THE HUMAN GESTATIONAL MEMBRANES AS A SITE OF POLYBROMINATED DIPHENYL ETHER TOXICITY

By

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to my family and friends,

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ABSTRACT

Preterm birth is a major public health concern impacting one in eight babies born in the U.S.A. Although a precise cause for preterm birth cannot be established in most cases, one of the most promising predictors of preterm birth is inflammation of the extra-placental gestational membranes. Gestational membrane production of pro-inflammatory cytokines has been shown to initiate term parturition pathways and is thought to play a role in preterm parturition as well.

Pregnant woman are exposed to a wide array of environmental pollutants, but few have been investigated epidemiologically for association with preterm birth and none have been satisfactorily investigated mechanistically. Polybrominated diphenyl ethers (PBDEs) are emerging toxicants of concern that have received limited attention regarding potential risks to human health and birth outcomes. Preliminary research suggests that PBDEs may stimulate the gestational membranes to secrete pro-inflammatory cytokines in vitro.

This dissertation tests the hypothesis that polybrominated diphenyl ethers bioaccumulate in the human gestational compartment and stimulate cytokine secretion from human gestational membranes. Total PBDE levels were measured in the human gestational membranes and found at levels of 17.4±3.9 pg/g tissue (5.6±1.3 ng/g lipid). Human amniotic fluid levels were 3795±1592

pg/ml fluid (404±126 ng/g lipid). Congener-specific profiles for the 21 congeners measured identified only tri- through hexa-BDEs in gestational membranes. In contrast, tri- through deca-BDE congeners were found in amniotic fluid. To assess stimulation of inflammatory responses in human gestational membranes, two tissue culture systems were compared for cytokine release into the culture medium: a biopsy punch explant culture system and a transwell mounted explant culture system. Although lipopolysacharide stimulated a robust increase of interleukins-1β, 6, 8, 10 and tumor necrosis factor-α in both systems, with an amplified release of interleukins-6, 8 and 10 in the punch culture system, no increase in cytokine release was observed in response to PBDE stimulation. This is the first report of PBDE accumulation in the human gestational membranes, providing a basis for future investigations of toxic action in this tissue and the first to suggest amniotic fluid as a significant route of PBDE exposure for both the gestational membranes and the developing fetus.

Chapter I

Introduction

Preterm birth - a public health crisis.

The scientific community's understanding of the mechanisms that regulate the timing of birth remains limited. Since 1981, the rate of preterm births, defined as delivery at less than 37 weeks completed gestation, has increased over 30% and now accounts for 12.7% of live births in the U.S.[1] The single largest cause of perinatal mortality and morbidity, preterm birth is associated with over one-third of infant deaths[2] and can lead to a host of developmental impairments including respiratory, gastrointestinal and neural abnormalities.[3] Average first-year medical costs are 10 times greater for preterm than for term infants, contributing to economic burdens in excess of $26.2 billion per year.[4] The emotional and societal burdens associated with the premature birth of one in every eight babies cannot be estimated.

Spontaneous preterm births due to preterm premature labor or preterm premature rupture of membranes (PPROM) account for 65-75% of all preterm births.[5] Labor following PPROM accounts for 25-30% of preterm births.[5, 6] PPROM is defined as the spontaneous rupture of the gestational membranes at less than 37 weeks gestation at least 1 hour prior to the onset of uterine contractions. Neither the pathogenesis of preterm delivery nor that of PPROM has been well elucidated. It has been suggested that these conditions may arise

due to the untimely idiopathic activation of normal labor processes, potentially as the result of pathological insults.[5]

In most cases a precise cause for preterm birth cannot be established. Therefore, associated risk factors have been sought to explain preterm labor and PPROM. Many social, environmental, and physical factors have been associated with increased risk of adverse birth outcomes. Previous preterm delivery, African-American ethnicity, multi-fetal pregnancies, conception through in vitro fertilization, low socioeconomic or educational status, inadequate nutrition, low and high maternal ages, cigarette smoking and bacterial infection have all been linked to preterm birth.[5] Of these, bacterial infection shows the strongest potential for direct mechanistic linkage for singleton births through the activation of pro-inflammatory cascades. Although infections as far reaching as periodontal disease have shown positive correlations with preterm birth,[7] it is infections of the extra-placental gestational membranes that provide the best clues to the etiology of preterm birth and PPROM.

As suggested by the trend of increasing preterm birth rates, current clinical interventions have proven relatively unsuccessful.[4] Although antibiotic treatment can prolong pregnancy for women experiencing PPROM at 34 or fewer weeks gestation,[8] many studies have shown that prophylactic administration of antibiotics does not reduce the risk of preterm birth.[9, 10] This leads to the conclusion that preterm birth does not fit the traditional “1 cause / 1 disease” paradigm. Instead, preterm birth should be considered a syndrome with many interrelated risk factors.

Inflammation and the role of cytokines in parturition.

Parturition is marked by three major changes within the gestational compartment: 1) sustained phasic contraction of the uterine muscle, 2) remodeling of the cervix, and 3) rupture of the extra-placental gestational membranes.

The gestational membranes are made up of three distinct layers: the amnion, the chorion and the decidua. The amnion is of fetal origin and faces the inner fetal compartment. The chorion, also of fetal origin, is found between the amnion and the decidua. The decidua is the maternal tissue derived from the endometrium and connecting the chorion to the uterine wall. The amnion and chorion are separated by a dense collagen layer that provides structural integrity to the membranes while maintaining elasticity. The chorion and decidua do not have a defined boundary and are often referred to as the choriodecidua. These gestational membranes surround the fetus and contain the amniotic fluid during pregnancy, creating a barrier between mother and fetus. The anatomy of the pregnant uterus is further detailed in Figure 1.1.

Normal term parturition is an inflammatory-like process mediated by endogenous cytokines and prostaglandins. Cytokines are small (8-30 kDa) hydrophilic signaling peptides and glycoproteins used in autocrine, paracrine and endocrine signaling throughout the body. In response to a pro-inflammatory stimulus, cytokines play an important role in T-cell and macrophage recruitment as well as activation of the innate and adaptive immune responses.[11-13] The

onset of labor is marked by a strong induction of cytokine secretion,[14-17] including secretion by each of the three layers of the extra-placental gestational membranes,[14, 18-22] leading to the activation of prostaglandins[23] and matrix metalloproteinases.[24]

The up-regulation of inflammatory genes[14, 15] and subsequent release of pro-inflammatory cytokines[17, 25-28] have been associated with parturition in many studies. Furthermore, increased levels of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) have each been associated with preterm birth.[29-32]

Clinical indications of bacterial infection and inflammation within the human gestational membranes suggest that 25-40% of preterm births may be attributable to bacterial causes.[33] Ascending microbes from the vaginal microflora are thought to infiltrate the uterine cavity via the cervix. This results in a progressive infection beginning in the decidua (deciduitis), advancing through the chorion and amnion (chorioamnionitis) and finally entering the amniotic fluid.[34] These bacteria stimulate pro-inflammatory cytokines which in turn recruit neutrophils, macrophages and lymphocytes to the gestational membranes. Traditionally, pathologic examination has been the gold standard for diagnosis of inflammation, but more recently it has been suggested that subclinical cases of inflammation are common and may play a role in preterm birth.[34-36] This is supported by molecular evidence that women with negative diagnoses of clinical infection but positive polymerase chain reaction screens for bacteria in the

amniotic fluid have similar rates of preterm birth as women with positive diagnoses of clinical infection.[37]

Non-infectious stimuli in preterm birth and pro-inflammatory cytokine induction.

Although bacterial infection and chorioamnionitis have strong associations with preterm birth, bacterial infections do not always induce preterm labor.[38, 39] Furthermore, spontaneous preterm birth and PPROM have been observed in the absence of identifiable bacterial infection. This supports the hypothesis that multiple factors may be responsible for these adverse birth outcomes and that etiological factors distinct from gestational infection play a role.

Pregnant woman are exposed to a wide array of environmental pollutants, but few have been investigated epidemiologically for association with preterm birth and none have been satisfactorily investigated mechanistically.[4] Although uninvestigated, it remains plausible that immunosuppression caused by toxicants may lead to increased incidence of infection and thereby increased risk of preterm birth.[40] Pregnant women are also continuously exposed to environmental toxicants reported to stimulate pro-inflammatory cytokines in a wide range of non-gestational tissues.[41-45] Polybrominated diphenyl ethers (PBDEs) are emerging toxicants of concern that have received limited attention regarding potential risks to human health and birth outcomes.[46-53]

PBDEs - exposure and impact.

PBDEs are commercially-produced flame retardants consisting of two benzene (phenyl) rings linked by an ether bond with variable hydrogen to bromine substitutions (Figure 1.2). Mixtures of PBDEs have been used in a wide array of consumer products including textiles, plastics, building materials, electronics and insulation to reduce the risk of spontaneous combustion and retard fire escalation. These compounds are not chemically bound to the products and thus may leech into the environment. Because of their chemical structure, several of the 209 PBDE congeners tend to be environmentally persistent and bioaccumulative.[54]

Because of their environmental persistence and toxicity, the US EPA has identified PBDEs as a priority human health concern.[55] Production of penta-BDE (tri- to hexa-BDE mixtures) and octa-BDE (hexa- to nona-BDE mixtures) ceased in the United States in 2004 by voluntary agreement of the manufacturer, and the European Union passed legislature to prohibit all penta- and octa-BDE use as of August 2004 (short-term deca-BDE extension granted Oct 2005).[56] Recently, the 4th meeting of the Convention of Parties of the Stockholm Convention on Persistent Organic Chemicals listed tetra-, penta-, hexa- and hepta-BDEs as persistent organic compounds, effectively banning their use in over 160 countries.[57] On the national level only Sweden has prohibited the use of deca-BDE, although at the state level Maine has limited its use in residential furniture.[58]

Although US and European production of PBDEs has been limited in recent years, PBDE exposure remains a pertinent risk to human health. The globalization of commerce has allowed the import and export of goods from regions with little or no regulation on PBDEs. Furthermore, a wide stock of products containing lower brominated congeners still exists, allowing continued exposures. Finally, many congeners have long environmental and biological half-lives. This allows continued exposure long after the products containing these chemicals have been discarded. Bioaccumulation through the food chain has been shown in many animal studies.[59-61] Occupational exposure at recycling facilities has also been shown to result in high levels of PBDEs within human samples.[62]

Since the 1970s, many researchers have reported PBDE concentrations and accumulation in human tissues (reviewed by Frederiksen 2009).[58] In human biomonitoring studies, the PBDE congeners BDE-47, 99, 100 and 153 are most often detected and comprise the majority of total PBDE loading,[48, 51, 53, 63, 64] but it is important to note that these are also the congeners most often assayed, potentially leaving other congeners underrepresented. The human biomonitoring data primarily focus on breast milk and sera with few studies focusing on accumulation or partitioning among gestational compartments. In a recent study conducted in the U.S., high levels of PBDEs were identified in fetal cord serum.[48] This is not unexpected, as PBDEs[65] and chemicals with structural similarity to PBDEs have been shown to cross the placenta,[66, 67] and North American serum levels are typically higher than other regions.[58]

To date, only two epidemiologic studies have been published detailing the effects of prenatal exposure to PBDEs. Main et al. report a significantly higher risk of cryptorchidism for sons born to mothers with elevated PBDE levels in breast milk.[68] In addition, Chao et al. found elevated levels of PBDEs in breast milk correlated with decreased infant birth weight, infant birth length, infant chest circumference and infant body mass index.[69]

Animal studies show that PBDEs exhibit neurodevelopmental,[70-73] hepatic,[74-79] immunological,[80, 81] reproductive[82, 83] and thyroid toxicity.[78, 81, 84] Rabbits orally exposed to PBDEs show decreased gestation length.[82] Although studies of PBDE developmental effects have predominantly used pharmacological doses, recent studies have demonstrated that doses relevant for humans at critical developmental windows can have endocrine and neural effects.[85, 86] Further research is needed to identify how these animal findings translate to humans and to understand the dose-response relationships for both individual PBDEs and PBDE mixtures.

Unpublished studies from our laboratory have shown that PBDEs act as pro-inflammatory stimulants of cytokine release from human gestational membrane cell isolates. Isolated human amnion epithelial cells were shown to secrete IL-6 into the surrounding medium in response to treatment with 500 nM concentrations of BDE-47, BDE-99, BDE-100 or BDE-153. Primary cultures of human amnion fibroblast cells showed similar, but less sensitive effects. Treatments of 10 uM BDE-153, 60uM BDE-47, or 60 uM BDE-100 all showed significantly elevated IL-6 secretion into the surrounding media compared to the

levels seen in solvent treated controls. Although compelling, these results were obtained with a low *n* and are yet to be confirmed.

Because of their environmental persistence, ubiquitous exposure, bioaccumulation and toxcicity, PBDEs warrant further investigations to identify their potential involvement in adverse birth outcomes.

Research objectives.

The research contained in this dissertation is designed to test the hypothesis that ***polybrominated diphenyl ethers bioaccumulate in the human gestational compartment and stimulate cytokine secretion from human gestational membranes.*** The specific aims of this research are to 1) Identify congener-specific PBDE abundances in the human gestational membranes, and amniotic fluid; and 2) Evaluate lipopolysaccharide- and PBDE-induced cytokine secretion from human gestational membranes using punch and transwell-mounted in vitro explant culture systems. Together these background data provide support for this investigation into the induction of inflammatory cytokines within the gestational membranes using PBDEs in an in vitro explant culture system. In the long term, the results from these investigations will hopefully provide the basis for future research into the toxicological mechanisms of preterm birth and PPROM, resulting in enhanced prediction and prevention of adverse birth outcomes.

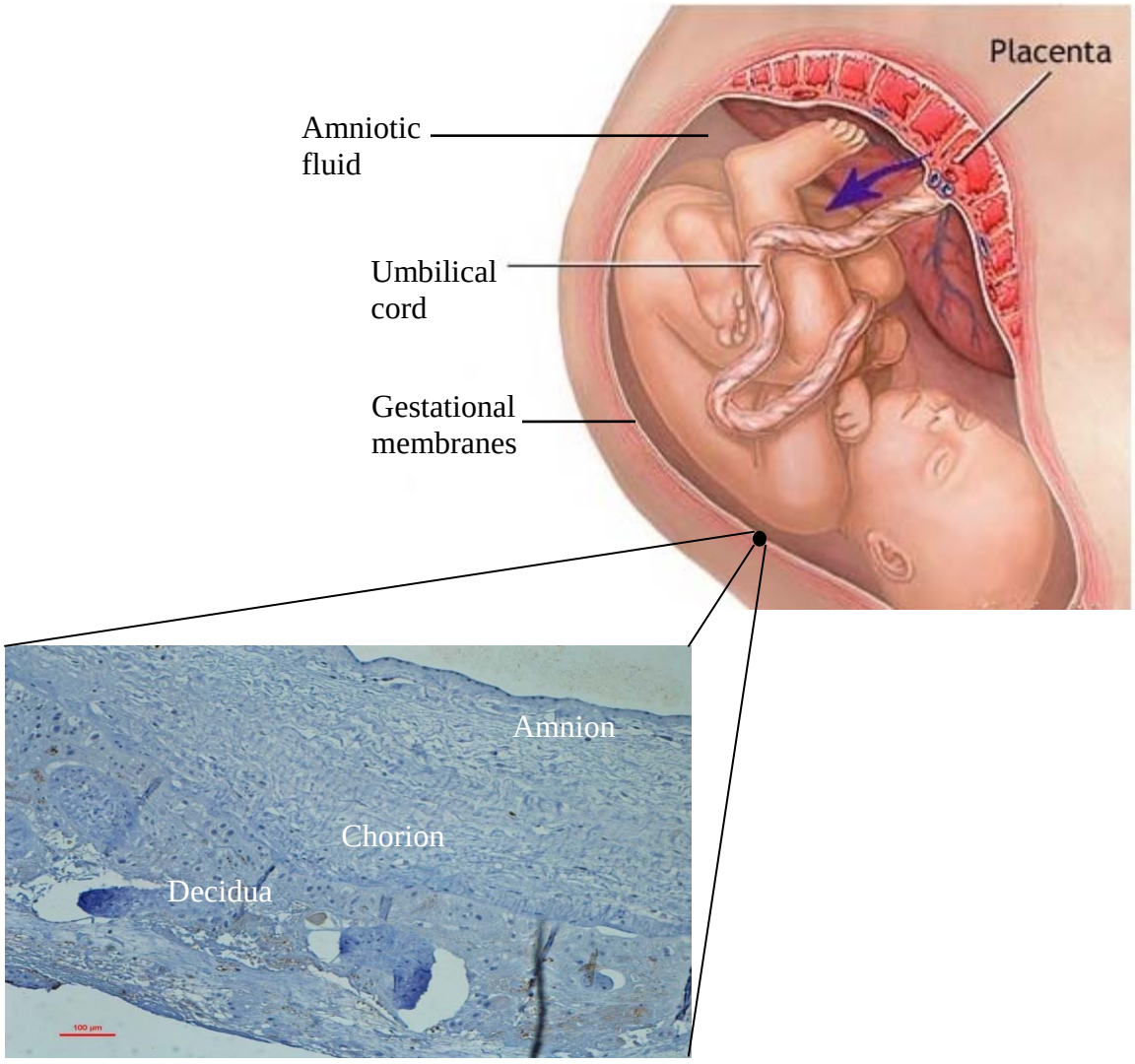

Figure 1.1. Anatomy of the pregnant uterus. Inset: histological section of gestational membrane. Diagram of pregnant uterus adapted from A.D.A.M. Interactive Anatomy 2009. Histological image by Mark Miller.

