and A. T. Grazul-Bilska (2002). "Gap junctional intercellular communication of bovine granulosa and thecal cells from antral follicles: effects of luteinizing hormone and follicle-stimulating hormone." Endocrine **18**(3): 261-270.

Juneja, S. C., K. J. Barr, G. C. Enders and G. M. Kidder (1999). "Defects in the germ line and gonads of mice lacking connexin43." Biol Reprod **60**(5): 1263-1270.

Kahn, L. G. and L. Trasande (2018). "Environmental Toxicant Exposure and Hypertensive Disorders of Pregnancy: Recent Findings." Curr Hypertens Rep **20**(10): 87.

Kalkunte, S., Z. Huang, E. Lippe, S. Kumar, L. W. Robertson and S. Sharma (2017). "Polychlorinated biphenyls target Notch/Dll and VEGF R2 in the mouse placenta and human trophoblast cell lines for their anti-angiogenic effects." Sci Rep **7**: 39885.

Kanemitsu, M. Y. and A. F. Lau (1993). "Epidermal growth factor stimulates the disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase." Mol Biol Cell **4**(8): 837-848.

Kang, K. S., M. R. Wilson, T. Hayashi, C. C. Chang and J. E. Trosko (1996). "Inhibition of gap junctional intercellular communication in normal human breast epithelial cells after treatment with pesticides, PCBs, and PBBs, alone or in mixtures." Environ Health Perspect **104**(2): 192-200.

Karol, M. D. (1990). "Mean residence time and the meaning of AUMC/AUC." Biopharm Drug Dispos **11**(2): 179-181.

Karrer, C., T. Roiss, N. von Goetz, D. Gramec Skledar, L. Peterlin Masic and K. Hungerbuhler (2018). "Physiologically Based Pharmacokinetic (PBPK) Modeling of the Bisphenols BPA, BPS, BPF, and BPAF with New Experimental Metabolic Parameters: Comparing the Pharmacokinetic Behavior of BPA with Its Substitutes." Environ Health Perspect **126**(7): 077002.

Kasa-Vubu, J. Z., G. E. Dahl, N. P. Evans, L. A. Thrun, S. M. Moenter, V. Padmanabhan and F. J. Karsch (1992). "Progesterone blocks the estradiol-induced gonadotropin discharge in the ewe by inhibiting the surge of gonadotropin-releasing hormone." Endocrinology **131**(1): 208-212.

Kashif, M., A. Hellwig, A. Kolleker, K. Shahzad, H. Wang, S. Lang, J. Wolter, M. Thati, I. Vinnikov, A. Bierhaus, P. P. Nawroth and B. Isermann (2011). "p45NF-E2 represses Gcm1 in trophoblast cells to regulate syncytium formation, placental vascularization and embryonic growth." Development **138**(11): 2235-2247.

Kawamoto, Y., W. Matsuyama, M. Wada, J. Hishikawa, M.P. Chan, A. Nakayama and S. Morisawa (2007). "Development of a physiologically based pharmacokinetic model for bisphenol A in pregnant mice." Toxicol Appl Pharmacol **224**(2): 182-191.

Ke, A. B., R. Greupink and K. Abduljalil (2018). "Drug Dosing in Pregnant Women: Challenges and Opportunities in Using Physiologically Based Pharmacokinetic Modeling and Simulations." CPT Pharmacometrics Syst Pharmacol **7**(2): 103-110.

Kelland, K. (2010). "Experts demand European action on plastics chemical." Retrieved 5 December 2019, from https://www.reuters.com/article/us-chemical-bpa-health/experts-demand-european-action-on-plastics-chemical-idUSTRE65L6JN20100622.

Kendall, N. R., P. Marsters, L. Guo, R. J. Scaramuzzi and B. K. Campbell (2006). "Effect of copper and thiomolybdates on bovine theca cell differentiation in vitro." J Endocrinol **189**(3): 455-463.

Kezios, K. L., X. Liu, P. M. Cirillo, B. A. Cohn, O. I. Kalantzi, Y. Wang, M. X. Petreas, J. S. Park and P. Factor-Litvak (2013). "Dichlorodiphenyltrichloroethane (DDT), DDT metabolites and pregnancy outcomes." Reprod Toxicol **35**: 156-164.

Kidder, G. M. and A. A. Mhawi (2002). "Gap junctions and ovarian folliculogenesis." Reproduction **123**(5): 613-620.

Kidder, G. M. and B. C. Vanderhyden (2010). "Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence." Can J Physiol Pharmacol **88**(4): 399-413.

Kilkenny, C., W. J. Browne, I. C. Cuthill, M. Emerson and D. G. Altman (2010). "Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research." PLoS Biol **8**(6): e1000412.

Kim, S., Y. H. Cho, I. Lee, W. Kim, S. Won, J. L. Ku, H. B. Moon, J. Park, S. Kim, G. Choi and K. Choi (2018). "Prenatal exposure to persistent organic pollutants and methylation of LINE-1 and imprinted genes in placenta: A CHECK cohort study." Environ Int **119**: 398-406.

Kim, Y. S., K. A. Hwang, S. H. Hyun, K. H. Nam, C. K. Lee and K. C. Choi (2015). "Bisphenol A and nonylphenol have the potential to stimulate the migration of ovarian cancer cells by inducing epithelial-mesenchymal transition via an estrogen receptor dependent pathway." Chem Res Toxicol **28**(4): 662-671.

Kinch, C. D., K. Ibhazehiebo, J. H. Jeong, H. R. Habibi and D. M. Kurrasch (2015). "Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish." Proc Natl Acad Sci U S A **112**(5): 1475-1480.

Klaunig, J. E., R. J. Ruch and C. M. Weghorst (1990). "Comparative effects of phenobarbital, DDT, and lindane on mouse hepatocyte gap junctional intercellular communication." Toxicol Appl Pharmacol **102**(3): 553-563.

Koch, H. M., H. Drexler and J. Angerer (2003). "An estimation of the daily intake of di(2-ethylhexyl)phthalate (DEHP) and other phthalates in the general population." Int J Hyg Environ Health **206**(2): 77-83.

Kohan-Ghadr, H. R., G. Fecteau, L. C. Smith, B. D. Murphy and R. C. Lefebvre (2011). "Endocrine profiles of somatic nuclear transfer-derived pregnancies in dairy cattle." Theriogenology **76**(5): 911-920.

Kokkinos, M. I., P. Murthi, R. Wafai, E. W. Thompson and D. F. Newgreen (2010). "Cadherins in the human placenta--epithelial-mesenchymal transition (EMT) and placental development." Placenta **31**(9): 747-755.

Kolatorova, L., J. Vitku, R. Hampl, K. Adamcova, T. Skodova, M. Simkova, A. Parizek, L. Starka and M. Duskova (2018). "Exposure to bisphenols and parabens during pregnancy and relations to steroid changes." Environ Res **163**: 115-122.

Kolla, S., M. Morcos, B. Martin and L. N. Vandenberg (2018). "Low dose bisphenol S or ethinyl estradiol exposures during the perinatal period alter female mouse mammary gland development." Reprod Toxicol **78**: 50-59.

Konieczna, A., A. Rutkowska and D. Rachon (2015). "Health risk of exposure to Bisphenol A (BPA)." Rocz Panstw Zakl Hig **66**(1): 5-11.

Koshi, K., Y. Suzuki, Y. Nakaya, K. Imai, M. Hosoe, T. Takahashi, K. Kizaki, T. Miyazawa and K. Hashizume (2012). "Bovine trophoblastic cell differentiation and binucleation involves enhanced endogenous retrovirus element expression." Reprod Biol Endocrinol **10**: 41.

Krishna, R., K. W. Riggs, E. Kwan, H. Wong, A. Szeitz, M. P. Walker and D. W. Rurak (2002). "Clearance and disposition of indometacin in chronically instrumented fetal lambs following a 3-day continuous intravenous infusion." J Pharm Pharmacol **54**(6): 801-808.

Kumar, P. and N. Magon (2012). "Hormones in pregnancy." Niger Med J **53**(4): 179-183.

Kummu, M., E. Sieppi, J. Koponen, L. Laatio, K. Vahakangas, H. Kiviranta, A. Rautio and P. Myllynen (2015). "Organic anion transporter 4 (OAT 4) modifies placental transfer of perfluorinated alkyl acids PFOS and PFOA in human placental ex vivo perfusion system." Placenta **36**(10): 1185-1191.

Kurtenbach, S., S. Kurtenbach and G. Zoidl (2014). "Gap junction modulation and its implications for heart function." Front Physiol **5**: 82.

Kwak, Y. T., S. Muralimanoharan, A. A. Gogate and C. R. Mendelson (2019). "Human Trophoblast Differentiation Is Associated With Profound Gene Regulatory and Epigenetic Changes." Endocrinology **160**(9): 2189-2203.

Lan, X., L. J. Fu, J. Zhang, X. Q. Liu, H. J. Zhang, X. Zhang, M. F. Ma, X. M. Chen, J. L. He, L. B. Li, Y. X. Wang and Y. B. Ding (2017). "Bisphenol A exposure promotes HTR-8/SVneo cell migration and

impairs mouse placentation involving upregulation of integrin-beta1 and MMP-9 and stimulation of MAPK and PI3K signaling pathways." Oncotarget **8**(31): 51507-51521.

LaPlante, C. D., M. C. Catanese, R. Ruby Bansal and L. N. Vandenberg (2017). " Bisphenol S alters the lactating mammary gland and nursing behaviors in mice exposed during pregnancy and lactation " Endocrinology **en.2017-00437**.

Lee, B. K., Y. J. Jang, M. Kim, L. LeBlanc, C. Rhee, J. Lee, S. Beck, W. Shen and J. Kim (2019). "Super-enhancer-guided mapping of regulatory networks controlling mouse trophoblast stem cells." Nat Commun **10**(1): 4749.