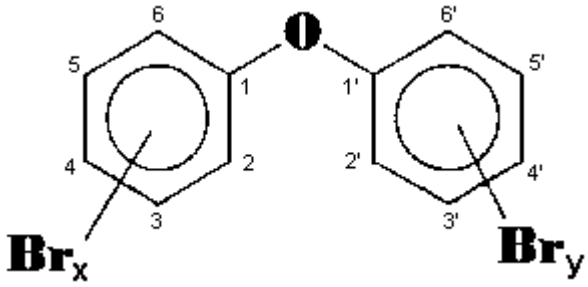

Figure 1.2. Generic structure of polybrominated diphenyl ethers. Hydrogen to bromine substitutions at each of the 10 open positions around the phenyl rings allow 209 distinct congeners.

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Chapter II

Polybrominated diphenyl ethers (PBDEs) in human gestational membranes from women in Southeast Michigan (USA)

Abstract

Polybrominated diphenyl ethers (PBDEs) have been incorporated into many consumer products as flame retardants. Due to their persistence and ability to bioaccumulate, PBDEs are ubiquitous in human blood and breast milk samples from industrialized nations. Although there exists a potential for environmental pollutants such as PBDEs to adversely impact birth outcomes and perinatal health, reports of PBDE levels in human reproductive tissues are limited. The aim of the current study is to evaluate the total levels and congener-specific profiles of PBDEs from human extraplacental gestational membranes. Gestational membranes from five term pregnancies were obtained from non-laboring caesarian deliveries at the University of Michigan Women's Hospital Birth Center. Duplicate samples were extracted and analyzed by GC-MS for twenty-one PBDE congeners. Total PBDE loading was 17.4 ± 3.9 pg/g tissue (5.62 ± 1.28 ng/g lipid). Seventy-eight percent of the total measurable PBDE

loading was due to BDEs 47, 49, 99, 100, and 153, with measured values of 3.63, 3.15, 3.05, 1.74, and 1.90 pg/g tissue (1170, 1018, 983, 561, and 612 pg/g lipid), respectively. The remaining 28% was comprised of BDEs 17, 28, 66, 71, 85, and 154. No octa-, nona- or deca- BDEs were identified. Although previously unreported in the human gestational compartment, BDE 49 comprised 17% of the total PBDE level. This work establishes baseline accumulated levels of PBDEs in gestational membranes of women in Southeast Michigan.

Introduction

Polybrominated diphenyl ethers (PBDEs) are commercially produced synthetic flame retardants consisting of two phenyl rings linked by an ether bond with variable hydrogen to bromine substitutions. Mixtures of PBDEs have been used in textiles, plastics, building materials and insulation. Because of their chemical structure, several of the 209 PBDE congeners tend to be environmentally persistent and bioaccumulative.[1] In human measurement studies, congeners BDE-47, 99, 100 and 153 are most often detected and comprise the majority of total PBDE loading.[2-4] Since 1970, over 50 reports have identified PBDEs in human adipose, liver, breast, whole blood, serum, breast milk, fetal cord blood and placenta (reviewed by Hites).[1] Furthermore, several studies have begun to address gestational compartment dynamics through paired sampling of maternal blood, fetal blood and placenta,[3, 5-8] although none to date has addressed the extra-placental maternal/fetal gestational membranes.

In animal studies, PBDEs exhibit neurodevelopmental, [9, 10] hepatic,[11, 12] immunological[13, 14] and thyroid toxicities.[12] Rabbits orally exposed to PBDEs show decreased gestation length.[15] Because of their environmental persistence and toxicity, the US EPA has identified PBDEs as a priority human health concern.[16] Production of penta-and octa-substituted congeners ceased in 2005 in the USA, and the European Union passed legislation to prohibit all PBDE use after July 2006 (a short-term extension for deca-BDE was granted in October 2005). Although U.S. and European production of PBDEs has ceased except for BDE 209, many PBDEs remain a pertinent risk to human health due to production in other regions, import/export of goods containing PBDEs, the wide stock of PBDE-containing materials in-use, and environmental persistence.[17]

Extraplacental gestational membranes are composed of the amniotic, chorionic and decidual layers that surround the fetus and create a protective barrier during gestation. Minimally vascularized and containing dense collagen, the extraplacental membranes are distinctly different from the highly vascularized placental disk used to transfer oxygen, nutrients and waste products between maternal and fetal blood. Additionally, the extraplacental membranes play an important role in parturition, producing both cytokines and prostaglandins in all three layers during labor.[18, 19] Cytokines and prostaglandins have been closely linked to the biological processes of birth including dynamic cervical remodeling,[20] uterine contractility[21] and gestational membrane rupture.[22] Rupture of these membranes plays a key role in term and preterm parturition. Recent increases in the rate of preterm premature rupture of membranes

(PPROM) has made it a leading cause of preterm birth, particularly in African-American women, and often results in fetal morbidity or mortality.[23] The causes of PPROM are still to be determined, and the role of environmental pollutants such as PBDEs has yet to be addressed.

Americans are ubiquitously exposed to PBDEs, primarily through dust and diet.[24] The 2003-2004 National Health and Nutrition Examination Survey (NHANES) identified measurable PBDE levels in 89% (2040 of 2305) of randomly selected blood serum samples. Once in the maternal blood circulation, PBDEs may access the gestational membranes directly or indirectly. The spiral arteries of the uterus supply blood to the decidua, providing a direct route of transfer to the membranes. In addition, previous studies have shown that PBDEs can enter the gestational compartment and fetal blood circulation, presumably by crossing the placental blood interface.[25, 26] It is also possible that PBDEs reach the gestational membranes indirectly from the fetal compartment through the amniotic fluid. Although no studies to date have assessed PBDE levels in the amniotic fluid, studies of structurally similar compounds (polychlorinated biphenyls) have shown high levels in human amniotic fluid.[27] Because of the likely PBDE exposure of gestational membranes and their critical role in parturition, an assessment of PBDE deposition in the human gestational membranes was undertaken.

The aim of this study was to determine the total PBDE concentrations and congener-specific deposition profiles in human extra-placental gestational membranes from women in southeast Michigan. Twenty-one PBDE congeners

(BDEs 17, 28, 47, 49, 66, 71, 75, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203, 206, 207, 208 and 209 [deca-]) were measured in the present study.

Materials and Methods

Sample collection.

From November 2007 through January 2008 duplicate extraplacental membranes samples were obtained from five healthy non-laboring women undergoing scheduled caesarean section deliveries at 37-39 completed weeks gestation at the University of Michigan Women's Hospital Birth Center in Ann Arbor, Michigan. Exclusion criteria included cigarette smoking, prescription of antibiotics in the past two weeks, collagen vascular disease, immunocompromised conditions, bacterial vaginosis or clinical chorioamnionitis (as noted in the chart or suspected by attending physician), cervical cerclage, third trimester bleeding, major maternal medical conditions (e.g., chronic renal disease, sarcoidosis, hepatitis, HIV), pre-eclampsia, diabetes, multifetal pregnancy, or any other condition which would require the tissue to be sent to pathology. Personal identifiable information was not collected, in compliance with the University of Michigan Institutional Review Board requirements. The investigators had no direct interaction with the human subjects and the tissues collected would have been otherwise discarded.

Following delivery, placentae with attached membranes were transported to the laboratory. Full thickness membranes were separated from the placental

disk, allowing a 3 cm margin to prevent sampling from the transitional zone. Each 1 g sample was compiled from 5-7 random collections of full-thickness membranes and was placed into a glass sample vials with Teflon® lined caps. To prevent contamination of samples, all stainless steel instruments and glassware used for collection were baked at 500 ^{0}C and rinsed with homogenization solvent immediately prior to use. Laboratory practices followed universal safety precautions for handling human tissue (e.g., personnel vaccination for hepatitis B and wearing of laboratory safety glasses, gloves, face mask and lab coat when handling tissues).

Analysis of lipid content.

Replicate 1 g tissue samples were homogenized using a Polytron® PT2100 tissue homogenizer (Kinematica, Bohemia, NY) in 25 ml hexane/ethyl acetate (9:1 v/v), or for comparison, in 25 ml hexane/carbon tetrachloride (4:1 v/v). HPLC grade hexane, carbon tetrachloride and ethyl acetate were purchased from Fisher Scientific (Fair Lawn, NJ), Acros Organics (Geel, Belgium) and Sigma (St. Louis, MO), respectively. Samples were centrifuged and the organic fraction was transferred to pre-weighed glass beakers. The solvent was volatilized under a N_2 (Cryogenic Gasses, Detroit, MI) stream, and the remaining lipid was maintained overnight at 100 °C to remove any remaining water. The glass beakers were then reweighed to determine the lipid content of the initial sample, which was expressed as percent of total tissue weight.

PBDE analysis.

Replicate 1 g samples from each caesarean delivery were homogenized using a Polytron® PT2100 tissue homogenizer in 25 ml hexane/ethyl acetate (9:1 v/v). The organic fractions were transferred to volumetric test tubes and evaporated to 1 ml under a N_2 stream. Samples were spiked with internal standards (CB IUPAC Nos 136 and 204). Each sample (2 µl injection) was analyzed for 21 PBDE congeners by GC-MS (Agilent 6890/5973, Palo Alto, CA, USA) using negative chemical ionization mode and a DB-5 column (30 m, 0.25 mm id, 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). The carrier gas was helium (flow rate of 0.7 mL/min, inlet pressure of 5.43 psi, average velocity of 31 cm/s), and methane was the reagent gas. In all runs, the injector was set at 280 °C. The oven temperature started at 80 °C, held for 2 min, then ramped at 10 °C/min to 300 °C, and held for 46 min. A separate run was made for BDE-209 using a temperature program that avoided fragmentation. In the latter case, the initial temperature again was 80 °C, held for 2 min, then ramped at 50 °C/min to 300 °C and held for 40 min. Calibration standards included BDEs 17, 28, 47, 49, 66, 71, 75, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203, 206, 207, 208 and 209. Method detection limits (MDLs) were estimated based on three times the standard deviation of seven low concentration standards and then dividing by the collected mass (g) of the samples. The estimated MDLs were 0.010-0.020 ng/g for tri- through hexa-BDEs, 0.020-0.040 ng/g for hepta- through nona-BDEs, and 2.5 ng/g for deca-BDE (assuming a 1 g tissue sample).

Data analysis.

Data for BDEs 17, 28, 47, 49, 66, 71, 85, 99, 100, 153, and 154 are represented as the mean ± SEM. Two replicate sample measures of BDE congeners were averaged and a grand mean was calculated for each BDE congener (N=5). Measurements below the MDL were assigned a value of one-half the MDL. Congener concentration as a percent of total PBDE loading was calculated by averaging the percent each congener constituted from each independent sample. BDEs 75, 138, 166, 183, 190, 203, 206, 207, 208 and 209 (deca) were not detected in any samples and have been excluded from subsequent data analysis.

Results and Discussion

The lipid content of 1-g gestational membrane samples using the hexane/carbon tetrachloride solvent averaged 0.30%. Comparable results (0.31%) were obtained using hexane/ethyl acetate. Based on these results, hexane/ethyl acetate was used for tissue extractions prior to PBDE analysis. The lipid content of the human gestational membranes appears lower than the lipid content reported for the human placenta.[6, 28]

On a tissue weight basis, the mean total PBDE concentration in the gestational membranes was 17.41 ±3.98 pg/g (n=5; Table 2.1.). Values for each sample are the average of two replicate measures. From this a grand mean was calculated based on the sample means (N=5). On a lipid weight basis, the total

PBDE level was 5.62 ± 1.28 ng/g (range from 3.06 to 9.53 ng/g) in the gestational membranes (Table 2.1.). Lipid contents for all human gestational membranes measured were 0.31%.

The individual congener profile is shown in Figure 2.1. Congeners exceeding 0.5 ng/g lipid were BDEs 47 (1.17 ng/g), 49 (1.02 ng/g), 99 (0.98 ng/g), 100 (0.56 ng/g) and 153 (0.61 ng/g). Congeners detected at lower concentrations were BDEs 17 (0.15 ng/g), 28 (0.14 ng/g), 66 (0.06 ng/g), 71 (0.32 ng/g), 85 (0.25 ng/g) and 154 (0.36 ng/g). BDEs 75, 138, 166, 183, 190, 203, 206, 207, 208 and 209 (deca) were not identified in any samples.

Individual congener contributions to total PBDEs were assessed for each sample. Percent of total (mean±SEM; N=5) is shown in Figure 2.2. Of the total 5.62 ng/g lipid PBDE loading, BDEs 47, 99, 49, 100, and 153 comprised 22, 21, 17, 12 and 11 %, respectively, for a total of 83%. Additional congeners identified and contributing to the total tissue concentration include BDEs 17 (2%), 28 (2%), 66 (<1%), 71 (6%), 85 (3%) and 154 (4%). BDEs 75, 138, 166, 183, 190, 203, 206, 207, 208 and deca-BDE were below the limit of detection in all samples tested and were therefore excluded from this analysis. When comparing the major contributing congeners analyzed in various studies, this composition is similar to the profiles observed in fish,[29] birds,[30] air,[31] and sediment[32-34] in the Great Lakes region.

This is the first report of PBDE levels in human extraplacental gestational membranes. These PBDE levels are several orders of magnitude lower than those reported by Doucet et al. in placenta proper from North America.[35] Doucet

et al. report highly variable levels ranging from >20,000 ng/g lipid in 2004 to ~200 ng/g lipid in 2005. Increased vascularization may lead to increased burdens in the placenta, although the relative PBDEs in the placental tissue versus blood remaining in the placental sample has yet to be addressed. The impact of lipid weight adjustment from tissue weight could not be addressed because placental lipid content was not reported. A similar study of European women showed significant variation in placental lipid content between Denmark and Finland.[8] When compared with placenta proper from European women (reviewed by Frederiksen et al.) the PBDE levels reported in this study are 3-5 fold higher those previously measured (1.18-1.9 ng/g lipid).[24] This is not unexpected as previous studies suggest biotic and abiotic PBDE concentrations in North America may be several orders of magnitude higher than those found in Europe[1, 24] and levels in gestational membranes appear to be several orders of magnitude lower than placenta. It should also be noted that BDE congeners selected for measurement vary between studies and may contribute to the variation seen in total PBDE levels between studies.

Previous research has suggested BDEs with greater numbers of bromine atoms have shorter biological half lives.[36] Thuresson et al. have estimated the biological half-lives of these large BDEs to range from 15 days (deca-BDE) to 97 days (BDE 183) in human serum. Relatively short biological half-lives in combination with minimal vascularization and potential steric hindrances of infiltration into the dense gestational membranes may contribute to our failure to identify higher order congeners in this study.

The tetra-brominated congener BDE-49 has been identified as a major contributor to PBDE accumulation in fish,[37, 38] including one report from the Great Lakes that found it to be the most abundant congener.[39] Interestingly, our data also show high levels of BDE-49, which has not been previously reported in the gestational compartment. BDE-49 comprised 17% of the total PBDE concentration. Because BDE-49 is often unreported (and presumably unmeasured) in the vast majority of human research, our results suggest that PBDE levels may be being underestimated by as much as 14-19%.

Oral exposure to PBDEs decreases gestational length in rabbits,[15] but a mechanism explaining this finding is lacking. Because the extra-placental gestational membranes play critical roles in pregnancy and parturition,[18-22] they may be important targets of PBDE activity. Current research suggests a possible mechanism by which PBDE stimulation of the gestational membranes may activate parturition pathways via reactive oxygen species generation. Penta-BDE increases reactive oxygen species in neutrophil granulocytes in vitro.[40] Reactive oxygen species are potent activators of the nuclear factor-kappa B (NF-κB) nuclear transcription pathway, which activates cytokine and prostaglandin production (reviewed by Schoonbroodt and Piette).[41] Because cytokines and prostaglandins are important stimulants of parturition,[42-44] premature activation of this pathway by PBDEs could contribute to preterm birth. Further research is needed to investigate this hypothesis, and to assess whether a link can be made between PBDE exposure and preterm birth in women. The identification of total

and congener-specific PBDE levels within the human gestational membranes in the present report is an important first step for further research.

This research provides evidence of accumulation for individual PBDE congeners in human gestational membranes. These initial data indicate a need for further investigation into the partitioning of specific PBDE congeners within the fetal membranes and the greater gestational compartment. Furthermore, these data suggest that evaluation of PBDE effects on gestational membranes may be warranted. The levels reported here for human tissue accumulation will allow assessment of the relevance of PBDE concentrations on gestational membrane responses in future experiments.

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Table 2.1. Total PBDE loading in human maternal- fetal membranes. Total measured concentrations of PBDEs represented as pg/g tissue and ng/g lipid (mean±SEM). All process blanks were below the limit of detection. N=5.

		Sample:						
PBDE Congener	**MDL**	**1**	**2**	**3**	**4**	**5**	**Mean (SEM)**	
17	0.01	0.91	0.86	0.55	<0.01[a]	<0.01[a]	0.46	(0.21)
28	0.01	1.07	0.89	0.17	<0.01[a]	<0.01[a]	0.43	(0.17)
49	0.01	5.07	4.16	2.71	1.34	2.50	3.15	(0.68)
71	0.02	1.23	1.46	0.49	0.97	0.83	0.99	(0.16)
47	0.02	5.49	5.50	2.28	2.30	2.58	3.63	(0.54)
66	0.02	0.53	0.35	<0.02[a]	<0.02[a]	<0.02[a]	0.18	(0.12)
100	0.02	2.88	1.86	1.43	1.05	1.48	1.74	(0.22)
99	0.02	4.17	3.26	2.77	2.62	2.41	3.05	(0.32)
85	0.02	1.68	2.11	<0.02[a]	<0.02[a]	<0.02[a]	0.76	(0.32)
154	0.01	3.83	1.38	0.31	0.01	0.01	1.11	(0.53)
153	0.01	2.68	2.32	1.58	1.16	1.75	1.90	(0.36)
	∑PBDE	29.56	24.14	12.29	9.47	11.58	17.41	(3.98)
	ng/g LW[b]	9.53	7.79	3.97	3.06	3.73	5.62	(1.28)

[a] values falling below the MDL were assigned a value one-half the MDL.
[b] LW= lipid weight; Lipid content for all membranes measured were 0.31%.

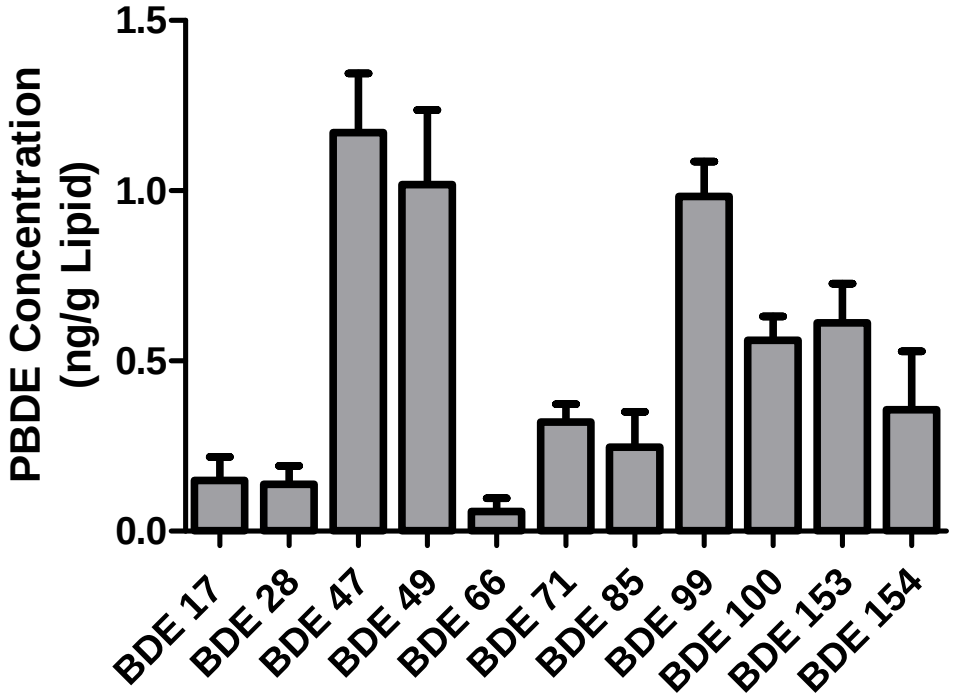

Figure 2.1. PBDE congener concentrations in human gestational membranes. Data are presented as mean±SEM. N=5

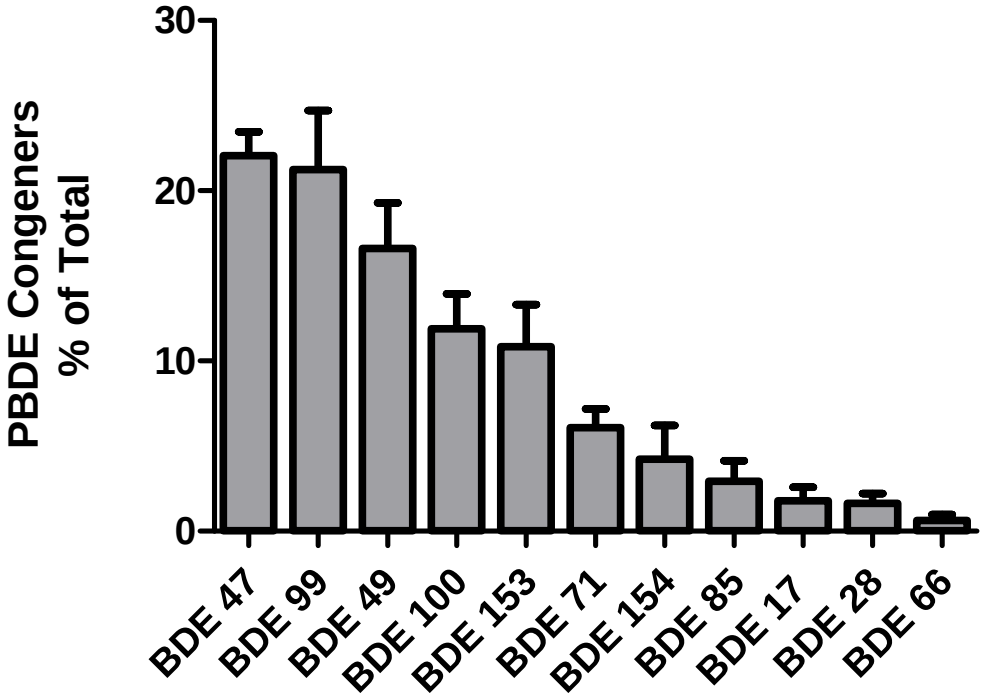

Figure 2.2. PBDE congener concentration as percent of total PBDE loading. Data are presented as mean±SEM. N=5

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Chapter III

Concentrations and speciation of polybrominated diphenyl ethers (PBDEs) in human amniotic fluid

Abstract

Polybrominated diphenyl ethers (PBDEs) are persistent organic chemicals used as flame retardants in textiles, plastics, and consumer products. Many of the 209 PBDE congeners have been classified as persistent organic pollutants at the 4th meeting of the Convention of Parties of the Stockholm Convention on Persistent Organic Chemicals. Although PBDE accumulation in humans has been noted since the 1970s, few studies have investigated PBDEs within the gestational compartment and none to date has identified levels in amniotic fluid. This study reports congener-specific PBDE concentrations in second-trimester amniotic fluid from fifteen women in southeast Michigan, USA. BDEs 17, 28, 47, 49, 66, 71, 75, 85, 99, 100, 138, 153, 154, 166, 183, 190, 203, 206, 207, 208 and 209 were measured by GC/MS. The average total PBDE concentration was 3,795 pg/ml amniotic fluid (range 337 – 21,842 pg/ml). High levels of both higher and lower brominated PBDEs were identified. The most

abundant congeners were BDE-138 (15% of the total BDE), 207 (15%), 47 (10%), 208 (9.5%), and deca (8.4%). BDEs 47 and 99 were identified in each sample. These results show that high PBDE concentrations in amniotic fluid are common, and they suggest that amniotic fluid may participate in the transfer of PBDEs to the fetus, a previously undocumented exposure route. The relatively high levels of PBDEs found in the human gestational compartment warrant further investigations of exposure pathways and potential impacts to birth outcomes and perinatal health.

Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of widely used brominated flame retardants that have been incorporated into many consumer electronics, textiles and furniture. These compounds are not chemically bound within the products and thus may leech into the environment. Although production of penta-BDE (tri- to hexa-BDE mixtures) and octa-BDE (hexa- to nona-BDE mixtures) ceased in the United States in 2004, PBDE exposure remains a concern due to production in other regions, import/export of goods containing PBDEs, the wide stock of PBDE-containing materials remaining in use, and PBDE environmental persistence.[1] In addition, deca-BDE (nona- to deca-BDE mixtures) production continues at increasing rates.

Because of their environmental persistence and toxicity, the US EPA has identified PBDEs as a priority human health concern.[2] Recently, the 4th meeting

of the Convention of Parties of the Stockholm Convention on Persistent Organic Chemicals listed tetra-, penta-, hexa- and hepta-BDEs as persistent organic compounds, effectively banning their use in over 160 countries.[3] Currently, the human reproductive and developmental health risks associated with PBDE exposure have not been thoroughly addressed and many uncertainties remain. Animal studies show that PBDEs exhibit neurodevelopmental,[4, 5] hepatic,[6, 7] immunological[8, 9] and thyroid toxicity.[7] Rabbits orally exposed to PBDEs show decreased gestation length.[10] Further research is needed to identify how these animal findings translate to humans and to understand the dose-response relationships for both individual PBDEs and PBDE mixtures.

Human amniotic fluid plays an important role in gestation. Amniotic fluid begins to collect within the amniotic cavity during the third week of gestation and increases in volume through the 35th week of gestation. During the first 20 weeks of gestation amniotic fluid is primarily formed through maternal secretion. The volume of fluid increases throughout the second trimester from approximately 200 ml at 16 weeks gestation to a peak of 1000 ml at 28 weeks gestation.[11] Amniotic fluid surrounds the fetus until birth, providing nourishment from early stages of pregnancy until birth. Throughout gestation, the fetus is continuously swallowing and "inhaling" amniotic fluid. Intake into the gastrointestinal and respiratory tracks along with dermal exposure provide a direct route of transport into the fetus for toxicants that enter the amniotic compartment. Historically, amniotic fluid analysis has been used to confirm fetal exposure to nicotine and cotinine, but is not a suitable method for routine

assessment of prenatal drug/toxicant exposure due to the invasive nature of sample collection potentially resulting in harm to the fetus.[12]

Since the 1970s, many researchers have reported PBDE concentrations and accumulation in human tissues (reviewed by Frederiksen 2009).[13] Although the human biomonitoring data primarily focus on breast milk and sera, several studies have begun to address the partitioning among and between gestational compartments through paired sampling of maternal blood, fetal blood and placenta.[14-18] Recently, we identified PBDE accumulation in human gestational membranes.[19] To date, however, the potential for PBDEs to accumulate in human amniotic fluid has not been addressed.

The aim of this study was to measure PBDE profiles, including congener-specific and total PBDE concentrations, in amniotic fluid collected from women in southeast Michigan, USA. This information will help to evaluate the significance of PBDE transport into amniotic fluid and from the amniotic fluid into other gestational matrices, including the developing fetus.

Materials and Methods

Sample collection.

In February and March 2009, excess clinical amniotic fluid samples, typically 15 ml, were collected at the University of Michigan Women's Hospital in Ann Arbor, Michigan, USA. Deidentified samples containing excess amniotic fluid from 15 women undergoing clinically-indicated amniocentesis for

identification of fetal genetic aberrations during their second trimester were analyzed for PBDE profiles. An average volume of 15 ml of amniotic fluid per sample was received in the cytogenetics laboratory and centrifuged at 280 g for 10 min to remove the cellular component. The excess supernatant was transferred into tubes without patient identification and stored at -80 °C. The resulting supernatant was used for all analyses.

The 15 women represent an anonymous sample where personal identifiable data were not collected, in compliance with the University of Michigan Institutional Review Board requirements. The investigators had no direct interaction with the human subjects. All amniotic fluid specimens used in this study would have been otherwise discarded.

Analysis of lipid content.

Amniotic fluid supernatants were evaluated independently for lipid content. For each amniotic fluid sample, 1 ml of amniotic fluid was denatured with 1 ml HCl followed by 6 ml isopropyl alcohol. Samples were then extracted using 5 ml methyl tert-butyl ether and hexane (1:1). Sample fractions were separated by centrifugation and the organic fraction collected. The extraction process was repeated three times and organic fractions were combined in a previously weighed beaker. The organic fraction was volatilized under a flowing nitrogen stream until dry, and then baked at 100°C for 12 h to remove all water. Beakers were reweighed and lipid weight per ml amniotic fluid was calculated.

PBDE analysis.

Amniotic fluid supernatants were evaluated for 21 PBDE congeners. For each amniotic fluid sample, 7 ml of amniotic fluid was denatured with 2 ml HCl, followed by 12 ml isopropyl alcohol. Samples were then extracted using 10 ml methyl tert-butyl ether and hexane (1:1). Sample fractions were separated by centrifugation and the organic fraction collected. The extraction process was repeated three times and organic fractions were combined and volatilized under a flowing nitrogen stream until nearly dry. Samples were resuspended in 5 ml hexane and cleaned with 3 ml sulfuric acid. The organic fraction was removed and neutralized using sodium carbonate.

Instrumental analyses used GC/MS (Agilent 6890, Palo Alto, CA, USA), negative chemical ionization mode, a DB-5 column (30m, 0.25 mm i.d., 0.25 μm film thickness, J&W Sci, Folsom, CA, USA) and a 2 μl splitless injection. The carrier gas was helium (0.7 ml/min, inlet pressure 5.43 psi, average velocity 37 cm/s), and methane was the reagent gas. The injector was set at 280 ^{o}C. The oven temperature started at 80 ^{o}C, held for 2 min, ramped at 10 ^{o}C/min to 300 ^{o}C, and held for 40 min. Calibration standards included BDEs 17, 28, 75, 49, 71, 47, 66, 100, 99, 85, 154, 153, 138, 166, 183, 190, 203, 208, 207, 206 and 209 (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). Standards were run for a wide range of concentrations (100 to 5000 ng/ml) that encompassed the range expected in field samples. The MS was operated in selected ion monitoring mode, and quality matching routines included at least two ions. Structural verification used additional ions and confirmed spectra by requiring

proper ratios of ions for each analyte. Complete details of the instrumental analysis have been published previously.[20]

Quality assurance.