Lee, C. K., S. G. Kang, J. T. Lee, S. W. Lee, J. H. Kim, D. H. Kim, B. C. Son, K. H. Kim, C. H. Suh, S. Y. Kim and Y. B. Park (2015). "Effects of perfluorooctane sulfuric acid on placental PRL-family hormone production and fetal growth retardation in mice." Mol Cell Endocrinol **401**: 165-172.

Lee, G. H., B. Jang, H. S. Choi, H. J. Kim, J. H. Park, Y. C. Jeon, R. I. Carp, Y. S. Kim and E. K. Choi (2016). "Upregulation of Connexin 43 Expression Via C-Jun N-Terminal Kinase Signaling in Prion Disease." J Alzheimers Dis **49**(4): 1005-1019.

Lee, S. G., J. Y. Kim, J. Y. Chung, Y. J. Kim, J. E. Park, S. Oh, Y. D. Yoon, K. S. Yoo, Y. H. Yoo and J. M. Kim (2013). "Bisphenol A exposure during adulthood causes augmentation of follicular atresia and luteal regression by decreasing 17beta-estradiol synthesis via downregulation of aromatase in rat ovary." Environ Health Perspect **121**(6): 663-669.

Lehmler, H. J., B. Liu, M. Gadogbe and W. Bao (2018). "Exposure to Bisphenol A, Bisphenol F, and Bisphenol S in U.S. Adults and Children: The National Health and Nutrition Examination Survey 2013-2014." ACS Omega **3**(6): 6523-6532.

Leithe, E. and E. Rivedal (2004). "Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment." J Biol Chem **279**(48): 50089-50096.

Leonetti, C., C. M. Butt, K. Hoffman, M. L. Miranda and H. M. Stapleton (2016). "Concentrations of polybrominated diphenyl ethers (PBDEs) and 2,4,6-tribromophenol in human placental tissues." Environ Int **88**: 23-29.

Leonetti, C., C. M. Butt, K. Hoffman, S. C. Hammel, M. L. Miranda and H. M. Stapleton (2016b). "Brominated flame retardants in placental tissues: associations with infant sex and thyroid hormone endpoints." Environ Health **15**(1): 113.

Levario-Carrillo, M., M. E. Olave, D. C. Corral, J. G. Alderete, S. M. Gagioti and E. Bevilacqua (2004). "Placental morphology of rats prenatally exposed to methyl parathion." Exp Toxicol Pathol **55**(6): 489-496.

Li, L. X., L. Chen, X. Z. Meng, B. H. Chen, S. Q. Chen, Y. Zhao, L. F. Zhao, Y. Liang and Y. H. Zhang (2013). "Exposure levels of environmental endocrine disruptors in mother-newborn pairs in China and their placental transfer characteristics." PLoS One **8**(5): e62526.

Li, Q., M. A. Kappil, A. Li, P. S. Dassanayake, T. H. Darrah, A. E. Friedman, M. Friedman, L. Lambertini, P. Landrigan, C. J. Stodgell, Y. Xia, J. A. Nanes, K. M. Aagaard, E. E. Schadt, J. C. Murray, E. B. Clark, N. Dole, J. Culhane, J. Swanson, M. Varner, J. Moye, C. Kasten, R. K. Miller and J. Chen (2015). "Exploring the associations between microRNA expression profiles and environmental pollutants in human placenta from the National Children's Study (NCS)." Epigenetics **10**(9): 793-802.

Li, X., L. Ye, Y. Ge, K. Yuan, Y. Zhang, Y. Liang, J. Wei, C. Zhao, Q. Q. Lian, X. Zhu and R. S. Ge (2016). "In utero perfluorooctane sulfonate exposure causes low body weights of fetal rats: A mechanism study." Placenta **39**: 125-133.

Li, Y., T. Fletcher, D. Mucs, K. Scott, C. H. Lindh, P. Tallving and K. Jakobsson (2018). "Half-lives of PFOS, PFHxS and PFOA after end of exposure to contaminated drinking water." Occup Environ Med **75**(1): 46-51.

Li, Z. M., D. Hernandez-Moreno, K. M. Main, N. E. Skakkebaek, H. Kiviranta, J. Toppari, U. Feldt-Rasmussen, H. Shen, K. W. Schramm and M. De Angelis (2018). "Association of In Utero Persistent Organic Pollutant Exposure With Placental Thyroid Hormones." Endocrinology **159**(10): 3473-3481.

Liao, C. and K. Kannan (2013). "Concentrations and profiles of bisphenol A and other bisphenol analogues in foodstuffs from the United States and their implications for human exposure." J Agric Food Chem **61**(19): 4655-4662.

Liao, C. and K. Kannan (2014). "A survey of bisphenol A and other bisphenol analogues in foodstuffs from nine cities in China." Food Addit Contam Part A Chem Anal Control Expo Risk Assess **31**(2): 319-329.

Liao, C., F. Liu, H. Alomirah, V. D. Loi, M. A. Mohd, H. B. Moon, H. Nakata and K. Kannan (2012). "Bisphenol S in urine from the United States and seven Asian countries: occurrence and human exposures." Environ Sci Technol **46**(12): 6860-6866.

Liao, C., F. Liu, Y. Guo, H. B. Moon, H. Nakata, Q. Wu and K. Kannan (2012b). "Occurrence of eight bisphenol analogues in indoor dust from the United States and several Asian countries: implications for human exposure." Environ Sci Technol **46**(16): 9138-9145.

Lim, T. C., B. Wang, J. Huang, S. Deng and G. Yu (2011). "Emission inventory for PFOS in China: review of past methodologies and suggestions." ScientificWorldJournal **11**: 1963-1980.

Lin, D., J. Zhou, P. S. Zelenka and D. J. Takemoto (2003). "Protein kinase Cgamma regulation of gap junction activity through caveolin-1-containing lipid rafts." Invest Ophthalmol Vis Sci **44**(12): 5259-5268.

Liu, J. Y., J. G. Li, Y. N. Wu, Y. F. Zhao, F. J. Luo, S. M. Li, L. Yang, E. K. Moez, I. Dinu and J. W. Martin (2017). "Bisphenol A Metabolites and Bisphenol S in Paired Maternal and Cord Serum." Environmental Science & Technology **51**(4): 2456-2463.

Liu, J., J. Li, Y. Liu, H. M. Chan, Y. Zhao, Z. Cai and Y. Wu (2011). "Comparison on gestation and lactation exposure of perfluorinated compounds for newborns." Environ Int **37**(7): 1206-1212.

Liu, S., J. Wang, H. Zou and X. Huang (2013). "Effect of apigenin on gap junctional intercellular communication in human Tenon's capsule fibroblasts." Eye Sci **28**(2): 62-67.

Long, A. C., C. M. Colitz and J. A. Bomser (2007). "Regulation of gap junction intercellular communication in primary canine lens epithelial cells: role of protein kinase C." Curr Eye Res **32**(3): 223-231.

Longnecker, M. P., W. J. Rogan and G. Lucier (1997). "The human health effects of DDT (dichlorodiphenyltrichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health." Annu Rev Public Health **18**: 211-244.

Lopez-Arellano, P., K. Lopez-Arellano, J. Luna, D. Flores, J. Jimenez-Salazar, G. Gavia, M. Teteltitla, J. J. Rodriguez, A. Dominguez, E. Casas, I. Bahena, M. Betancourt, C. Gonzalez, Y. Ducolomb and E. Bonilla (2019). "Perfluorooctanoic acid disrupts gap junction intercellular communication and induces reactive oxygen species formation and apoptosis in mouse ovaries." Environ Toxicol **34**(1): 92-98.

Luo, H., C. Li, H. Fang and X. Wang (2016). "Comparative study of the interactions between bisphenol analogues and serum albumins by electrospray mass spectrometry and fluorescence spectroscopy." Rapid Commun Mass Spectrom **30 Suppl 1**: 162-167.

Machala, M., L. Blaha, J. Vondracek, J. E. Trosko, J. Scott and B. L. Upham (2003). "Inhibition of gap junctional intercellular communication by noncoplanar polychlorinated biphenyls: inhibitory potencies and screening for potential mode(s) of action." Toxicol Sci **76**(1): 102-111.

Machtinger, R., J. Zhong, A. Mansur, M. Adir, C. Racowsky, R. Hauser, K. Brennan, O. Karlsson and A. A. Baccarelli (2018). "Placental lncRNA Expression Is Associated With Prenatal Phthalate Exposure." Toxicol Sci **163**(1): 116-122.

Magata, F., M. Horiuchi, A. Miyamoto and T. Shimizu (2014). "Lipopolysaccharide (LPS) inhibits steroid production in theca cells of bovine follicles in vitro: distinct effect of LPS on theca cell function in pre- and post-selection follicles." J Reprod Dev **60**(4): 280-287.

Majewska, M., A. Lipka, G. Panasiewicz, M. Gowkielewicz, M. Jozwik, M. K. Majewski and B. Szafranska (2017). "Identification of Novel Placentally Expressed Aspartic Proteinase in Humans." Int J Mol Sci **18**(6).

Marciniak, A., J. Patro-Malysza, Z. Kimber-Trojnar, B. Marciniak, J. Oleszczuk and B. Leszczynska-Gorzelak (2017). "Fetal programming of the metabolic syndrome." Taiwan J Obstet Gynecol **56**(2): 133-138.

Marqueno, A., E. Perez-Albaladejo, C. Flores, E. Moyano and C. Porte (2019). "Toxic effects of bisphenol A diglycidyl ether and derivatives in human placental cells." Environ Pollut **244**: 513-521.

Marsee, K., T. J. Woodruff, D. A. Axelrad, A. M. Calafat and S. H. Swan (2006). "Estimated daily phthalate exposures in a population of mothers of male infants exhibiting reduced anogenital distance." Environ Health Perspect **114**(6): 805-809.

Marsit, C. J. (2016). "Placental Epigenetics in Children's Environmental Health." Semin Reprod Med **34**(1): 36-41.

Masten, S. J., M. Tian, B. L. Upham and J. E. Trosko (2001). "Effect of selected pesticides and their ozonation by-products on gap junctional intercellular communication using rat liver epithelial cell lines." Chemosphere **44**(3): 457-465.

Mehlmann, L. M. (2005). "Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation." Reproduction **130**(6): 791-799.

Merida-Ortega, A., S. J. Rothenberg, L. Torres-Sanchez, L. Schnaas, C. Hernandez-Alcaraz, M. E. Cebrian, R. M. Garcia-Hernandez, R. Ogaz-Gonzalez and L. Lopez-Carrillo (2019). "Polyunsaturated fatty acids and child neurodevelopment among a population exposed to DDT: a cohort study." Environ Health **18**(1): 17.

Mersha, M. D., B. M. Patel, D. Patel, B. N. Richardson and H. S. Dhillon (2015). "Effects of BPA and BPS exposure limited to early embryogenesis persist to impair non-associative learning in adults." Behav Brain Funct **11**: 27.

Meruvu, S., J. Zhang and M. Choudhury (2016). "Mono-(2-ethylhexyl) Phthalate Increases Oxidative Stress Responsive miRNAs in First Trimester Placental Cell Line HTR8/SVneo." Chem Res Toxicol **29**(3): 430-435.

Meruvu, S., J. Zhang, Y. S. Bedi and M. Choudhury (2016b). "Mono-(2-ethylhexyl) phthalate induces apoptosis through miR-16 in human first trimester placental cell line HTR-8/SVneo." Toxicol In Vitro **31**: 35-42.

Mese, G., G. Richard and T. W. White (2007). "Gap junctions: basic structure and function." J Invest Dermatol **127**(11): 2516-2524.

Metz, C. M. (2016). "Bisphenol A: Understanding the Controversy." Workplace Health Saf **64**(1): 28-36; quiz 37.

Midic, U., B. Goheen, K. A. Vincent, C. A. VandeVoort and K. E. Latham (2018). "Changes in gene expression following long-term in vitro exposure of Macaca mulatta trophoblast stem cells to biologically relevant levels of endocrine disruptors." Reprod Toxicol **77**: 154-165.

Mitro, S. D., T. Johnson and A. R. Zota (2015). "Cumulative Chemical Exposures During Pregnancy and Early Development." Curr Environ Health Rep **2**(4): 367-378.

Molina-Molina, J. M., E. Amaya, M. Grimaldi, J. M. Saenz, M. Real, M. F. Fernandez, P. Balaguer and N. Olea (2013). "In vitro study on the agonistic and antagonistic activities of bisphenol-S and other bisphenol-A congeners and derivatives via nuclear receptors." Toxicol Appl Pharmacol **272**(1): 127-136.

Morrish, D. W., J. Dakour and H. Li (2001). "Life and death in the placenta: new peptides and genes regulating human syncytiotrophoblast and extravillous cytotrophoblast lineage formation and renewal." Curr Protein Pept Sci **2**(3): 245-259.

Morrison, D. K. (2012). "MAP kinase pathways." Cold Spring Harb Perspect Biol **4**(11).

Mourier, E., A. Tarrade, J. Duan, C. Richard, C. Bertholdt, M. Beaumont, O. Morel and P. Chavatte-Palmer (2017). "Non-invasive evaluation of placental blood flow: lessons from animal models." Reproduction **153**(3): R85-R96.

Muller, J. E., N. Meyer, C. G. Santamaria, A. Schumacher, E. H. Luque, M. L. Zenclussen, H. A. Rodriguez and A. C. Zenclussen (2018). "Bisphenol A exposure during early pregnancy impairs uterine spiral artery remodeling and provokes intrauterine growth restriction in mice." Sci Rep **8**(1): 9196.

Muller, M. H. B., A. Polder, O. B. Brynildsrud, R. Gronnestad, M. Karimi, E. Lie, W. B. Manyilizu, R. H. Mdegela, F. Mokiti, M. Murtadha, H. E. Nonga, J. U. Skaare, A. Solhaug and J. L. Lyche (2019). "Prenatal exposure to persistent organic pollutants in Northern Tanzania and their distribution between breast milk, maternal blood, placenta and cord blood." Environ Res **170**: 433-442.

Murray, J., B. Eskenazi, R. Bornman, F. W. Gaspar, M. Crause, M. Obida and J. Chevrier (2018). "Exposure to DDT and hypertensive disorders of pregnancy among South African women from an indoor residual spraying region: The VHEMBE study." Environ Res **162**: 49-54.

Mustieles, V., L. Minguez-Alarcon, G. Christou, J. B. Ford, I. Dimitriadis, R. Hauser, I. Souter, C. Messerlian, Environment and T. Reproductive Health Study (2019). "Placental weight in relation to maternal and paternal preconception and prenatal urinary phthalate metabolite concentrations among subfertile couples." Environ Res **169**: 272-279.

Naderi, M., M. Y. Wong and F. Gholami (2014). "Developmental exposure of zebrafish (Danio rerio) to bisphenol-S impairs subsequent reproduction potential and hormonal balance in adults." Aquat Toxicol **148**: 195-203.

Nahar, M. S., C. Liao, K. Kannan, C. Harris and D. C. Dolinoy (2015). "In utero bisphenol A concentration, metabolism, and global DNA methylation across matched placenta, kidney, and liver in the human fetus." Chemosphere **124**: 54-60.

Nakamura, Y., C. C. Chang, T. Mori, K. Sato, K. Ohtsuki, B. L. Upham and J. E. Trosko (2005). "Augmentation of differentiation and gap junction function by kaempferol in partially differentiated colon cancer cells." Carcinogenesis **26**(3): 665-671.

Nakamura, Y., N. Yoshikawa, I. Hiroki, K. Sato, K. Ohtsuki, C. C. Chang, B. L. Upham and J. E. Trosko (2005b). "beta-sitosterol from psyllium seed husk (Plantago ovata Forsk) restores gap junctional intercellular communication in Ha-ras transfected rat liver cells." Nutrition and Cancer-an International Journal **51**(2): 218-225.

Nakanishi, T., J. Kohroki, S. Suzuki, J. Ishizaki, Y. Hiromori, S. Takasuga, N. Itoh, Y. Watanabe, N. Utoguchi and K. Tanaka (2002). "Trialkyltin compounds enhance human CG secretion and aromatase activity in human placental choriocarcinoma cells." J Clin Endocrinol Metab **87**(6): 2830-2837.

Nakanishi, T., Y. Hiromori, H. Yokoyama, M. Koyanagi, N. Itoh, J. Nishikawa and K. Tanaka (2006). "Organotin compounds enhance 17beta-hydroxysteroid dehydrogenase type I activity in human choriocarcinoma JAr cells: potential promotion of 17beta-estradiol biosynthesis in human placenta." Biochem Pharmacol **71**(9): 1349-1357.

Nativelle-Serpentini, C., S. Richard, G. E. Seralini and P. Sourdaine (2003). "Aromatase activity modulation by lindane and bisphenol-A in human placental JEG-3 and transfected kidney E293 cells." Toxicol In Vitro **17**(4): 413-422.

NCBI. "Compound summary: methyl parathion, CID=4130." PubChem Database Retrieved 11/12/2019, from https://pubchem.ncbi.nlm.nih.gov/compound/Methyl-parathion.

Nevoral, J., Y. Kolinko, J. Moravec, T. Zalmanova, K. Hoskova, S. Prokesova, P. Klein, K. Ghaibour, P. Hosek, M. Stiavnicka, H. Rimnacova, Z. Tonar, J. Petr and M. Kralickova (2018). "Long-term exposure to very low doses of bisphenol S affects female reproduction." Reproduction **156**(1): 47-57.

Newnham, J. P., J. E. Dickinson, R. J. Hart, C. E. Pennell, C. A. Arrese and J. A. Keelan (2014). "Strategies to prevent preterm birth." Front Immunol **5**: 584.

Ngamprasertwong, P., M. Dong, J. Niu, R. Venkatasubramanian, A. A. Vinks and S. Sadhasivam (2016). "Propofol Pharmacokinetics and Estimation of Fetal Propofol Exposure during Mid-Gestational Fetal Surgery: A Maternal-Fetal Sheep Model." PLoS One **11**(1): e0146563.

Nippoldt, T. B., N. E. Reame, R. P. Kelch and J. C. Marshall (1989). "The roles of estradiol and progesterone in decreasing luteinizing hormone pulse frequency in the luteal phase of the menstrual cycle." J Clin Endocrinol Metab **69**(1): 67-76.

Nishii, K., Y. Shibata and Y. Kobayashi (2014). "Connexin mutant embryonic stem cells and human diseases." World J Stem Cells **6**(5): 571-578.

Nishikawa, M., H. Iwano, R. Yanagisawa, N. Koike, H. Inoue and H. Yokota (2010). "Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus." Environ Health Perspect **118**(9): 1196-1203.

Norris, R. P., M. Freudzon, L. M. Mehlmann, A. E. Cowan, A. M. Simon, D. L. Paul, P. D. Lampe and L. A. Jaffe (2008). "Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption." Development **135**(19): 3229-3238.

Nteeba, J., M. V. Sanz-Fernandez, R. P. Rhoads, L. H. Baumgard, J. W. Ross and A. F. Keating (2015). "Heat Stress Alters Ovarian Insulin-Mediated Phosphatidylinositol-3 Kinase and Steroidogenic Signaling in Gilt Ovaries." Biol Reprod **92**(6): 148.

Oh, J., J. W. Choi, Y. A. Ahn and S. Kim (2018). "Pharmacokinetics of bisphenol S in humans after single oral administration." Environ Int **112**: 127-133.

Ohtani, N., H. Iwano, K. Suda, E. Tsuji, K. Tanemura, H. Inoue and H. Yokota (2017). "Adverse effects of maternal exposure to bisphenol F on the anxiety- and depression-like behavior of offspring." J Vet Med Sci **79**(2): 432-439.

Okuma, A., A. Kuraoka, H. Iida, T. Inai, K. Wasano and Y. Shibata (1996). "Colocalization of connexin 43 and connexin 45 but absence of connexin 40 in granulosa cell gap junctions of rat ovary." J Reprod Fertil **107**(2): 255-264.