In parallel with the analyses, field, lab and method blanks were processed and shown to be clear of detectable contamination. Linearity and drift checks were analyzed with each sample batch. Repeat analysis of a standard injected every fifth sample varied by less than 10% and linearity plots produced r^2 values greater than 0.999. Surrogate spike recoveries ranged from 82-106%.

Data analysis.

Measurements falling below the method detection limit (MDL) were assigned a value of one-half the MDL. Descriptive statistics, including the mean, the standard error of the mean, median, range, and the percentage of observations above the MDL, were calculated for each congener. For each sample, the total PBDE (∑PBDE) concentration was calculated as the sum of the 21 congeners, and a grand mean was calculated as the average of the sample ∑PBDEs. Concentrations are expressed as volumetric and lipid-based fractions. Congener-specific abundances were calculated for each sample as the congener concentration divided by the total PBDE loading and expressed in percent. Sample statistics utilized the mean, median and sum of these abundances. The relationship of lipid content to BDE levels was evaluated using the Spearman

rank order correlation. Relationships among BDE levels were also evaluated using the Spearman rank order correlation.

Results and Discussion

Lipid content.

The lipid content averaged 0.94 ± 0.03% and ranged from 0.74 to 1.25%. The lipid content reported here is lower than a previous report that found values as high as 22.5 ± 1.2% in human amniotic fluid.[21] The difference between the studies is likely due to the use of amniotic fluid supernatant in the present study. Cellular debris, which is lipid rich, was removed by centrifugation prior to lipid and PBDE measurements.

PBDE concentrations.

Concentrations varied widely among the samples, but BDE-47 and 99 were found in all samples tested. On a volumetric basis, the ∑PBDE concentration in amniotic fluid averaged 3,795 ± 1,529 pg/ml (median = 1,253 pg/ml; n=15; Table 3.1), and individual samples ranged from 337 to 21,842 pg/ml. On a lipid basis, this is equivalent to a ∑PBDE concentration of 404 ± 163 ng/g lipid (median = 133 ng/g).

The correlation between lipid content and ∑PBDE concentration was low and not statistically significant (p=0.86). Similarly, no correlation was found between lipid content and any of the individual congeners (p=0.15-0.99). These

results are not unexpected given the small amount of variation in lipid content. For example, the highest ∑PBDE concentration (21,842 pg/ml = 2,324 ng/g lipid) was found in a specimen with a lipid content of 1.01%, just slightly above the study average.

Significantly, the highest ∑PBDE level in amniotic fluid is comparable to the highest U. S. reports of PBDE levels in breast milk (1,900 ng/g lipid),[22] although it is below that reported in adipose tissue (9,600 ng/g lipid).[23] Interestingly, tri- to octa-congeners accounted for 82% of the ∑PBDEs in the sample with the highest ∑PBDE level well above the 60% average for all samples.

Many factors can affect an individual's PBDE body burden and the concentrations of PBDEs found in amniotic fluid. These include variation in an individual's PBDE exposure before and during pregnancy, the number of previous children for each woman, the timing of amniocentesis within the second trimester, and the woman's age. Due to the nature of sample collection, these factors were not controlled in this study.

Congener abundances.

The speciation of PBDEs found in human amniotic fluid is shown in Figure 3.1. The median congener abundances, interquartile range and max/min values are displayed in Figure 3.2. We identified a wide range of congeners in this study, and all 21 target congeners were measured above MDLs in at least three of the fifteen samples (20%). As noted earlier, BDE-47 and 99 were identified in

all samples; additionally, BDE-100, 209, and 17 were identified in 93%, 80% and 80% of samples, respectively. Based on median abundances, the dominant congeners were BDE-206, 209, 203, 208 and 207, representing 23, 16, 12, 10 and 9%, respectively, of the ∑PBDEs.

The contribution of PBDE homologues are shown in Figure 3.3. The PBDE profile of the amniotic fluid shows an abundance of PBDE congeners with high numbers of bromine substitutions, representing a shift toward higher brominated congeners as compared to profiles reported for breast milk and serum.[13] This shift may be due to altered partitioning in amniotic fluid or differences in exposure for our sampling population. Although the lower brominated congeners (tri- through octa-BDEs) are no longer produced in the United States, the homologue profile shows that they still constitute the bulk (60%) of the ∑PBDE loading. Mobilization of body burdens, exposure from the import/export of goods containing PBDEs, the wide stock of PBDE-containing materials still in use, and environmental persistence may explain the legacy of these congeners we see accumulating in nascent amniotic fluid.

PBDE correlations.

The Spearman rank order correlations comparing the PBDE measurements from human amniotic fluid are detailed in Table 3.2. A strong correlation was seen between the deca- and nona-BDE congeners. This is not surprising since nona-BDEs are often found as contaminants of the deca-BDE commercial mixture, and therefore we would expect individuals exposed to deca-

BDE to also be exposed to nona-BDEs. Stapleton et. al have shown that deca-BDEs can break down into nona-congeners in fish.[24] Interestingly, BDE-71 also showed a strong correlation with the deca- and nona- congeners. Although not previously reported in any system, this correlation raises the question of whether the higher order congeners can break down into lower order congeners.

Strong correlations were seen among the specific homologues. Hexa-BDEs 138, 153 and 154 were all found to have significant correlations with each other. In addition, tetra-BDE congeners 49, 71 and 75 also showed significant correlations. This is expected because these BDE homologues are often found together in the commercial mixtures of PBDE used prior to 2004.

The present study is the first to report concentrations and congener abundances for PBDEs within human amniotic fluid. Because amniotic fluid surrounds the fetus during gestation, toxicant concentrations in amniotic fluid are likely correlated to exposure to the fetus. Although our sample size is modest, the measured concentrations appear significant, and suggest the need for further investigation into the partitioning of PBDEs among the amniotic fluid, the developing fetus, and the maternal circulation. Our results suggest the need for further investigation of an exposure pathway not considered previously: the amniotic fluid-to-fetus route.

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Table 3.1. PBDE Concentration in Human Amniotic Fluid. (N=15)

		Amniotic Fluid Concentration (pg/ml fluid)			
PBDE	**MDL**	**Mean (SEM)**	**Median**	**Range**	**N>MDL (%)**
17	0.01	108 (91)	11	<MDL-1237	80
28	0.02	206 (206)	<MDL	< MDL -1374	27
47	0.05	380 (295)	49	9.11-4491	100
49	0.02	50 (43)	<MDL	< MDL -655	40
66	0.02	11 (6)	4.92	< MDL -90	53
71	0.01	31 (29)	<MDL	< MDL -434	33
75	0.02	12 (8)	<MDL	< MDL -119	40
85	0.01	75 (25)	24	< MDL -308	73
99	0.06	188 (95)	31	4-1124	100
100	0.05	187 (127)	37	< MDL -1916	93
138	0.01	565 (312)	37	< MDL -4456	67
153	0.03	143 (77)	18	< MDL -1119	67
154	0.02	192 (140)	36	< MDL -2134	53
166	0.01	20 (18)	<MDL	< MDL -273	20
183	0.05	28 (17)	<MDL	< MDL -263	40
190	0.05	36 (25)	<MDL	< MDL -371	27
203	0.05	177 (61)	97	< MDL -839	60
206	0.22	149 (69)	79	< MDL -957	60
207	0.22	559 (346)	71	< MDL -5213	67
208	0.22	362 (181)	189	< MDL -2598	67
209	0.65	319 (102)	133	< MDL -1286	80
∑PBDE (pg/ml fluid)		3795 (1529)	1253	337-21842	
∑ Tri-octaBDEs		2407 (1183)	788	81-17977	
∑PBDE (ng/g lipid)		404 (163)	133	36-2324	
∑ Tri-octaBDEs		256 (126)	84	9-1912	

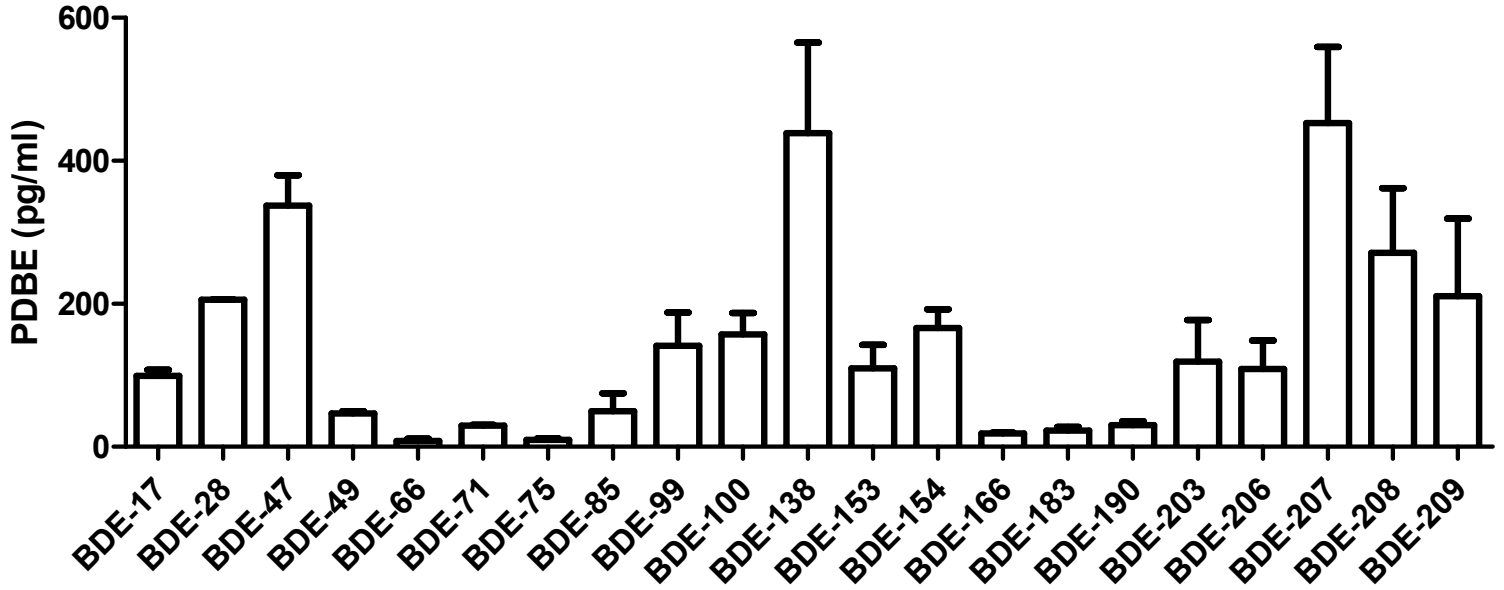

Figure 3.1. PBDE congener concentrations in human amniotic fluid. Data are presented as mean±SEM. N=15.

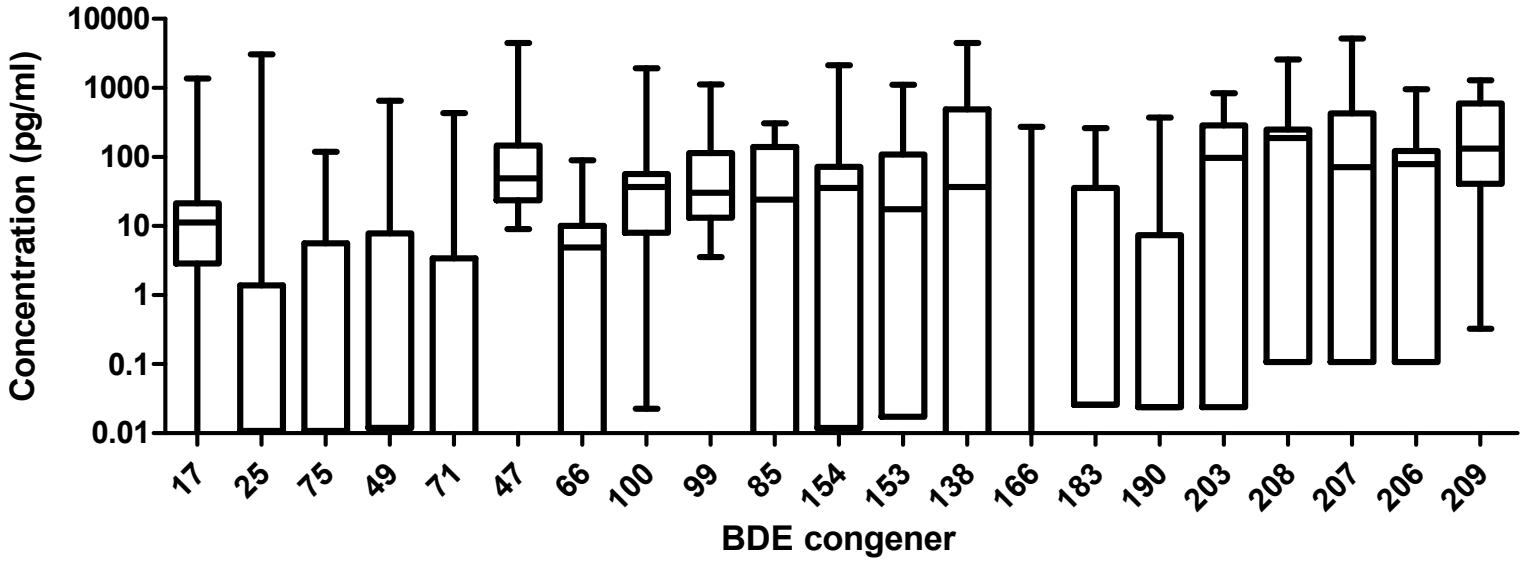

Figure 3.2. Congener profile for PBDEs in human amniotic fluid. Box plots present median and interquartile range. Whiskers represent maximum and minimum values. N=15.

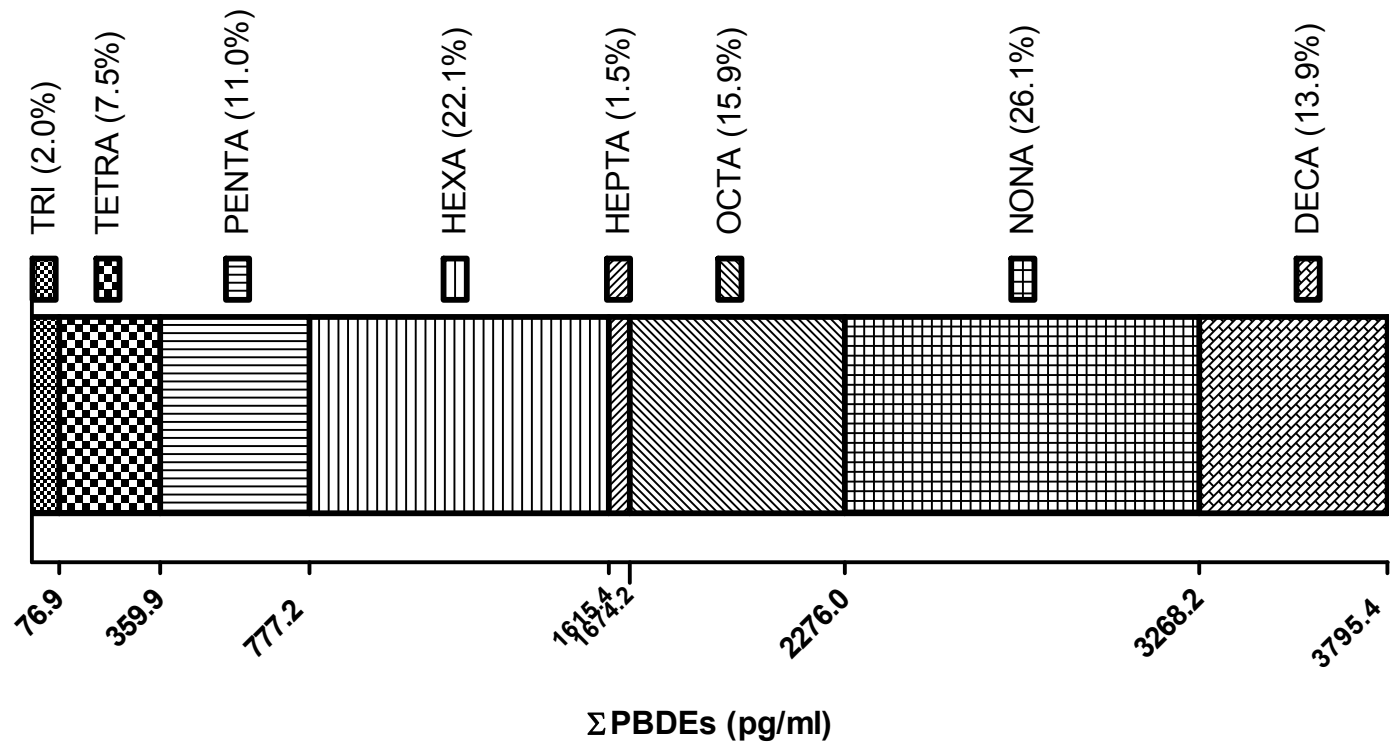

Figure 3.3. Profile of PBDE homologues in human amniotic fluid. The percent contribution based on median abundances for each homologue group (tri-, tetra-, penta-, etc.) is listed above the diagram. The PBDE median values (pg/ml) used to calculate these percentages are represented on the x-axis.