Orisaka, M., K. Tajima, B. K. Tsang and F. Kotsuji (2009). "Oocyte-granulosa-theca cell interactions during preantral follicular development." J Ovarian Res **2**(1): 9.

Osgood, R. S., B. L. Upham, P. R. Bushel, K. Velmurugan, K. N. Xiong and A. K. Bauer (2017). "Secondhand Smoke-Prevalent Polycyclic Aromatic Hydrocarbon Binary Mixture-Induced Specific Mitogenic and Pro-inflammatory Cell Signaling Events in Lung Epithelial Cells." Toxicol. Sci **157**(1): 156-171.

Osgood, R. S., B. L. Upham, T. Hill, III, K. L. Helms, K. Velmurugan, P. Babica and A. K. Bauer (2014). "Polycyclic aromatic hydrocarbon-induced signaling events relevant to inflammation and tumorigenesis in lung cells are dependent on molecular structure." PLoS. One **8**(6): e65150.

Pacifici, G. M. and R. Nottoli (1995). "Placental transfer of drugs administered to the mother." Clin Pharmacokinet **28**(3): 235-269.

Palaniappan, M., B. Menon and K. M. Menon (2013). "Stimulatory effect of insulin on theca-interstitial cell proliferation and cell cycle regulatory proteins through MTORC1 dependent pathway." Mol Cell Endocrinol **366**(1): 81-89.

Palioura, E. and E. Diamanti-Kandarakis (2015). "Polycystic ovary syndrome (PCOS) and endocrine disrupting chemicals (EDCs)." Rev Endocr Metab Disord **16**(4): 365-371.

Pan, F., T. Xu, L. Yang, X. Jiang and L. Zhang (2014). "Probing the binding of an endocrine disrupting compound-Bisphenol F to human serum albumin: insights into the interactions of harmful chemicals with functional biomacromolecules." Spectrochim Acta A Mol Biomol Spectrosc **132**: 795-802.

Park, H. R. and R. Loch-Caruso (2014). "Protective effect of nuclear factor E2-related factor 2 on inflammatory cytokine response to brominated diphenyl ether-47 in the HTR-8/SVneo human first trimester extravillous trophoblast cell line." Toxicol Appl Pharmacol **281**(1): 67-77.

Patel, S., C. Zhou, S. Rattan and J. A. Flaws (2015). "Effects of Endocrine-Disrupting Chemicals on the Ovary." Biol Reprod **93**(1): 20.

Patterson, T. A., N. C. Twaddle, C. S. Roegge, R. J. Callicott, J. W. Fisher and D. R. Doerge (2013). "Concurrent determination of bisphenol A pharmacokinetics in maternal and fetal rhesus monkeys." Toxicol Appl Pharmacol **267**(1): 41-48.

Peinado, H., D. Olmeda and A. Cano (2007). "Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" Nat Rev Cancer **7**(6): 415-428.

Pelch, K. E., Y. Li, L. Perera, K. A. Thayer and K. S. Korach (2019). "Characterization of Estrogenic and Androgenic Activities for Bisphenol A-like Chemicals (BPs): In Vitro Estrogen and Androgen Receptors Transcriptional Activation, Gene Regulation, and Binding Profiles." Toxicol Sci.

Peltier, M. R., N. G. Klimova, Y. Arita, E. M. Gurzenda, A. Murthy, K. Chawala, V. Lerner, J. Richardson and N. Hanna (2012). "Polybrominated diphenyl ethers enhance the production of proinflammatory cytokines by the placenta." Placenta **33**(9): 745-749.

Perez-Albaladejo, E., D. Fernandes, S. Lacorte and C. Porte (2017). "Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells." Toxicol In Vitro **38**: 41-48.

Petit, J., A. Wakx, S. Gil, T. Fournier, N. Auzeil, P. Rat and O. Laprevote (2018). "Lipidome-wide disturbances of human placental JEG-3cells by the presence of MEHP." Biochimie **149**: 1-8.

Petroff, M. G., T. A. Phillips, H. Ka, J. L. Pace and J. S. Hunt (2006). "Isolation and culture of term human trophoblast cells." Methods Mol Med **121**: 203-217.

Petrovic, M., E. Eljarrat, S. Diez, G. Kowalewska and D. Barcelo (2007). "Chemical Analysis of Contaminants in Sediments." Sustainable Management of Sediment Resources **1**: 61-129.

Philippat, C., B. Heude, J. Botton, N. Alfaidy, A. M. Calafat, R. Slama and E. M.-C. C. S. Group (2019). "Prenatal Exposure to Select Phthalates and Phenols and Associations with Fetal and Placental Weight among Male Births in the EDEN Cohort (France)." Environ Health Perspect **127**(1): 17002.

Philips, E. M., V. W. V. Jaddoe, A. G. Asimakopoulos, K. Kannan, E. A. P. Steegers, S. Santos and L. Trasande (2018). "Bisphenol and phthalate concentrations and its determinants among pregnant

women in a population-based cohort in the Netherlands, 2004-5." Environmental Research **161**: 562-572.

Pidoux, G., P. Gerbaud, J. Dompierre, B. Lygren, T. Solstad, D. Evain-Brion and K. Tasken (2014). "A PKA-ezrin-Cx43 signaling complex controls gap junction communication and thereby trophoblast cell fusion." J Cell Sci **127**(Pt 19): 4172-4185.

Pivnenko, K., G. A. Pedersen, E. Eriksson and T. F. Astrup (2015). "Bisphenol A and its structural analogues in household waste paper." Waste Manag **44**: 39-47.

Pohler, K. G., M. H. Pereira, F. R. Lopes, J. C. Lawrence, D. H. Keisler, M. F. Smith, J. L. Vasconcelos and J. A. Green (2016). "Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds." J Dairy Sci **99**(2): 1584-1594.

Pollock, T., L. J. Greville, R. E. Weaver, M. Radenovic and D. deCatanzaro (2018). "Bisphenol S modulates concentrations of bisphenol A and oestradiol in female and male mice." Xenobiotica: 1-9.

Ponniah, M., E. E. Billett and L. A. De Girolamo (2015). "Bisphenol A increases BeWo trophoblast survival in stress-induced paradigms through regulation of oxidative stress and apoptosis." Chem Res Toxicol **28**(9): 1693-1703.

Pottenger, L. H., J. Y. Domoradzki, D. A. Markham, S. C. Hansen, S. Z. Cagen and J. M. Waechter, Jr. (2000). "The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration." Toxicol Sci **54**(1): 3-18.

Practice Committee of the American Society for Reproductive, M. (2015). "Diagnostic evaluation of the infertile female: a committee opinion." Fertil Steril **103**(6): e44-50.

Prins, G. S., S. H. Ye, L. Birch, S. M. Ho and K. Kannan (2011). "Serum bisphenol A pharmacokinetics and prostate neoplastic responses following oral and subcutaneous exposures in neonatal Sprague-Dawley rats." Reprod Toxicol **31**(1): 1-9.

Pu, Y., J. Gingrich, J. P. Steibel and A. Veiga-Lopez (2017). "Sex-specific modulation of fetal adipogenesis by gestational bisphenol A and bisphenol S exposure." Endocrinology.

Pu, Y., S. Pearl, J. Gingrich, J. Jing, D. Martin, C. A. Murga-Zamalloa and A. Veiga-Lopez (2019). "Multispecies study: low-dose tributyltin impairs ovarian theca cell cholesterol homeostasis through the RXR pathway in five mammalian species including humans." Arch Toxicol.

PubChem. "PubChem, Open Chemistry database." Retrieved 12/12/2018, 2018, from https://pubchem.ncbi.nlm.nih.gov/.

Pulkkinen, M. and M. M. Hamalainen (1995). "Myometrial estrogen and progesterone receptor binding in pregnancy: inhibition by the detergent action of phospholipids." J Steroid Biochem Mol Biol **52**(3): 287-294.

Purves, R. D. (1992). "Optimum numerical integration methods for estimation of area-under-the-curve (AUC) and area-under-the-moment-curve (AUMC)." J Pharmacokinet Biopharm **20**(3): 211-226.

Putney, J. W. and T. Tomita (2012). "Phospholipase C signaling and calcium influx." Adv Biol Regul **52**(1): 152-164.

Pycke, B. F., L. A. Geer, M. Dalloul, O. Abulafia and R. U. Halden (2015). "Maternal and fetal exposure to parabens in a multiethnic urban U.S. population." Environ Int **84**: 193-200.

Qiu, W., M. Yang, S. Liu, P. Lei, L. Hu, B. Chen, M. Wu and K. J. Wang (2018). "Toxic Effects of Bisphenol S Showing Immunomodulation in Fish Macrophages." Environ Sci Technol **52**(2): 831-838.

Qiu, W., Y. Zhao, M. Yang, M. Farajzadeh, C. Pan and N. L. Wayne (2015). "Actions of Bisphenol A and Bisphenol S on the Reproductive Neuroendocrine System During Early Development in Zebrafish." Endocrinology: en20151785.

Rai, S. K., F. M. Duh, V. Vigdorovich, A. Danilkovitch-Miagkova, M. I. Lerman and A. D. Miller (2001). "Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation." Proc Natl Acad Sci U S A **98**(8): 4443-4448.

Rajakumar, C., H. Guan, D. Langlois, M. Cernea and K. Yang (2015). "Bisphenol A disrupts gene expression in human placental trophoblast cells." Reprod Toxicol **53**: 39-44.

Rantakokko, P., K. M. Main, C. Wohlfart-Veje, H. Kiviranta, R. Airaksinen, T. Vartiainen, N. E. Skakkebaek, J. Toppari and H. E. Virtanen (2014). "Association of placenta organotin concentrations with growth and ponderal index in 110 newborn boys from Finland during the first 18 months of life: a cohort study." Environ Health **13**(1): 45.

Ren, P. and R. J. Ruch (1996). "Inhibition of gap junctional intercellular communication by barbiturates in long-term primary cultured rat hepatocytes is correlated with liver tumour promoting activity." Carcinogenesis **17**(10): 2119-2124.

Reyna, L., J. Flores-Martin, M. E. Ridano, G. M. Panzetta-Dutari and S. Genti-Raimondi (2017). "Chlorpyrifos induces endoplasmic reticulum stress in JEG-3 cells." Toxicol In Vitro **40**: 88-93.

Reynolds, L. P., K. A. Vonnahme, C. O. Lemley, D. A. Redmer, A. T. Grazul-Bilska, P. P. Borowicz and J. S. Caton (2013). "Maternal stress and placental vascular function and remodeling." Curr Vasc Pharmacol **11**(5): 564-593.

Richards, J. S. and M. Ascoli (2018). "Endocrine, Paracrine, and Autocrine Signaling Pathways That Regulate Ovulation." Trends in Endocrinology and Metabolism **29**(5): 313-325.

Richter, W. F., S. G. Bhansali and M. E. Morris (2012). "Mechanistic determinants of biotherapeutics absorption following SC administration." AAPS J **14**(3): 559-570.

Ridano, M. E., A. C. Racca, J. Flores-Martin, S. A. Camolotto, G. M. de Potas, S. Genti-Raimondi and G. M. Panzetta-Dutari (2012). "Chlorpyrifos modifies the expression of genes involved in human placental function." Reprod Toxicol **33**(3): 331-338.

Rieke, S., S. Koehn, K. Hirsch-Ernst, R. Pfeil, C. Kneuer and P. Marx-Stoelting (2014). "Combination effects of (tri)azole fungicides on hormone production and xenobiotic metabolism in a human placental cell line." Int J Environ Res Public Health **11**(9): 9660-9679.

Roberts, J. N., K. J. May and A. Veiga-Lopez (2017). "Time-dependent changes in pregnancy-associated glycoproteins and progesterone in commercial crossbred sheep." Theriogenology **89**: 271-279.

Robinson, J. F., M. Kapidzic, E. G. Hamilton, H. Chen, K. W. Puckett, Y. Zhou, K. Ona, E. Parry, Y. Wang, J. S. Park, J. F. Costello and S. J. Fisher (2019). "Genomic Profiling of BDE-47 Effects on Human Placental Cytotrophoblasts." Toxicol Sci **167**(1): 211-226.

Rocha, B. A., A. G. Asimakopoulos, M. Honda, N. L. da Costa, R. M. Barbosa, F. Barbosa, Jr. and K. Kannan (2018). "Advanced data mining approaches in the assessment of urinary concentrations of bisphenols, chlorophenols, parabens and benzophenones in Brazilian children and their association to DNA damage." Environ Int **116**: 269-277.

Rock, K. D., B. Horman, A. L. Phillips, S. L. McRitchie, S. Watson, J. Deese-Spruill, D. Jima, S. Sumner, H. M. Stapleton and H. B. Patisaul (2018). "EDC IMPACT: Molecular effects of developmental FM 550 exposure in Wistar rat placenta and fetal forebrain." Endocr Connect **7**(2): 305-324.

Rosenfeld, C. S. (2015). "Sex-Specific Placental Responses in Fetal Development." Endocrinology **156**(10): 3422-3434.

Rosenkranz, H. S., N. Pollack and A. R. Cunningham (2000). "Exploring the relationship between the inhibition of gap junctional intercellular communication and other biological phenomena." Carcinogenesis **21**: 1007-1011.

Rosenmai, A. K., M. Dybdahl, M. Pedersen, B. M. Alice van Vugt-Lussenburg, E. B. Wedebye, C. Taxvig and A. M. Vinggaard (2014). "Are structural analogues to bisphenol a safe alternatives?" Toxicol Sci **139**(1): 35-47.

Rossi, R. O., J. J. Costa, A. W. Silva, M. V. Saraiva, R. Van den Hurk and J. R. Silva (2016). "The bone morphogenetic protein system and the regulation of ovarian follicle development in mammals." Zygote **24**(1): 1-17.

Ruch, R. J., J. E. Trosko and B. V. Madhukar (2001). "Inhibition of connexin43 gap junctional intercellular communication by TPA requires ERK activation." J Cell Biochem **83**(1): 163-169.

Ruis, M. T., K. D. Rock, S. M. Hall, B. Horman, H. B. Patisaul and H. M. Stapleton (2019). "PBDEs Concentrate in the Fetal Portion of the Placenta: Implications for Thyroid Hormone Dysregulation." Endocrinology **160**(11): 2748-2758.

Rundell, K. and B. Panchal (2017). "Preterm Labor: Prevention and Management." Am Fam Physician **95**(6): 366-372.

Rutkowska, A. Z. and E. Diamanti-Kandarakis (2016). "Polycystic ovary syndrome and environmental toxins." Fertil Steril **106**(4): 948-958.

Sai, K., J. Kanno, R. Hasegawa, J. E. Trosko and T. Inoue (2000). "Prevention of the down-regulation of gap junctional intercellular communication by green tea in the liver of mice fed pentachlorophenol." Carcinogenesis **21**(9): 1671-1676.

Salthammer, T. (2020). "Emerging indoor pollutants." Int J Hyg Environ Health **224**: 113423.

Sapbamrer, R., T. Prapamontol, O. Prakobvitayakit, Y. Vaneesorn, A. Mangklabruks and B. Hock (2008). "Placental transfer of DDT in mother-infant pairs from Northern Thailand." J Environ Sci Health B **43**(6): 484-489.

Sassone-Corsi, P. (2012). "The cyclic AMP pathway." Cold Spring Harb Perspect Biol **4**(12).

Saulsbury, M. D., S. O. Heyliger, K. Wang and D. Round (2008). "Characterization of chlorpyrifos-induced apoptosis in placental cells." Toxicology **244**(2-3): 98-110.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona (2012). "Fiji: an open-source platform for biological-image analysis." Nat Methods **9**(7): 676-682.

Schindler, A. E. (2005). "Endocrinology of pregnancy: consequences for the diagnosis and treatment of pregnancy disorders." J Steroid Biochem Mol Biol **97**(5): 386-388.

Schmalhofer, O., S. Brabletz and T. Brabletz (2009). "E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer." Cancer Metastasis Rev **28**(1-2): 151-166.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C-T method." Nature Protocols **3**(6): 1101-1108.

Schneider, J. E., J. M. Brozek and E. Keen-Rhinehart (2014). "Our stolen figures: the interface of sexual differentiation, endocrine disruptors, maternal programming, and energy balance." Horm Behav **66**(1): 104-119.

Sela-Abramovich, S., E. Chorev, D. Galiani and N. Dekel (2005). "Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles." Endocrinology **146**(3): 1236-1244.

Shamshirsaz, A. A., K. A. Fox, H. Erfani, S. L. Clark, S. K. Hui, A. A. Shamshirsaz, A. Rezaei, A. A. Nassr, Y. N. Lake, J. Teruya and M. A. Belfort (2019). "Coagulopathy in surgical management of placenta accreta spectrum." Eur J Obstet Gynecol Reprod Biol **237**: 126-130.

Shi, M., N. Sekulovski, J. A. MacLean, A. Whorton and K. Hayashi (2019). "Prenatal Exposure to Bisphenol A Analogues on Female Reproductive Functions in Mice." Toxicol Sci **168**(2): 561-571.

Shoaito, H., J. Petit, A. Chissey, N. Auzeil, J. Guibourdenche, S. Gil, O. Laprevote, T. Fournier and S. A. Degrelle (2019). "The Role of Peroxisome Proliferator-Activated Receptor Gamma (PPARgamma) in Mono(2-ethylhexyl) Phthalate (MEHP)-Mediated Cytotrophoblast Differentiation." Environ Health Perspect **127**(2): 27003.

Shoup, M., M. Gonen, M. D'Angelica, W. R. Jarnagin, R. P. DeMatteo, L. H. Schwartz, S. Tuorto, L. H. Blumgart and Y. Fong (2003). "Volumetric analysis predicts hepatic dysfunction in patients undergoing major liver resection." J Gastrointest Surg **7**(3): 325-330.

Shuhaibar, L. C., J. R. Egbert, R. P. Norris, P. D. Lampe, V. O. Nikolaev, M. Thunemann, L. Wen, R. Feil and L. A. Jaffe (2015). "Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles." Proc Natl Acad Sci U S A **112**(17): 5527-5532.

Siddiqi, M. A., R. H. Laessig and K. D. Reed (2003). "Polybrominated diphenyl ethers (PBDEs): new pollutants-old diseases." Clin Med Res **1**(4): 281-290.

Siegrist, K. J., D. Romo, B. L. Upham, M. Armstrong, K. Quinn, L. Vanderlinden, R. S. Osgood, K. Velmurugan, M. Elie, J. Manke, D. Reinhold, N. Reisdorph, L. Saba and A. K. Bauer (2019). "Early Mechanistic Events Induced by Low Molecular Weight Polycyclic Aromatic Hydrocarbons in Mouse Lung Epithelial Cells: A Role for Eicosanoid Signaling." Toxicol Sci **169**(1): 180-193.

Sieppi, E., K. Vahakangas, A. Rautio, F. Ietta, L. Paulesu and P. Myllynen (2016). "The xenoestrogens, bisphenol A and para-nonylphenol, decrease the expression of the ABCG2 transporter protein in human term placental explant cultures." Mol Cell Endocrinol **429**: 41-49.

Silva, M. J., D. B. Barr, J. A. Reidy, N. A. Malek, C. C. Hodge, S. P. Caudill, J. W. Brock, L. L. Needham and A. M. Calafat (2004). "Urinary levels of seven phthalate metabolites in the U.S. population from the National Health and Nutrition Examination Survey (NHANES) 1999-2000." Environ Health Perspect **112**(3): 331-338.

Sinyuk, M., E. E. Mulkearns-Hubert, O. Reizes and J. Lathia (2018). "Cancer Connectors: Connexins, Gap Junctions, and Communication." Front Oncol **8**: 646.