Table 3.2. Spearman Rank Order Correlations Matrix for PBDE Congeners in Human Amniotic Fluid[a]

	BDE-28	BDE-75	BDE-49	BDE-71	BDE-47	BDE-66	BDE-100	BDE-99	BDE-85	BDE-154	BDE-153	BDE-138	BDE-166	BDE-183	BDE-190	BDE-203	BDE-208	BDE-207	BDE-206	BDE-209	∑PBDE	∑PBDE-deca
BDE-17	0.09	**0.04**	**0.04**	0.12	**0.01**	0.35	**0.00**	**0.00**	0.47	**0.01**	**0.01**	0.31	0.45	0.58	**0.05**	0.80	0.08	0.06	0.11	0.21	**0.00**	**0.00**
BDE-28		**0.02**	0.06	**0.03**	0.38	0.73	0.17	0.29	0.50	0.19	0.91	0.81	**0.03**	**0.04**	0.21	0.57	0.18	0.18	0.22	0.16	0.37	0.70
BDE-75			**0.00**	**0.00**	0.31	0.36	**0.04**	0.16	0.61	0.37	0.32	0.40	0.13	0.22	0.43	0.29	0.11	0.06	0.07	0.09	**0.04**	0.30
BDE-49				**0.00**	0.36	0.29	**0.04**	0.19	0.63	0.41	0.26	0.37	0.23	0.26	0.29	0.37	0.15	0.11	0.10	0.18	**0.04**	0.28
BDE-71					0.51	0.58	0.10	0.22	1.00	0.71	0.76	0.94	0.17	0.58	0.83	0.66	**0.02**	**0.02**	**0.01**	**0.02**	0.12	0.84
BDE-47						**0.04**	**0.00**	**0.02**	0.08	**0.00**	**0.00**	**0.03**	0.30	0.29	0.21	0.43	0.77	0.51	0.40	0.39	**0.00**	**0.00**
BDE-66							0.20	0.31	**0.00**	**0.02**	0.05	**0.00**	0.10	**0.03**	**0.02**	0.25	0.29	0.17	0.42	0.87	0.09	**0.04**
BDE-100								**0.00**	0.14	**0.00**	**0.00**	0.11	0.27	0.51	0.07	0.37	0.23	0.10	0.11	0.15	**0.00**	**0.00**
BDE-99									0.17	**0.00**	**0.02**	0.17	0.09	0.57	0.31	0.36	0.13	0.07	0.13	0.08	**0.00**	**0.00**
BDE-85										**0.03**	0.08	**0.00**	0.05	**0.01**	0.08	0.45	0.03	0.12	0.15	0.57	0.28	**0.03**

BDE-154											**0.00**	**0.04**	0.09	0.06	0.11	0.21	0.71	0.65	0.62	0.33	**0.00**	**0.00**
BDE-153												**0.02**	0.33	0.31	0.08	0.90	1.00	0.55	0.49	0.55	**0.00**	**0.00**
DBE-138													0.16	0.10	0.13	0.99	**0.04**	0.17	0.15	0.38	0.07	**0.00**
BDE-166														**0.00**	0.45	0.70	0.79	0.73	0.65	0.05	0.18	0.13
BDE-183															0.17	0.79	0.41	0.48	0.64	0.43	0.58	0.21
BDE-190																0.82	0.58	0.47	0.73	0.42	0.16	0.05
BDE-203																	0.95	0.59	0.96	0.80	0.78	0.77
BDE-208																		**0.00**	**0.00**	**0.00**	0.15	0.95
BDE-207																			**0.00**	**0.00**	0.07	0.70
BDE-206																				**0.00**	0.07	0.86
BDE-209																					0.08	0.72
∑PBDE																						**0.00**

[a] P-values are displayed for each pair of congeners. Statistically significant P-values less than 0.05 are in bold. All correlations are positive.

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Chapter IV

Comparison of LPS-stimulated release of cytokines in punch versus transwell tissue culture systems of human gestational membranes

Abstract

Cytokine signaling within the amnion, chorion and decidua plays an important role in membrane rupture and therefore the timing of birth. The predominant in vitro explant culture system for evaluating cytokine induction in human gestational membranes has been the free-floating biopsy punch culture. Punch systems have been used to investigate the impact of various toxicants, pharmaceuticals and genetic variation on expression of pro-inflammatory cytokines. More recently, a dual compartment transwell culture system has been developed that more closely mimics the intrauterine compartment. The current study compares these two systems with respect to release of pro- and anti-inflammatory cytokines in response to lipopolysaccharide (LPS), a model stimulant. Tissue samples were exposed to 100 ng/ml LPS for 12 h and cytokines were measured by ELISA. Data are expressed as increase relative to non-treated controls. Levels of interleukin-6 increased in punch culture medium

samples (34.2 fold) to a significantly greater extent than in the medium from transwell cultures in the amnion (6.6 fold) or choriodecidual (7.1 fold) compartments. Interleukin-8 also showed a significantly greater induction in punch (4.8 fold) than transwell amnion (1.6 fold) or choriodecidual (1.7 fold) samples. The anti-inflammatory interleukin-10 showed a significant difference between punch (36.5 fold) and transwell amnion (15.4 fold) samples, but no difference was observed between punch and transwell choriodecidual (28.5 fold) samples. Neither interleukin-1β nor tumor necrosis factor-α (TNF-α) showed a significant difference between the punch and transwell samples. These results suggest that methodological factors in gestational membrane culture systems can cause differential responses to inflammatory stimuli, confounding comparisons between studies that use the different gestational membrane culture systems.

Introduction

Cultures of the extraplacental human gestational membranes (amnion, chorion and decidua) provide useful in vitro research models for inquiries into obstetric challenges such as inflammation, preterm premature rupture of membranes (PPROM) and preterm birth. Gestational membranes collected immediately after birth and cultured in vitro allow assessment of responses in tissues with an intact cellular matrix. Such cultures have been used to study stimulated production and release of cytokines and prostaglandins in

investigations of inflammatory responses of the membranes.[1-7] Two model systems used extensively to study human gestational membranes in vitro are a biopsy punch explant culture system[1-4, 6, 8-14] and a transwell mounted explant culture system.[5, 7, 15-19]

Biopsy punch explants from human gestational membranes have been used extensively to study responses of the intact full-thickness membranes [4, 10] as well as separated amnion [6, 9] or choriodecidua.[1, 12] In the punch explant culture system, an excised piece of gestational tissue is free-floating in culture medium. This single-compartment system has been used to investigate cytokine,[1, 3, 4, 6, 8, 9, 13, 14] prostaglandin,[1-3, 6, 12-14] adipokine[20] and protease[3, 10] regulation. In contrast, a transwell culture system employs gestational membrane explants attached to rigid frames and suspended in culture medium. The transwell creates a two-compartment system with discrete amniotic and choriodecidual chambers. The transwell system has been used to study cytokine and prostaglandin production induced by bacteria,[5, 7, 18, 21] yeast [19] or oxygen tension [15] as well as meconium interactions with the gestational membrane.[16] The two-compartment system has provided a model system in which investigators can expose and collect media samples from each side of the membrane independently.

To date no reports have directly compared the cytokine responses of traditional free-floating biopsy punch explants and transwell mounted explant cultures. The potential for different responses due to model-related restrictions suggests a need for further investigation. Therefore, the aim of this study is to

compare floating biopsy punch and transwell mounted explant culture systems for lipopolysaccharide (LPS)-induced cytokine responses.

Materials and Methods

Sample collection.

Human extraplacental gestational membranes were obtained from healthy non-laboring women undergoing scheduled caesarean section deliveries at 37-39 completed weeks gestation at the University of Michigan Women's Hospital Birth Center in Ann Arbor, Michigan. Exclusion criteria included cigarette smoking, prescription of antibiotics in the past two weeks, collagen vascular disease, immunocompromised conditions, bacterial vaginosis or clinical chorioamnionitis (as noted in the chart or suspected by attending physician), cervical cerclage, third trimester bleeding, major maternal medical conditions (e.g., chronic renal disease, sarcoidosis, hepatitis, HIV), pre-eclampsia, diabetes, multifetal pregnancy, or any other condition which would require the tissue to be sent to pathology. The investigators had no direct interaction with the human subjects and the tissues collected would have been otherwise discarded. Personal identifiable information was not collected, in compliance with the University of Michigan Institutional Review Board requirements.

Following delivery, placentae with attached membranes were transported to the laboratory in warm isotonic phosphate buffered solution (PBS). Full thickness membranes were dissected from the placental disk, allowing a 3-cm

margin to prevent sampling from the transitional zone. A 3-cm margin was also maintained around the site of incision. Laboratory practices followed universal safety precautions for handling human tissue (e.g., personnel vaccination for hepatitis B and wearing of laboratory safety glasses, gloves, face mask and lab coat when handling tissues).

Culture and treatment.

From each of seven gestational membranes, twelve punch explants and eight transwell explants were collected from throughout the membranes. Under sterile conditions, intact membranes were rinsed with isotonic PBS to remove adherent blood clots and randomly sampled for 12-mm punches and 3-cm x 3-cm squares of tissue. Individual punches were free-floated in 12 well polystyrene tissue culture plates (Becton Dickinson, Franklin Lakes, NJ). Tissue squares were mounted on polycarbonate transwell inserts (gift from Corning Inc., Corning, NY), attached with elastic latex bands. Excess tissue extending beyond the band was trimmed and the transwell insert was suspended in culture medium (Figure 4.1.). Tissues were mounted with the choriodecidua facing the inner chamber and the amnion facing the outer chamber, creating two discrete compartments.

Cultures were acclimated for 24 h in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin (HyClone, Logan, UT) and 1% fetal bovine serum (FBS) (Gibco, Grand Island, NY). Culture medium was changed after 6 h and 22 h during acclimation. After acclimation, six punch and four transwell cultures were

randomly assigned for culture in DMEM or DMEM supplemented with 100 ng/ml lipopolysaccharide (LPS; *Salmonella typhimurium,* ATCC #14028; List Biological Laboratories, Inc., Campbell, CA) for 12 h. Punch explants were exposed in 1 ml total volume of medium. In the transwell cultures, the inner chamber medium volume was 0.5 ml and the outer chamber volume was 1.5 ml.

Cytokine assessment.

Concentrations of the cytokines IL-1β, IL-6, IL-8, IL-10 and TNF-α were quantified by ELISA using the manufacturer's suggested protocol (Duosets, R&D systems, Minneapolis, MN) in the Immunology Core Facility at the University of Michigan Cancer Center. Immunohistochemistry was performed at the University of Michigan Research Histology & Immunohistochemistry Laboratory. Samples were fixed in 10% formalin and stored in 70% ethanol until processing. Each sample was paraffin embedded, sectioned and labeled with IL-6 and IL-8 specific polyclonal primary antibodies and biotinylated secondary antibodies (Abcam Inc., Cambridge, MA).

Data analysis.

Data are represented as concentration mean ± SEM for each treatment within a culture method. Because the culture methods used different medium volumes, data are also represented as fold-increase over control to allow comparison of LPS activation between culture methods (mean±SEM). Differences between control and LPS-stimulated groups were analyzed using t-

tests. Mann-Whitney rank sum tests were used for data sets that were not normally distributed. Differences between fold increases of the culture methods were analyzed using one-way analysis of variance with Student-Newman-Keuls method for posthoc pairwise comparisons.

Results

Table 4.1. provides the cytokine concentrations in the medium of control and LPS-exposed human gestational membranes in biopsy punch and transwell cultures. Non-treated controls showed no differences in basal release of IL-1β, IL-6, IL-10 and TNF-α between the amniotic and choriodecidual compartments of the transwell cultures. In contrast, IL-8 had greater unstimulated secretion into the choriodecidual compartment (236.48±32.46 ng/ml) than into the amniotic compartment (154.30±29.15 ng/ml) in untreated controls ($p<0.001$). LPS induced a significant increase of IL-1β, IL-6, IL-8, IL-10, and TNF-α in the tissue punch cultures as well as on each sides of the transwell cultures compared with respective controls ($p<0.05$; Table 4.1.).

We evaluated five pro- and anti-inflammatory cytokines in this study and identified LPS-induced secretion increases of all cytokines tested. In the punch biopsy explant cultures, LPS increased IL-1β 6.9 (±1.4) fold , IL-6 34.2 (±11.7) fold, IL-8 4.8 (±1.4) fold, IL-10 36.5 (±6.0) fold, and TNF-α 222.3 (±47.1) fold over control explants. In the transwell cultures, LPS increased secretion of IL-1β 10.3 (±5.6) fold, IL-6 6.6 (±1.8) fold, IL-8 1.6 (±0.1) fold, IL-10 15.4 (±4.5) fold, and

TNF-α 258.2 (±115.5) fold on the amniotic side of the membrane and IL-1β 6.4 (±2.8) fold, IL-6 7.1 (±2.3) fold, IL-8 1.7 (±0.2) fold, IL-10 28.5 (±4.9) fold, and TNF-α 254.1 (±105.2) fold on the choriodecidual side of the membrane (Figure 4.2.).

By representing LPS induction within a treatment model as a fold increase over non-treated control, we allow a comparison between treatment models that is not biased by variations in tissue mass or medium volume (Figure 4.2.). On the basis of fold increase over control, LPS-induced secretion of both IL-6 and IL-8 was greater in the punch culture system than in either compartment of the transwell culture system ($p<0.05$). Interestingly, IL-10 showed a significantly lower fold increase on the amniotic but not the choriodecidual chamber of the transwell culture when compared to punch cultures ($p=0.015$ and $p=0.227$, respectively). Furthermore, within the transwell culture system, the IL-10 increase in the amniotic chamber was significantly lower than the IL-10 increase in the choriodecidual chamber ($p<0.042$). There were no statistically significant differences for IL-1β and TNF-α secretion between transwell and punch cultures.

Immunohistochemical labeling of IL-6 and IL-8 was performed on paraffin embedded sections to identify cellular origins of these cytokines within the amnion, chorion and decidua. IL-6 and IL-8 were selected for evaluation due to their significant secretion differences between punch cultures and both compartments of the transwell cultures. Figure 4.3. shows representative immunohistological sections of control and LPS treated membranes from punch and transwell culture systems. The IL-6 staining was confined to the decidual

region and primarily localized to the intercellular space. No positive staining was observed in the dense collagen layer or in the amnion (Figure 4.3.). Increased IL-6 labeling was observed within the chorion and decidua following treatment with LPS and the labeling continued to be distributed in the intercellular space. Similarly, IL-8 is identified in the chorionic and decidual regions, but is also visualized in the sub-epithelial amnion (Figure 4.4.). Unstimulated punch and transwell membranes show IL-8 positive labeling localized intracellularly within the cytoplasm of histiocytes. After stimulation with LPS, intracellular IL-8 can still be visualized but in addition intercellular signal is also present in the chorion and decidua.