Skakkebaek, N. E., J. Toppari, O. Soder, C. M. Gordon, S. Divall and M. Draznin (2011). "The exposure of fetuses and children to endocrine disrupting chemicals: a European Society for Paediatric Endocrinology (ESPE) and Pediatric Endocrine Society (PES) call to action statement." J Clin Endocrinol Metab **96**(10): 3056-3058.

Small, C. M., K. Cheslack-Postava, M. Terrell, H. M. Blanck, P. Tolbert, C. Rubin, A. Henderson and M. Marcus (2007). "Risk of spontaneous abortion among women exposed to polybrominated biphenyls." Environ Res **105**(2): 247-255.

Sohl, G. and K. Willecke (2004). "Gap junctions and the connexin protein family." Cardiovasc Res **62**(2): 228-232.

Sommersberg, B., A. Bulling, U. Salzer, U. Frohlich, R. E. Garfield, A. Amsterdam and A. Mayerhofer (2000). "Gap junction communication and connexin 43 gene expression in a rat granulosa cell line: Regulation by follicle-stimulating hormone." Biol. Reprod **63**(6): 1661-1668.

Song, W., M. Puttabyatappa, L. Zeng, D. Vazquez, S. Pennathur and V. Padmanabhan (2019). "Developmental programming: Prenatal bisphenol A treatment disrupts mediators of placental function in sheep." Chemosphere **243**: 125301.

Sotogaku, N., S. E. Tully, C. I. Gama, H. Higashi, M. Tanaka, L. C. Hsieh-Wilson and A. Nishi (2007). "Activation of phospholipase C pathways by a synthetic chondroitin sulfate-E tetrasaccharide promotes neurite outgrowth of dopaminergic neurons." J Neurochem **103**(2): 749-760.

Sousa, A., R. Pastorinho, S. Takahashi and S. Tanabe (2014). "History on organotin compounds, from snails to humans." Environmental Chemistry Letters **12**.

Sousa, N. M., A. Ayad, J. F. Beckers and Z. Gajewski (2006). "Pregnancy-associated glycoproteins (PAG) as pregnancy markers in the ruminants." J Physiol Pharmacol **57 Suppl 8**: 153-171.

Sovadinova, I., P. Babica, H. Boeke, E. Kumar, A. Wilke, J.-S. Park, J. E. Trosko and B. L. Upham (2015). "Phosphatidylcholine Specific PLC-Induced Dysregulation of Gap Junctions, a Robust Cellular Response to Environmental Toxicants, and Prevention by Resveratrol in a Rat Liver Cell Model." Plos One **10**(5).

Spagnoletti, A., L. Paulesu, C. Mannelli, L. Ermini, R. Romagnoli, M. Cintorino and F. Ietta (2015). "Low concentrations of Bisphenol A and para-Nonylphenol affect extravillous pathway of human trophoblast cells." Mol Cell Endocrinol **412**: 56-64.

Speidel, J. T., M. Xu and S. Z. Abdel-Rahman (2018). "Bisphenol A (BPA) and bisphenol S (BPS) alter the promoter activity of the ABCB1 gene encoding P-glycoprotein in the human placenta in a haplotype-dependent manner." Toxicol Appl Pharmacol **359**: 47-54.

Srinivas, M., V. K. Verselis and T. W. White (2018). "Human diseases associated with connexin mutations." Biochim Biophys Acta Biomembr **1860**(1): 192-201.

Stel, J. and J. Legler (2015). "The Role of Epigenetics in the Latent Effects of Early Life Exposure to Obesogenic Endocrine Disrupting Chemicals." Endocrinology **156**(10): 3466-3472.

Stowell, C. L., K. K. Barvian, P. C. M. Young, R. M. Bigsby, D. E. Verdugo, C. R. Bertozzi and T. S. Widlanski (2006). "A role for sulfation-desulfation in the uptake of bisphenol A into breast tumor cells." Chemistry & Biology **13**(8): 891-897.

Strakovsky, R. S. and S. L. Schantz (2018). "Impacts of bisphenol A (BPA) and phthalate exposures on epigenetic outcomes in the human placenta." Environ Epigenet **4**(3): dvy022.

Svobodova, K., M. Plackova, V. Novotna and T. Cajthaml (2009). "Estrogenic and androgenic activity of PCBs, their chlorinated metabolites and other endocrine disruptors estimated with two in vitro yeast assays." Sci Total Environ **407**(22): 5921-5925.

Tai, M. H., B. L. Upham, L. K. Olson, M. S. Tsao, D. N. Reed, Jr. and J. E. Trosko (2007). "Cigarette smoke components inhibited intercellular communication and differentiation in human pancreatic ductal epithelial cells." Int J Cancer **120**(9): 1855-1862.

Tait, S., R. Tassinari, F. Maranghi and A. Mantovani (2015). "Bisphenol A affects placental layers morphology and angiogenesis during early pregnancy phase in mice." J Appl Toxicol **35**(11): 1278-1291.

Tanaka, Y., A. Otsuka, H. Tanaka and K. Shigenobu (1999). "Glycyrrhetinic acid-sensitive mechanism does not make a major contribution to non-prostanoid, non-nitric oxide mediated endothelium-dependent relaxation of rat mesenteric artery in response to acetylcholine." Res Commun Mol Pathol Pharmacol **103**(3): 227-239.

Tang, J. and J. X. Zhai (2017). "Distribution of polybrominated diphenyl ethers in breast milk, cord blood and placentas: a systematic review." Environ Sci Pollut Res Int **24**(27): 21548-21573.

Tarafdar, A. and A. M. Michie (2014). "Protein kinase C in cellular transformation: a valid target for therapy?" Biochem Soc Trans **42**(6): 1556-1562.

Tetz, L. M., D. M. Aronoff and R. Loch-Caruso (2015). "Mono-ethylhexyl phthalate stimulates prostaglandin secretion in human placental macrophages and THP-1 cells." Reprod Biol Endocrinol **13**: 56.

Thayer, K. A., D. R. Doerge, D. Hunt, S. H. Schurman, N. C. Twaddle, M. I. Churchwell, S. Garantziotis, G. E. Kissling, M. R. Easterling, J. R. Bucher and L. S. Birnbaum (2015). "Pharmacokinetics of bisphenol A in humans following a single oral administration." Environ Int **83**: 107-115.

Thoma, M. E., A. C. McLain, J. F. Louis, R. B. King, A. C. Trumble, R. Sundaram and G. M. Buck Louis (2013). "Prevalence of infertility in the United States as estimated by the current duration approach and a traditional constructed approach." Fertil Steril **99**(5): 1324-1331 e1321.

Thompson, I. M., S. Tao, J. Branen, A. D. Ealy and G. E. Dahl (2013). "Environmental regulation of pregnancy-specific protein B concentrations during late pregnancy in dairy cattle." J Anim Sci **91**(1): 168-173.

Tong, D., T. Y. Li, K. E. Naus, D. Bai and G. M. Kidder (2007). "In vivo analysis of undocked connexin43 gap junction hemichannels in ovarian granulosa cells." J Cell Sci **120**(Pt 22): 4016-4024.

Trosko, J. E. (2011). "The gap junction as a "Biological Rosetta Stone": implications of evolution, stem cells to homeostatic regulation of health and disease in the Barker hypothesis." J. Cell Commun. Signal **5**(1): 53-66.

Tsuji, M., Y. Aiko, T. Kawamoto, T. Hachisuga, C. Kooriyama, M. Myoga, C. Tomonaga, F. Matsumura, A. Anan, M. Tanaka, H. S. Yu, Y. Fujisawa, R. Suga and E. Shibata (2013). "Polychlorinated biphenyls (PCBs) decrease the placental syncytiotrophoblast volume and increase Placental Growth Factor (PlGF) in the placenta of normal pregnancy." Placenta **34**(7): 619-623.

Turusov, V., V. Rakitsky and L. Tomatis (2002). "Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks." Environ Health Perspect **110**(2): 125-128.

Upham, B. L. (2011). "Role of integrative signaling through gap junctions in toxicology." Curr Protoc Toxicol **Chapter 2**: Unit2 18.

Upham, B. L., A. M. Rummel, J. M. Carbone, J. E. Trosko, Y. Ouyang, R. B. Crawford and N. E. Kaminski (2003). "Cannabinoids inhibit gap junctional intercellular communication and activate ERK in a rat liver epithelial cell line." Int J Cancer **104**(1): 12-18.

Upham, B. L., J. S. Park, P. Babica, I. Sovadinova, A. M. Rummel, J. E. Trosko, A. Hirose, R. Hasegawa, J. Kanno and K. Sai (2009). "Structure-activity-dependent regulation of cell communication by perfluorinated fatty acids using in vivo and in vitro model systems." Environ. Health Perspect **117**(4): 545-551.

Upham, B. L., L. Blaha, P. Babica, J. S. Park, I. Sovadinova, C. Pudrith, A. M. Rummel, L. M. Weis, K. Sai, P. K. Tithof, M. Guzvic, J. Vondracek, M. Machala and J. E. Trosko (2008). "Tumor promoting properties of a cigarette smoke prevalent polycyclic aromatic hydrocarbon as indicated by the inhibition of gap junctional intercellular communication via phosphatidylcholine-specific phospholipase C." Cancer Sci **99**(4): 696-705.

Upham, B. L., M. Guzvic, J. Scott, J. M. Carbone, L. Blaha, C. Coe, L. L. Li, A. M. Rummel and J. E. Trosko (2007). "Inhibition of gap junctional intercellular communication and activation of mitogen-activated protein kinase by tumor-promoting organic peroxides and protection by resveratrol." Nutr. Cancer **57**(1): 38-47.

Upham, B. L., N. D. Deocampo, B. Wurl and J. E. Trosko (1998). "Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail." Int. J. Cancer **78**(4): 491-495.

Van Calster, B., S. Bobdiwala, S. Guha, K. Van Hoorde, M. Al-Memar, R. Harvey, J. Farren, E. Kirk, G. Condous, S. Sur, C. Stalder, D. Timmerman and T. Bourne (2016). "Managing pregnancy of unknown location based on initial serum progesterone and serial serum hCG levels: development and validation of a two-step triage protocol." Ultrasound Obstet Gynecol **48**(5): 642-649.

van den Berg, H. (2009). "Global status of DDT and its alternatives for use in vector control to prevent disease." Environ Health Perspect **117**(11): 1656-1663.

van den Berg, H., G. Manuweera and F. Konradsen (2017). "Global trends in the production and use of DDT for control of malaria and other vector-borne diseases." Malar J **16**(1): 401.

Vandenberg, L. N., I. Chahoud, J. J. Heindel, V. Padmanabhan, F. J. Paumgartten and G. Schoenfelder (2010). "Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A." Environ Health Perspect **118**(8): 1055-1070.

Varshavsky, J., A. Smith, A. Wang, E. Hom, M. Izano, H. Huang, A. Padula and T. J. Woodruff (2019). "Heightened susceptibility: A review of how pregnancy and chemical exposures influence maternal health." Reprod Toxicol.

Vaughan, O. R., A. N. Sferruzzi-Perri, P. M. Coan and A. L. Fowden (2011). "Environmental regulation of placental phenotype: implications for fetal growth." Reprod Fertil Dev **24**(1): 80-96.

Veiga-Lopez, A., K. Kannan, C. Liao, W. Ye, S. E. Domino and V. Padmanabhan (2015). "Gender-Specific Effects on Gestational Length and Birth Weight by Early Pregnancy BPA Exposure." J Clin Endocrinol Metab **100**(11): E1394-1403.

Veiga-Lopez, A., L. J. Luense, L. K. Christenson and V. Padmanabhan (2013). "Developmental programming: gestational bisphenol-A treatment alters trajectory of fetal ovarian gene expression." Endocrinology **154**(5): 1873-1884.

Veiga-Lopez, A., Y. Pu, J. Gingrich and V. Padmanabhan (2018). "Obesogenic Endocrine Disrupting Chemicals: Identifying Knowledge Gaps." Trends Endocrinol Metab **29**(9): 607-625.

Velmurugan, K., R. S. Osgood, K. N. Xiong, J. Xiong, B. L. Upham and A. K. Bauer (2015). "Secondhand Smoke Prevalent Low Molecular Weight Polycyclic Aromatic Hydrocarbon Effects On Lung Epithelial Cells." American Journal of Respiratory and Critical Care Medicine **191**.

Vinken, M., T. Doktorova, E. Decrock, L. Leybaert, T. Vanhaecke and V. Rogiers (2009). "Gap junctional intercellular communication as a target for liver toxicity and carcinogenicity." Crit Rev Biochem Mol Biol **44**(4): 201-222.

Vinken, M., T. Henkens, T. Vanhaecke, P. Papeleu, A. Geerts, E. Van Rossen, J. K. Chipman, P. Meda and V. Rogiers (2006). "Trichostatin a enhances gap junctional intercellular communication in primary cultures of adult rat hepatocytes." Toxicol Sci **91**(2): 484-492.

Vitku, J., L. Starka, M. Bicikova, M. Hill, J. Heracek, L. Sosvorova and R. Hampl (2016). "Endocrine disruptors and other inhibitors of 11beta-hydroxysteroid dehydrogenase 1 and 2: Tissue-specific consequences of enzyme inhibition." J Steroid Biochem Mol Biol **155**(Pt B): 207-216.

Volkel, W., T. Colnot, G. A. Csanady, J. G. Filser and W. Dekant (2002). "Metabolism and kinetics of bisphenol a in humans at low doses following oral administration." Chem Res Toxicol **15**(10): 1281-1287.

vom Saal, F. S., L. J. Guillette Jr., J. P. Myers and S. H. Swan (2008). Endocrine disruptors: effect in wildlife and laboratory animals. Encyclopedia of Ecology. S. E. Jorgensen and B. D. Fath, Oxford**:** 1261-1264.

vom Saal, F. S., M. M. Clark, B. G. Galef Jr, L. C. Drickamer and J. G. Vandenbergh (1999). The intrauterine position (IUP) phenomenon. Encyclopedia of reproduction. E. Knobil and J. Neill. New York, Academic Press. **2:** 893-900.

Vrooman, L. A., F. Xin and M. S. Bartolomei (2016). "Morphologic and molecular changes in the placenta: what we can learn from environmental exposures." Fertil Steril **106**(4): 930-940.

Wallace, R. M., K. G. Pohler, M. F. Smith and J. A. Green (2015). "Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy." Reproduction **149**(3): R115-126.

Wan, Y., W. Huo, S. Xu, T. Zheng, B. Zhang, Y. Li, A. Zhou, Y. Zhang, J. Hu, Y. Zhu, Z. Chen, S. Lu, C. Wu, M. Jiang, Y. Jiang, H. Liu, X. Yang and W. Xia (2018). "Relationship between maternal exposure to bisphenol S and pregnancy duration." Environ Pollut **238**: 717-724.

Wan, Y., W. Xia, S. Yang, X. Pan, Z. He and K. Kannan (2018). "Spatial distribution of bisphenol S in surface water and human serum from Yangtze River watershed, China: Implications for exposure through drinking water." Chemosphere **199**: 595-602.

Wang, G. Y., Y. G. Bi, X. D. Liu, J. F. Han, M. Wei and Q. Y. Zhang (2017). "Upregulation of connexin 43 and apoptosisassociated protein expression by high glucose in H9c2 cells was improved by resveratrol via the autophagy signaling pathway." Mol Med Rep **16**(3): 3262-3268.

Wang, H. X., D. Tong, F. El-Gehani, F. R. Tekpetey and G. M. Kidder (2009). "Connexin expression and gap junctional coupling in human cumulus cells: contribution to embryo quality." J Cell Mol Med **13**(5): 972-984.

Wang, H., Y. Pu, L. Luo, Y. Li, Y. Zhang and Z. Cao (2018). "Membrane receptor-independent inhibitory effect of melatonin on androgen production in porcine theca cells." Theriogenology **118**: 63-71.

Wang, X. K., M. Agarwal, N. Parobchak, A. Rosen, A. M. Vetrano, A. Srinivasan, B. Wang and T. Rosen (2016). "Mono-(2-Ethylhexyl) Phthalate Promotes Pro-Labor Gene Expression in the Human Placenta." PLoS One **11**(1): e0147013.

Wang, Z. Y., J. Lu, Y. Z. Zhang, M. Zhang, T. Liu and X. L. Qu (2015). "Effect of Bisphenol A on invasion ability of human trophoblastic cell line BeWo." Int J Clin Exp Pathol **8**(11): 14355-14364.

Warn-Cramer, B. J., P. D. Lampe, W. E. Kurata, M. Y. Kanemitsu, L. W. Loo, W. Eckhart and A. F. Lau (1996). "Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein." J Biol Chem **271**(7): 3779-3786.

Whigham, C. A., T. M. MacDonald, S. P. Walker, N. J. Hannan, S. Tong and T. J. Kaitu'u-Lino (2019). "The untapped potential of placenta-enriched molecules for diagnostic and therapeutic development." Placenta **84**: 28-31.

Whyatt, R. M., D. B. Barr, D. E. Camann, P. L. Kinney, J. R. Barr, H. F. Andrews, L. A. Hoepner, R. Garfinkel, Y. Hazi, A. Reyes, J. Ramirez, Y. Cosme and F. P. Perera (2003). "Contemporary-use

pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns." Environ Health Perspect **111**(5): 749-756.

Williams, S. A., D. Blache, G. B. Martin, R. Foot, M. A. Blackberry and R. J. Scaramuzzi (2001). "Effect of nutritional supplementation on quantities of glucose transporters 1 and 4 in sheep granulosa and theca cells." Reproduction **122**(6): 947-956.

Winterhager, E. and G. M. Kidder (2015). "Gap junction connexins in female reproductive organs: implications for women's reproductive health." Hum Reprod Update **21**(3): 340-352.

Wojciechowska, A., J. Mlynarczuk and J. Kotwica (2017). "Changes in the mRNA expression of structural proteins, hormone synthesis and secretion from bovine placentome sections after DDT and DDE treatment." Toxicology **375**: 1-9.

Wojciechowska, A., J. Mlynarczuk and J. Kotwica (2018). "The protein expression disorders of connexins (Cx26, Cx32 and Cx43)and keratin 8 in bovine placenta under the influence of DDT, DDE and PCBs." Pol J Vet Sci **21**(4): 721-729.

Wojtowicz, A. K., T. Milewicz and E. L. Gregoraszczuk (2007). "DDT and its metabolite DDE alter steroid hormone secretion in human term placental explants by regulation of aromatase activity." Toxicol Lett **173**(1): 24-30.

Wojtowicz, A. K., T. Milewicz and E. L. Gregoraszczuk (2008). "Time-dependent action of DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) and its metabolite DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene) on human chorionic gonadotropin and progesterone secretion." Gynecol Endocrinol **24**(1): 54-58.

Wooding, P., G. Burton and SpringerLink (Online service) (2008). Comparative Placentation : Structures, Functions and Evolution. Berlin, Heidelberg, Springer Berlin Heidelberg,**:** 1 online resource (XI, 301 p.

Woodruff, T. J., A. R. Zota and J. M. Schwartz (2011). "Environmental chemicals in pregnant women in the United States: NHANES 2003-2004." Environ Health Perspect **119**(6): 878-885.

Worley, R. R., S. M. Moore, B. C. Tierney, X. Ye, A. M. Calafat, S. Campbell, M. B. Woudneh and J. Fisher (2017). "Per- and polyfluoroalkyl substances in human serum and urine samples from a residentially exposed community." Environ Int **106**: 135-143.

Wright, C. S., D. L. Becker, J. S. Lin, A. E. Warner and K. Hardy (2001). "Stage-specific and differential expression of gap junctions in the mouse ovary: connexin-specific roles in follicular regulation." Reproduction **121**(1): 77-88.

Wu, F., F. J. Tian and Y. Lin (2015). "Oxidative Stress in Placenta: Health and Diseases." Biomed Res Int **2015**: 293271.