Discussion

Cytokines are important protein mediators of cell signaling within the human gestational membranes. Pro-inflammatory cytokine genes are up-regulated during labor,[22, 23] leading to increased cytokine secretion and triggering the induction of prostaglandins[24] and matrix metalloproteinases[25] that stimulate uterine contraction,[26] cervical dilation[27] and the rupture of the gestational membranes.[28] Elevated IL-1β, IL-6 and TNF-α levels in the amniotic fluid are associated with both term[29] and preterm[30] parturition.

In vitro cytokine secretion into explant culture medium has been widely used as a measure of inflammation for human gestational membranes.[1-8] Published studies have adopted two major model systems, the biopsy punch

explant culture system and the transwell mounted explant culture system. In the present study, we compared LPS-stimulated cytokine release for these two model systems. Consistent with previously published reports,[4, 7, 8] elevated secretion of all cytokines tested (IL-1β, IL-6, IL-8, IL-10 and TNF-α) was observed in response to endotoxic lipopolysaccharide stimulation. Although both systems have been widely used, the present report is the first direct comparison of the culture systems and demonstrates that the pattern of LPS-stimulated cytokine secretion differs between these culture methods.

Elevated IL-6 concentrations in the amniotic fluid have been used as a marker of membrane inflammation [31] and are associated with preterm labor [32] and PPROM.[33] After stimulation with LPS, the medium concentrations of IL-6 were five-fold greater in punch cultures when compared to transwell secretion into the amniotic or choriodecidual chambers. The IL-6 media concentrations suggest an amplified cytokine response to LPS in the punch cultures, possibly due to positive feedback regulation of IL-6.

Similar to previous reports showing intense IL-6 staining of decidual and chorionic trophoblasts,[34, 35] the immunohistochemical labeling of IL-6 reported in this study was confined to the choriodecidual region, with no positive staining observed in the dense collagen layer or in the amnion. In contrast, this study reports IL-6 primarily localized to the intercellular space where others have reported intracellular localization.[34, 35] Intercellular IL-6 would suggest that high levels of IL-6 are rapidly released from choriodecidual cells and diffuse into the surrounding tissue. Previous findings support the choriodecidua as the primary

site of IL-6 production in non-laboring membranes, with little or no production in the amnion.[6, 14] In a series of papers by Simpson et al., separated human amnion or choriodecidua from non-laboring deliveries were stimulated using LPS (5 ug/ml) and IL-6 secretion was measured by ELISA. Results showed a significant 3 fold increase from the choriodecidual explants and no effect in the amniotic explants.

Interestingly, we observed similar levels of IL-6 in the amniotic and choriodecidual media, as reported by Zaga-Clavellina et al. after 24 h of stimulation with cervicovaginal derived *E. coli.*[18] Taken together the histological data and transwell culture medium concentrations establish the choriodecidua as the primary site of IL-6 production. Cytokines have been previously shown to cross the gestational membranes in vitro but at rates much lower than those necessary to allow equal choriodecidual and amniotic levels of IL-6 after 12 h of stimulation.[17] Therefore, we conclude that IL-6 crosses the choriodecidual-amniotic barrier at rates much greater than previously suggested.

Although levels of IL-8 secretion from gestational membranes have not been well documented, our LPS-induction appears similar to a previous report.[4] The current study reports a 4.8 (±1.4) fold increase using the punch system, similar to the 3.4 fold increases reported previously by Menon et al. after 24-h stimulation with 100 ng/ml LPS using a punch explants from intact membranes.[4] The mean IL-8 concentration seen in the present study was nearly three-fold greater in punch cultures compared with transwell culture medium in the amniotic or choriodecidual chambers. It should be noted that these modest fold increases

represent relatively large changes in release and local concentration due to the high unstimulated secretion of IL-8. The immunohistochemistry provides evidence of stimulation in all three layers of membrane, suggesting both amniotic and choriodecidual secretion.

The LPS-induced secretion of IL-10 was 15.4 (±4.5) fold and 28.5 (±4.9) fold greater than untreated controls in the transwell amniotic and choriodecidual compartments, respectively. This significant difference is the first report of IL-10 being directionally secreted from the gestational membranes in vitro.

IL-10 is an important anti-inflammatory cytokine, inhibiting immune function and suppressing pro-inflammatory cytokine production.[36] Induced by other cytokines and prostaglandins,[36, 37] IL-10 has emerged as a possible mediator of pregnancy maintenance.[38] Through its anti-inflammatory effects and the suppression of the cytokine cascade that leads to prostaglandin and matrix metalloproteinase production, Il-10 may prevent gestational membrane rupture and maintain quiescence of the myometrium. Preferential secretion of IL-10 from the chorioamniotic face of the gestational membranes suggests the gestational membranes may play an important role in maintaining myometrial quiescence within the uterus.

Increased IL-8 concentrations were observed in the choriodecidual compartment medium compared to the amniotic compartment medium in unstimulated control transwell cultures, suggesting directional secretion of cytokines from the human gestational membranes. The qualitative histological findings in this study support the cytokine measurement data, with positive IL-8

labeling being observed primarily in the decidual region of the untreated transwells. The ability of IL-8 to cross the gestational membranes as a functional protein has yet to be investigated.

We suggest that the differences in cytokine response between culture systems reflects the lack of compartmentalization in the punch explant system. In contrast to the single compartment punch system, the transwell culture system maintains separated amniotic and choriodecidual compartments that more closely mimics the physiologic separation of fetal and maternal compartments in vivo. Cytokines are regulated through receptor-mediated activation of second messenger and nuclear transcription pathways that are susceptible to positive feedback.[39] Secretion of cytokines into the single-compartment punch system may allow direct non-physiologic signaling between the amnion and choriodecidua through the shared medium, leading to increased positive feedback on both sides of the membrane and causing the rapid cytokine surge seen at 12 h. As such, the amplified response seen in punch explant cultures exposed to LPS may not accurately reflect physiologic responses as would occur in vivo. In contrast, the two-compartment transwell culture system prevents signaling through the medium between the amnion and the choriodecidua, thereby providing a more physiologic in vitro model of cytokine response of gestational membranes.

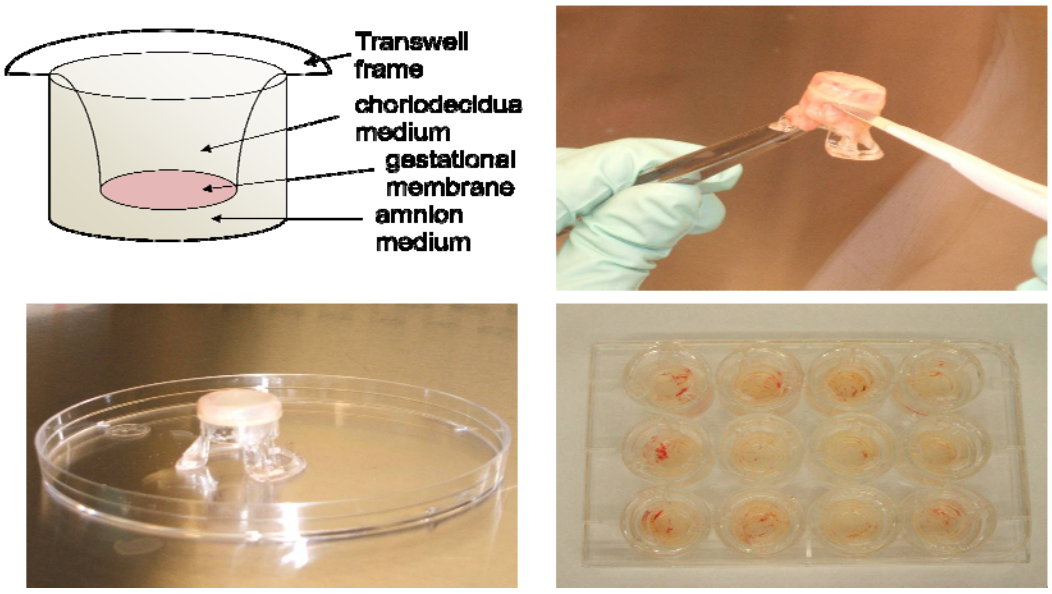


Figure 4.1. Transwell explant culture system. Gestational membranes were mounted to transwell frames and suspended in 12-well culture plates. The membrane created a barrier maintaining an internal choriodecidual compartment and an external amniotic compartment

Table 4.1. In vitro Cytokine Secretion into Culture Media by Human Gestational Membranes using a Biopsy Punch Explant Culture System or a Transwell Mounted Explant Culture System[a]

	Punch		Transwell Amnion		Transwell Choriodecidua	
	Control	LPS[b]	Control	LPS[b]	Control	LPS[b]
Interleukin-1β (pg/ml)	10.66 (1.68)	66.50 (13.87)	30.54 (10.40)	203.10 (81.60)	36.72 (18.91)	126.72 (42.35)
Interleukin-6 (ng/ml)	4.42 (1.24)	78.83 (20.27)	15.22 (5.33)	80.34 (30.10)	23.75 (7.33)	104.19 (23.81)
Interleukin-8 (ng/ml)	62.41 (14.23)	225.37 (48.57)	154.30[c] (29.15)	238.46 (43.81)	236.48[c] (32.46)	377.94 (55.59)
Interleukin-10 (pg/ml)	12.61 (2.78)	335.11 (50.05)	15.42 (4.21)	166.57 (69.59)	15.34 (4.13)	290.66 (70.21)
TNF-α (pg/ml)	21.37 (3.44)	5026.48 (1419.42)	33.11 (4.31)	8394.66 (4192.91)	50.04 (13.50)	11109.32 (4789.19)

Abbreviations: LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

[a] Cytokines were measured 12 h after incubation with or without 100 ng/ml LPS. Transwell culture cytokine concentrations are provided separately for the amniotic and choriodecidual compartments. Data are presented as mean (SEM). (N= 7 membranes)

[b] LPS treatments induced a significant increase of all cytokines from their respective controls ($P<0.05$).

[c] Basal secretion of Interleukin-8 differed significantly between the amniotic and choriodecidual sides of the transwell culture ($P<0.001$).

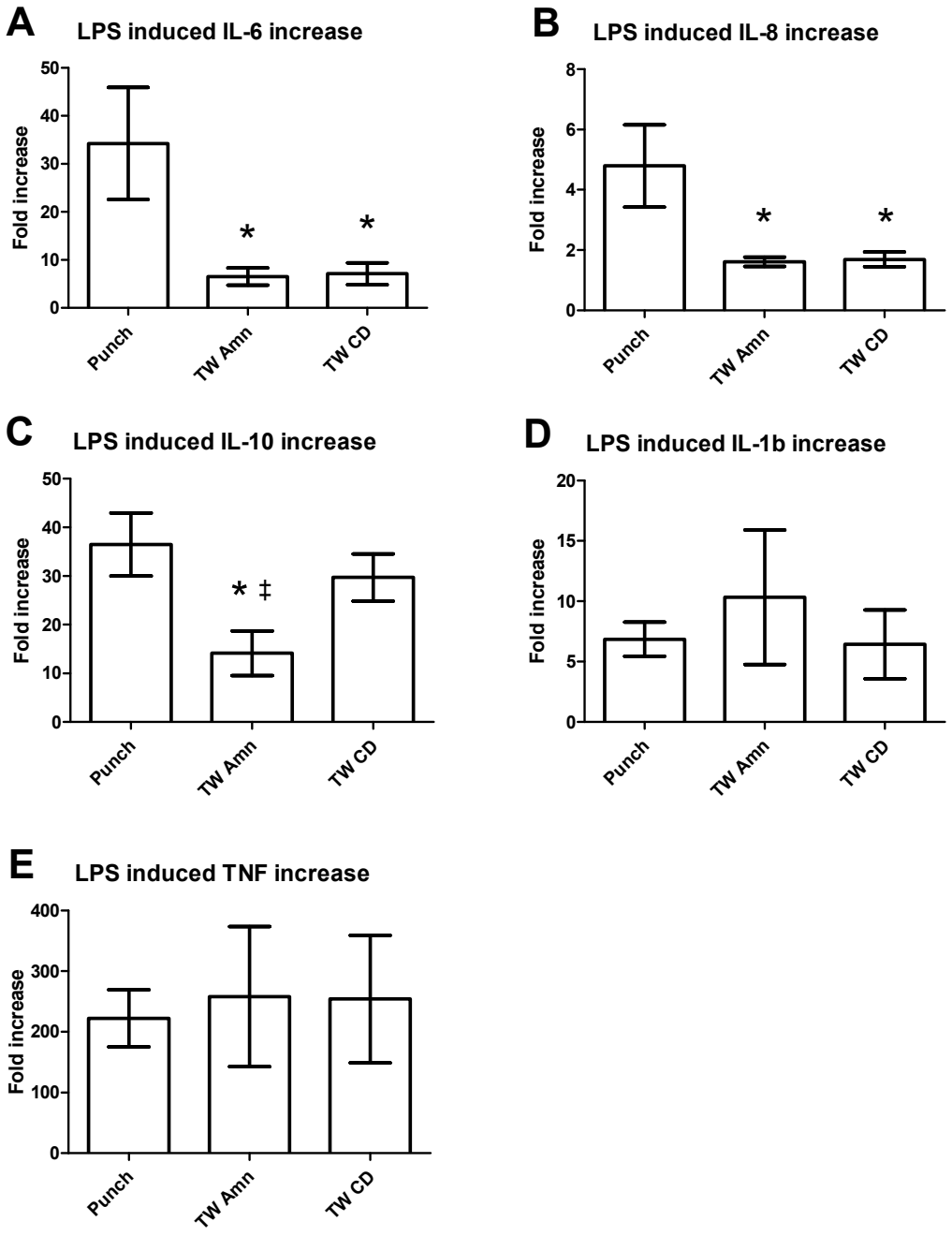
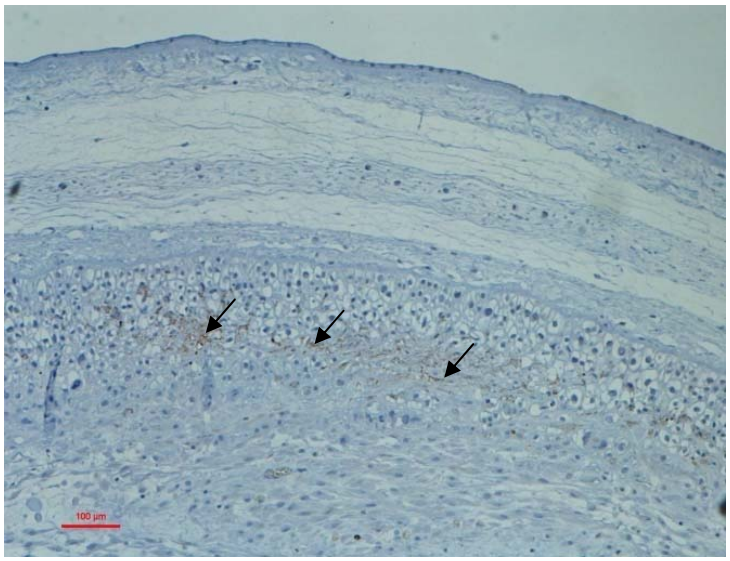
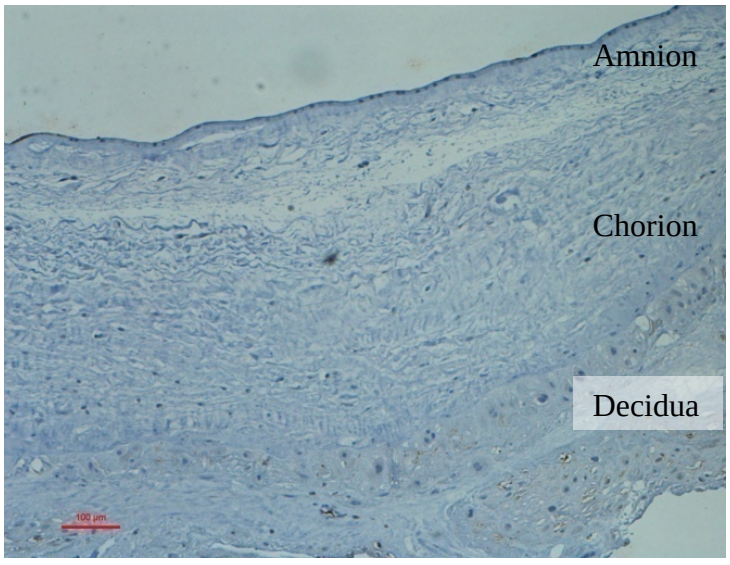


Figure 4.2. Lipopolysaccharide (LPS)-induced release of (A) interleukin-6 (IL-6), (B) interleukin-8 (IL-8), (C) interleukin-10 (IL-10), (D) interleukin-1β (IL-1β), and (E) tumor necrosis factor-α (TNF-α) into culture medium. Data are represented as fold increase over non-treated control samples ± SEM. Transwell culture cytokine increases are provided separately for the amniotic and choriodecidual compartments. * Indicates significant fold-increase difference from punch culture ($P<0.05$). ‡ Indicates significant difference from TW-CD ($P<0.05$). TW Amn indicates transwell media from amniotic chamber; TW CD indicates transwell media from choriodecidual chamber. N=7 membranes.

A



B



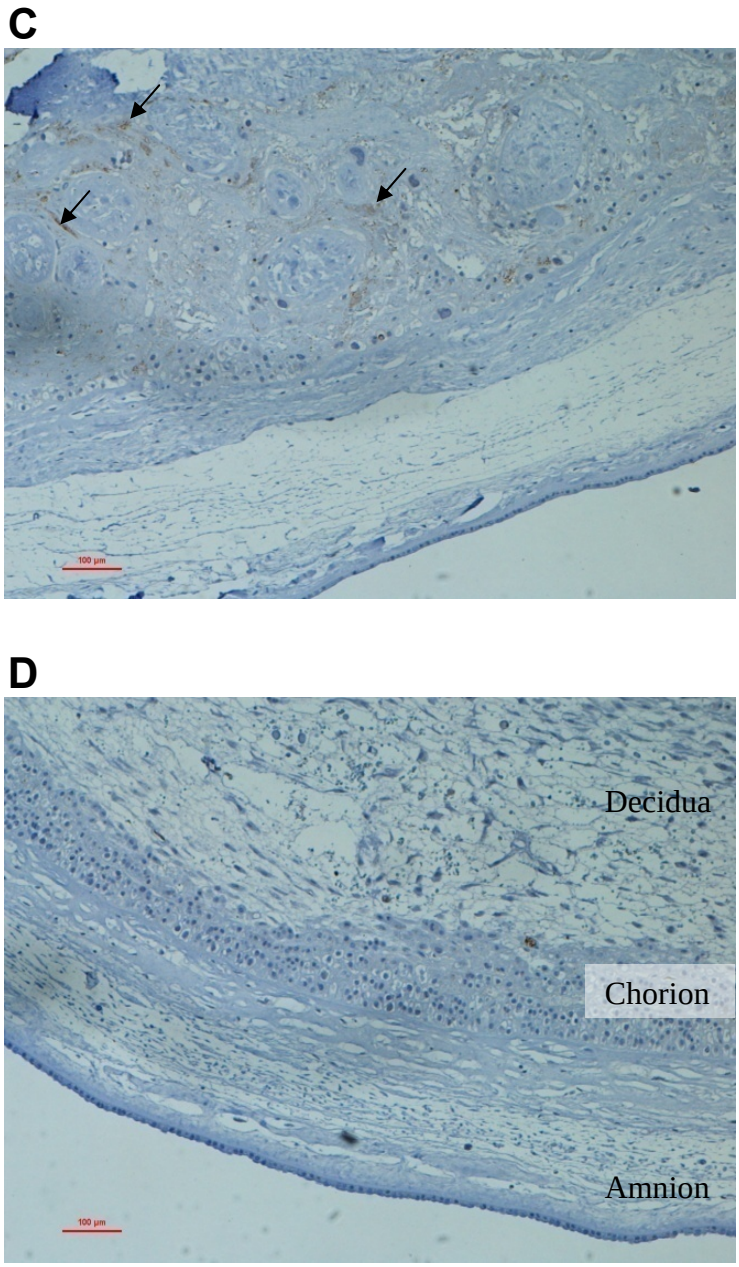
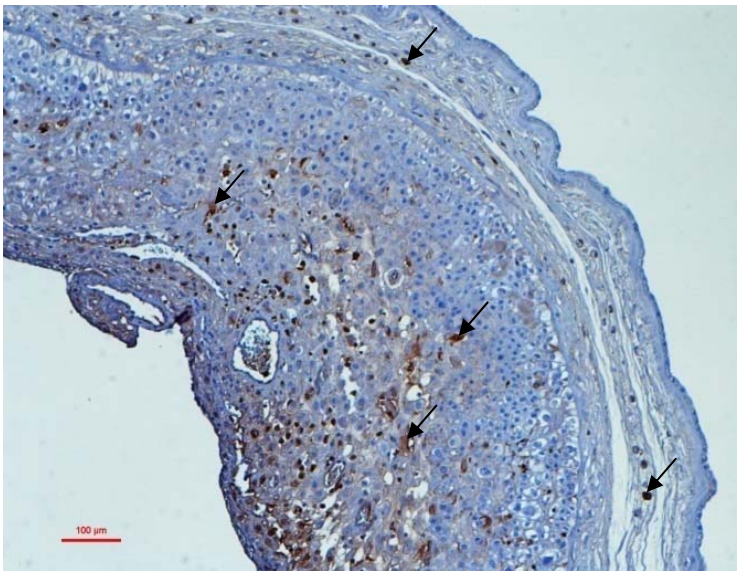


Figure 4.3. Immunohistochemical analysis of Interleukin-6 (IL-6) expression in human gestational membranes. Increased induction of IL-6 by lipopolysacchride (LPS) was identified within intercellular regions of the chorion and decidua of free-floating punch explants (A) and transwell mounted explants (C), compared to non-treated control (B, D respectively).

A



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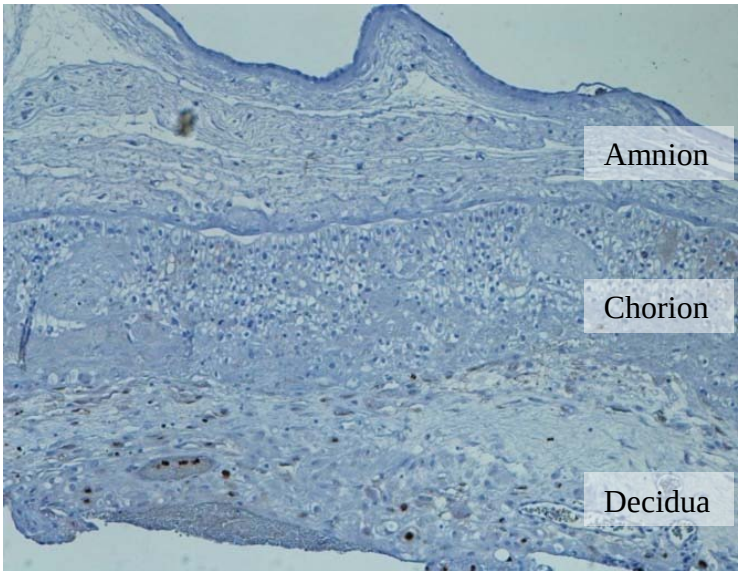

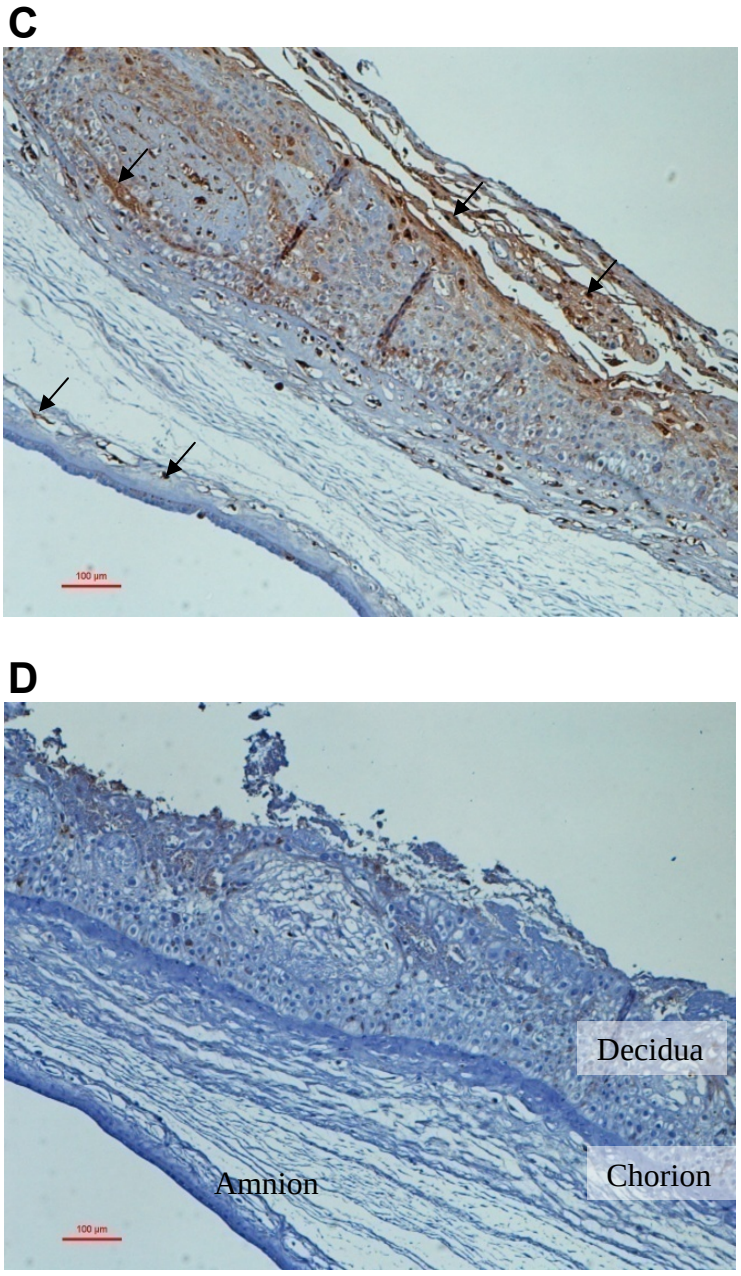

Figure 4.4. Immunohistochemical analysis of Interleukin-8 (IL-8) expression in human gestational membranes. Increased induction of IL-8 by lipopolysacchride (LPS) was identified within intercellular regions of the chorion, decidua and sub-epithelial amnion of free-floating punch explants (A) and transwell mounted explants (C), compared to non-treated control (B, D respectively).

Appendix A

Evaluation of PBDE-stimulated release of cytokines from human extraplacental membranes

Methods and Materials

Sample collection.

Sample collection was as described in Chapter IV.

Culture and treatment.

Biopsy punch culture methods were as described in Chapter IV. Human amniotic epithelial and fibroblast cells were isolated as described in the Loch-Caruso Laboratory Standard Operating Procedures with modifications noted.

Briefly, freshly collected amnion was carefully separated from the choriodecidual membranes. Tissue was rinsed with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) and cut into 2 in^2 sections. Tissue was added to 250 ml incubation bottles containing 100 ml DMEM supplemented with 100 units/ml penicillin, 100ug/ml streptomycin (Hyclone, Logan, UT), where amnion epithelial cells were dissociated at 37°C using 0.125 g trypsin and 0.02 g DNAse I for 25 minutes. Fifty milliliters of dissociation solution was removed and

dissociation was repeated. After the second dissociation, tissues pieces were removed and supernatants from first and second dissociations were composited. Epithelial cells were isolated from the digested tissue using a 5-20-40-60% Percoll (GE Healthcare, Upsalla, Sweden) gradient.

After trypsin digestion, tissue pieces were transferred to 100 ml DMEM supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin and with 10% fetal bovine serum (FBS) to inactivate trypsin, rinsed and transferred to a new incubation bottle. To this mixture, 0.100 g collagenase and 0.02 g DNAse I were added and incubated for 50 min at 37°C to release amniotic fibroblast cells. After incubation, the remaining tissue was discarded and the released cells were pelleted and rinsed. Resuspended fibroblast cells were then isolated using a 5-20-40-60% Percoll gradient.

After isolation, cells were acclimated for 24 h in 100 mm culture plates containing DMEM supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin and with 10% FBS, before being used in experiments.

Tissue and cells were treated for 12 or 24 h with DMEM supplemented with 100 units/ml penicillin, 100 ug/ml streptomycin and with 10% FBS with or without 100 ng/ml lipopolysaccaride (positive control), 0.2% dimethyl sulfoxide (DMSO; solvent control), BDE-47, BDE-100 or BDE-153.

Cytokine assessment.

Cytokine concentrations of interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) were determined as described in Chapter IV.

Data analysis.

Data for biopsy punch experiments are represented as mean ± SEM of the membrane means for each treatment (N=4-5). Membrane means were calculated by averaging the individual well concentrations for each membrane (N=3-4). Well concentrations were calculated by averaging replicate ELISA determinations for each treatment well.

Data for isolated primary cell experiments are represented as mean ± SEM of the individual membrane fold increase over non-treated solvent control (0.2% DMSO) (N=3-5). For each membrane, individual treatment fold increases over solvent control were calculated for each treatment group by dividing the mean treatment level by the mean solvent control concentration (N=4).

Differences between treatments were tested by analysis of variance with Student-Newman-Keuls method for posthoc pairwise comparisons.

Results

Based on the LPS-stimulated cytokine release data described in Chapter IV, the biopsy punch explant cultures method was selected for these studies to identify the stimulatory effects of PBDEs on this tissue. Although transwell mounted explant culture methods were shown to be more physiologically relevant, the amplified effects seen in the Punch culture system were desirable in preliminary experiments to identify PBDE–induced effects.

The PBDE treatment of punch cultures showed no statistical difference from the solvent control for any of the congeners tested. LPS-induced positive control values were significantly elevated in all punch culture experiments. In addition, no difference was observed between the 0.2% DMSO treated solvent control and the untreated control.

To further evaluate the stimulatory effects of PBDEs on cytokine secretion, isolated amniotic epithelial or fibroblast cells were treated with BDEs-47, 100 and 153. This replicated previous work in the lab that showed a positive increase in cytokine secretion in response to PBDE stimulation (Dr. K. Brant, unpublished data).

The PBDE treatment of neither amnion epithelial nor amnion fibroblast cells resulted in a significant increase in IL-6 secretion into the surrounding medium.