Wu, J. F., J. Ji, S. Y. Dong, B. B. Li, M. L. Yu, D. D. Wu, L. Tao and X. H. Tong (2016). "Gefitinib enhances oxaliplatin-induced apoptosis mediated by Src and PKC-modulated gap junction function." Oncol Rep **36**(6): 3251-3258.

Wu, K., X. Xu, J. Liu, Y. Guo and X. Huo (2011). "In utero exposure to polychlorinated biphenyls and reduced neonatal physiological development from Guiyu, China." Ecotoxicol Environ Saf **74**(8): 2141-2147.

Wu, L. H., X. M. Zhang, F. Wang, C. J. Gao, D. Chen, J. R. Palumbo, Y. Guo and E. Y. Zeng (2018). "Occurrence of bisphenol S in the environment and implications for human exposure: A short review." Science of the Total Environment **615**: 87-98.

Xiao, J., G. Zhang, P. Qiu, X. Liu, Y. Wu, B. Du, J. Li, J. Zhou, J. Li and Y. Tan (2013). "Tanshinone IIA increases the bystander effect of herpes simplex virus thymidine kinase/ganciclovir gene therapy via enhanced gap junctional intercellular communication." PLoS One **8**(7): e67662.

Xie, H. Y., Y. Cui, F. Deng and J. C. Feng (2015). "Connexin: a potential novel target for protecting the central nervous system?" Neural Regen Res **10**(4): 659-666.

Xu, H., X. Zhang, Y. Ye and X. Li (2019). "Bisphenol A affects estradiol metabolism by targeting CYP1A1 and CYP19A1 in human placental JEG-3 cells." Toxicol In Vitro **61**: 104615.

Xu, Y., G. T. Knipp and T. J. Cook (2006). "Effects of di-(2-ethylhexyl)-phthalate and its metabolites on the lipid profiling in rat HRP-1 trophoblast cells." Arch Toxicol **80**(5): 293-298.

Xue, J., Q. Wu, S. Sakthivel, P. V. Pavithran, J. R. Vasukutty and K. Kannan (2015). "Urinary levels of endocrine-disrupting chemicals, including bisphenols, bisphenol A diglycidyl ethers, benzophenones, parabens, and triclosan in obese and non-obese Indian children." Environ Res **137**: 120-128.

Yabe, S., A. P. Alexenko, M. Amita, Y. Yang, D. J. Schust, Y. Sadovsky, T. Ezashi and R. M. Roberts (2016). "Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas." Proc Natl Acad Sci U S A **113**(19): E2598-2607.

Yang, C., G. Song and W. Lim (2019). "A mechanism for the effect of endocrine disrupting chemicals on placentation." Chemosphere **231**: 326-336.

Yang, C., W. Lim, F. W. Bazer and G. Song (2018). "Butyl paraben promotes apoptosis in human trophoblast cells through increased oxidative stress-induced endoplasmic reticulum stress." Environ Toxicol **33**(4): 436-445.

Yang, L., J. Li, J. Lai, H. Luan, Z. Cai, Y. Wang, Y. Zhao and Y. Wu (2016). "Placental Transfer of Perfluoroalkyl Substances and Associations with Thyroid Hormones: Beijing Prenatal Exposure Study." Sci Rep **6**: 21699.

Yang, S., H. Li, Q. Ge, L. Guo and F. Chen (2015). "Deregulated microRNA species in the plasma and placenta of patients with preeclampsia." Mol Med Rep **12**(1): 527-534.

Yang, Y., S. K. Qin, Q. Wu, Z. S. Wang, R. S. Zheng, X. H. Tong, H. Liu, L. Tao and X. D. He (2014). "Connexin-dependent gap junction enhancement is involved in the synergistic effect of sorafenib and all-trans retinoic acid on HCC growth inhibition." Oncol Rep **31**(2): 540-550.

Ye, X., L. Y. Wong, J. Kramer, X. Zhou, T. Jia and A. M. Calafat (2015). "Urinary Concentrations of Bisphenol A and Three Other Bisphenols in Convenience Samples of U.S. Adults during 2000-2014." Environ Sci Technol **49**(19): 11834-11839.

Ye, Y., Y. Tang, Y. Xiong, L. Feng and X. Li (2019). "Bisphenol A exposure alters placentation and causes preeclampsia-like features in pregnant mice involved in reprogramming of DNA methylation of WNT2." FASEB J **33**(2): 2732-2742.

Yoshikawa, Y., A. Hayashi, M. Inai, A. Matsushita, N. Shibata and K. Takada (2002). "Permeability characteristics of endocrine-disrupting chemicals using an in vitro cell culture model, Caco-2 cells." Current Drug Metabolism **3**(5): 551-557.

Young, J. M. and A. S. McNeilly (2010). "Theca: the forgotten cell of the ovarian follicle." Reproduction **140**(4): 489-504.

Yu, Z., Y. Han, R. Shen, K. Huang, Y. Y. Xu, Q. N. Wang, S. S. Zhou, D. X. Xu and F. B. Tao (2018). "Gestational di-(2-ethylhexyl) phthalate exposure causes fetal intrauterine growth restriction through disturbing placental thyroid hormone receptor signaling." Toxicol Lett **294**: 1-10.

Zbucka-Kretowska, M., U. Lazarek, W. Miltyk, I. Sidorkiewicz, P. Pierzynski, R. Milewski, S. Wolczynski and J. Czerniecki (2019). "Simultaneous analysis of bisphenol A fractions in maternal and fetal compartments in early second trimester of pregnancy." J Perinat Med **47**(7): 765-770.

Zhai, D., J. He, X. Li, L. Gong and Y. Ouyang (2016). "Bisphenol A regulates Snail-mediated epithelial-mesenchymal transition in hemangioma cells." Cell Biochem Funct **34**(6): 441-448.

Zhang, H., Q. Wei, Z. Gao, C. Ma, Z. Yang, H. Zhao, C. Liu, J. Liu, X. Zhao and B. Ma (2019). "G protein-coupled receptor 30 mediates meiosis resumption and gap junction communications downregulation in goat cumulus-oocyte complexes by 17beta-estradiol." J Steroid Biochem Mol Biol **187**: 58-67.

Zhang, N., W. S. Wang, W. J. Li, C. Liu, Y. Wang and K. Sun (2015). "Reduction of progesterone, estradiol and hCG secretion by perfluorooctane sulfonate via induction of apoptosis in human placental syncytiotrophoblasts." Placenta **36**(5): 575-580.

Zhang, N., W. Wang, W. Li, C. Liu, Y. Chen, Q. Yang, Y. Wang and K. Sun (2015b). "Inhibition of 11beta-HSD2 expression by triclosan via induction of apoptosis in human placental syncytiotrophoblasts." J Clin Endocrinol Metab **100**(4): E542-549.

Zhang, Q., S. Wu, L. Liu, X. Hou, J. Jiang, X. Wei and W. Hao (2019). "Effects of bisphenol A on gap junctions in HaCaT cells as mediated by the estrogen receptor pathway." J Appl Toxicol **39**(2): 271-281.

Zhang, X., X. Wu, B. Lei, Y. Jing, Z. Jiang, X. Zhang, X. Fang and Y. Yu (2018). "Transplacental transfer characteristics of organochlorine pesticides in paired maternal and cord sera, and placentas and possible influencing factors." Environ Pollut **233**: 446-454.

Zhao, F., G. Jiang, P. Wei, H. Wang and S. Ru (2018). "Bisphenol S exposure impairs glucose homeostasis in male zebrafish (Danio rerio)." Ecotoxicol Environ Saf **147**: 794-802.

Zhao, M., Y. Zhang, S. Zhuang, Q. Zhang, C. Lu and W. Liu (2014). "Disruption of the hormonal network and the enantioselectivity of bifenthrin in trophoblast: maternal-fetal health risk of chiral pesticides." Environ Sci Technol **48**(14): 8109-8116.

Zhao, X., S. Peng, Y. Xiang, Y. Yang, J. Li, Z. Shan and W. Teng (2017). "Correlation between Prenatal Exposure to Polybrominated Diphenyl Ethers (PBDEs) and Infant Birth Outcomes: A Meta-Analysis and an Experimental Study." Int J Environ Res Public Health **14**(3).

Zhao, Y., H. J. Shi, C. M. Xie, J. Chen, H. Laue and Y. H. Zhang (2015). "Prenatal phthalate exposure, infant growth, and global DNA methylation of human placenta." Environ Mol Mutagen **56**(3): 286-292.

Zhao, Y., P. Liu, J. Wang, X. Xiao, X. Meng and Y. Zhang (2016). "Umbilical cord blood PBDEs concentrations are associated with placental DNA methylation." Environ Int **97**: 1-6.

Zhao, Y., X. Ruan, Y. Li, M. Yan and Z. Qin (2013). "Polybrominated diphenyl ethers (PBDEs) in aborted human fetuses and placental transfer during the first trimester of pregnancy." Environ Sci Technol **47**(11): 5939-5946.

Zhou, Y., S. J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M. Wheelock and C. H. Damsky (1997). "Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion?" J Clin Invest **99**(9): 2139-2151.

Zhu, H. S., Z. Qian, H. L. Liu and E. D. Bao (2016). "ACTH-induced stress in weaned sows impairs LH receptor expression and steroidogenesis capacity in the ovary." Reprod Biol Endocrinol **14**(1): 80.

Zhu, Y., Y. Q. Tan, C. C. Wang and L. K. Leung (2017). "The flame retardant 2,2',4,4'-Tetrabromodiphenyl ether enhances the expression of corticotropin-releasing hormone in the placental cell model JEG-3." Chemosphere **174**: 499-505.

Zhuang, X. and C. Lu (2016). "PBPK modeling and simulation in drug research and development." Acta Pharm Sin B **6**(5): 430-440.

Zong, L., Y. Zhu, R. Liang and H. B. Zhao (2016). "Gap junction mediated miRNA intercellular transfer and gene regulation: A novel mechanism for intercellular genetic communication." Sci Rep **6**: 19884.