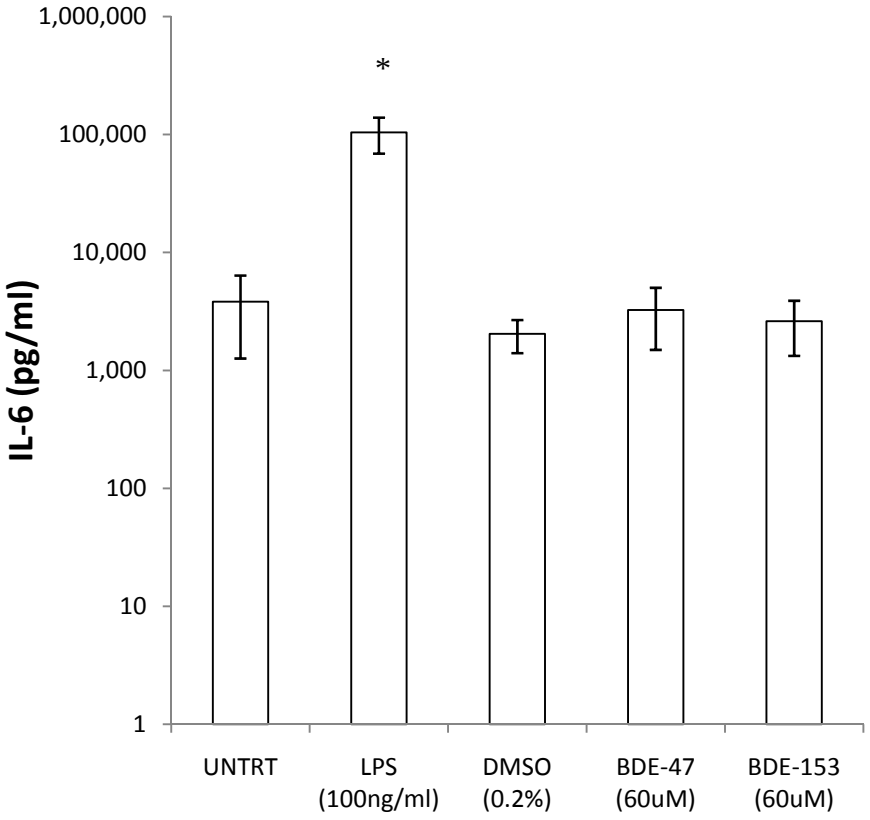

Figure A.1. PBDE-stimulated interleukin-6 (Il-6) secretion into culture media by human gestational membranes biopsy punch explants. Il-6 was measured after a 12-h incubation with untreated medium, 0.2% dimethyl sulfoxide (DMSO; solvent control), lipopolysaccharide (LPS; positive control), 60 uM BDE-47 or 60 uM BDE153. IL-6 was measured by enzyme-linked immunosorbent assay (ELISA). Bars represent mean ± SEM. N=5 membranes; 3-4 wells/membrane/treatment.

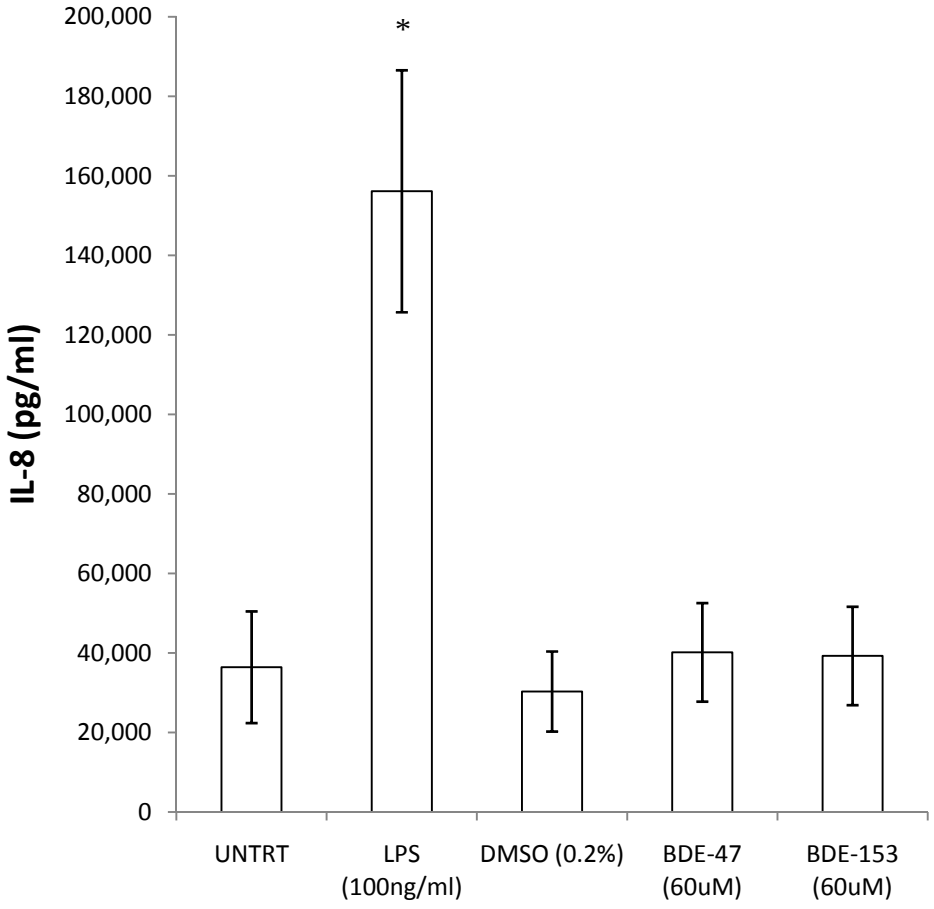

Figure A.2. PBDE-stimulated interleukin-8 (Il-8) secretion into culture media by human gestational membranes biopsy punch explants. Il-8 was measured after a 12-h incubation with untreated medium, 0.2% dimethyl sulfoxide (DMSO; solvent control), lipopolysaccharide (LPS; positive control), 60 uM BDE-47 or 60 uM BDE153. IL-8 was measured by enzyme-linked immunosorbent assay (ELISA). Bars represent mean ± SEM. N=5 membranes; 3-4 wells/membrane/treatment.

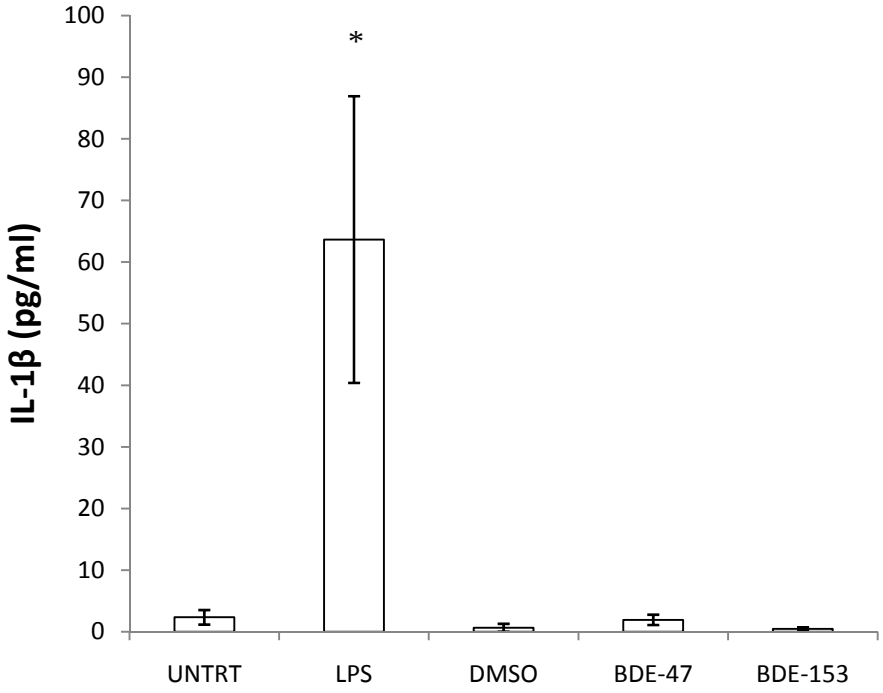

Figure A.3. PBDE-stimulated interleukin-1β (Il-1β) secretion into culture media by human gestational membranes biopsy punch explants. Il-1β was measured after a 12-h incubation with untreated medium, 0.2% dimethyl sulfoxide (DMSO; solvent control), lipopolysaccharide (LPS; positive control), 60 uM BDE-47 or 60 uM BDE153. IL-1β was measured by enzyme-linked immunosorbent assay (ELISA). Bars represent mean ± SEM. N=4 membranes; 3-4 wells/membrane/treatment.

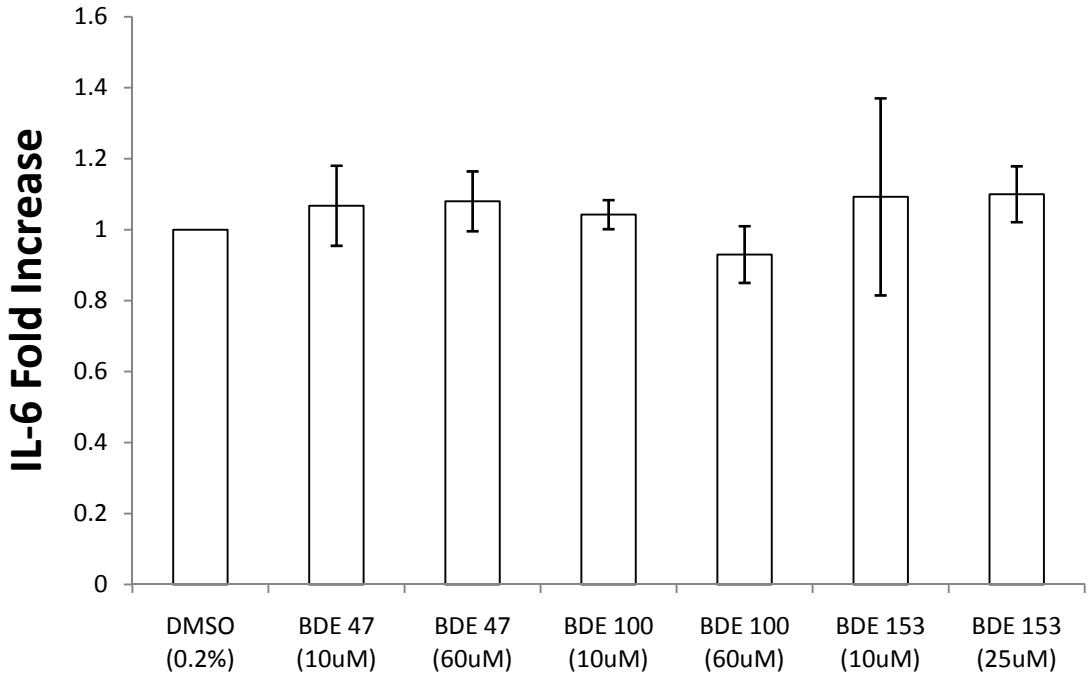

Figure A.4. PBDE-stimulated interleukin-6 (Il-6) secretion into culture media by primary amniotic epithelial cell isolates. Il-6 was measured after a 24-h incubation with 0.2% dimethyl sulfoxide (DMSO; solvent control), BDE-47 (10 uM, 60 uM), BDE 100 (10 uM, 60 uM) or BDE153 (10 uM, 60 uM). IL-6 was measured by enzyme-linked immunosorbent assay (ELISA). Bars represent the mean ± SEM of the fold increases over solvent control within each experiment. Lipopolysaccharide (LPS) positive control induced a significant increase in IL-6 (data not shown). N=3-5 membranes; 4 wells/membrane/treatment.

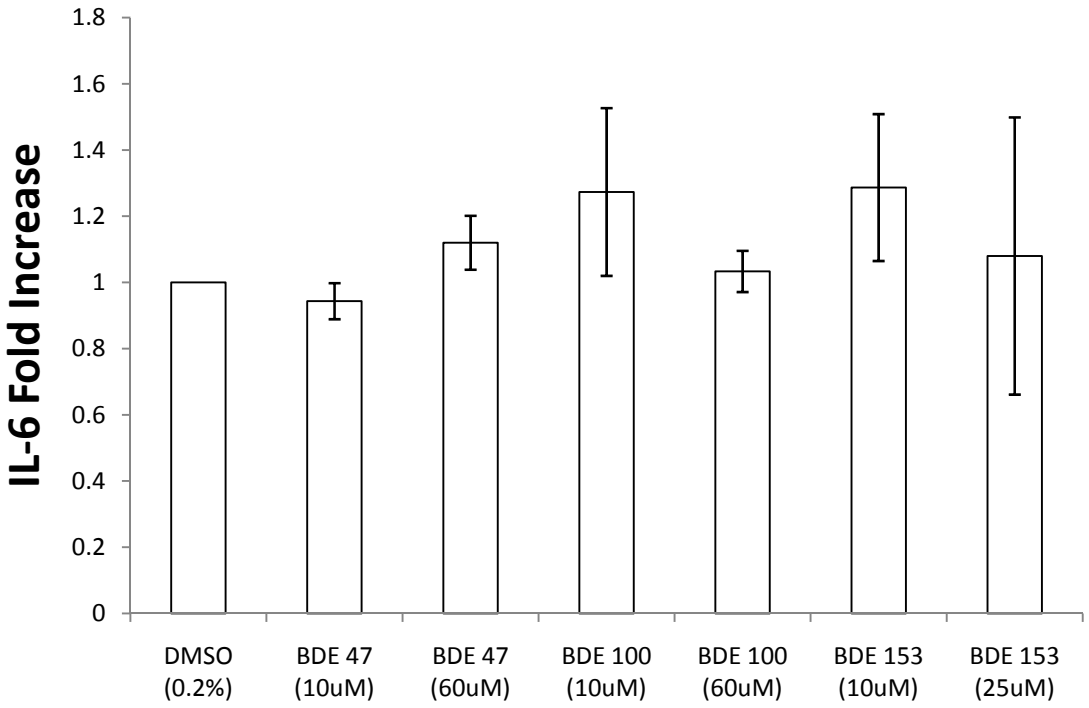

Figure A.5. PBDE-stimulated interleukin-6 (Il-6) secretion into culture media by primary amniotic fibroblast cell isolates. Il-6 was measured after a 24-h incubation with 0.2% dimethyl sulfoxide (DMSO; solvent control), BDE-47 (10 uM, 60 uM), BDE 100 (10 uM, 60 uM) or BDE153 (10 uM, 60 uM). IL-6 was measured by enzyme-linked immunosorbent assay (ELISA). Bars represent the mean ± SEM of the fold increases over solvent control within each experiment. Lipopolysaccharide (LPS) positive control induced a significant increase in IL-6 (data not shown). N=3-5 membranes; 4 wells/membrane/treatment.

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Chapter V

Discussion

PBDE abundances in human tissue and fluids from the United States are among the highest in the world (reviewed by Frederiksen).[1] Further, these levels seem to be continuously rising in the United States while PBDE levels in other parts of the world are remaining static or decreasing.[2-4] PBDEs raise significant health and regulatory concerns due to persistence, bioaccumulation and developmental toxicities of PBDEs (reviewed by the Institute of Medicine of the National Academies).[5] Perinatal exposure to PBDEs is of particular concern due to reports of higher PBDE abundances in neonatal infants than in adults, and a lack of reports for prenatal infants.[6-9]

Scientific research has established that human gestational membranes have significant roles in the timing of birth through the production of cytokines and prostaglandins. Therefore, investigations into the impact of toxicants on the human gestational membranes and their role in preterm birth and PPROM are warranted. The research contained in this dissertation is the first to show bioaccumulation of PBDEs in the human gestational membranes, identify a potential route of gestational membrane exposure through the amniotic fluid and

compare culture methods for the investigation of gestational membrane inflammatory response in vitro.

Total burdens of PBDEs in the human gestational membranes of women in Southeast Michigan were 3.3-8.8 fold greater than levels previously reported for placental disk samples collected in Denmark, Finland or Spain,[10-13] but considerably less than fetal liver samples and maternal or fetal sera samples from the United States.[14-18] Although most studies monitor 5-10 PBDE congeners, the present study assessed 21 congeners in the human gestational membranes. This 21-congener study is one of the most inclusive biomonitoring reports of PBDE congener profiles in humans. By analyzing a broad range of congeners, I identified BDE-49 as a major constituent of the total PBDE levels in this population, comprising 17% of the total PBDE loading. This was the first report of BDE-49 in the human reproductive compartment. This latter finding suggests that other studies that do not measure BDE-49 could be significantly underestimating the total PBDE accumulation.

Very little is known about the distribution of persistent organic pollutants within the human gestational compartment. In order to identify the sources of PBDE exposure for gestational membranes, I evaluated congener-specific abundances within the amniotic fluid and gestational membranes. Gestational membranes are supplied with blood from the spiral arteries of the uterus. These arteries are part of the maternal circulation and provide a direct route of PBDE exposure to the decidual face of the gestational membranes. Because the

gestational membranes encapsulate the amniotic fluid there is also potential for direct transfer of PBDEs across the amnion-amniotic fluid boundary.

The amniotic fluid levels of PBDEs were much higher than gestational membrane levels on a tissue/fluid basis (amniotic fluid- 3.8 ng/ml; gestational membrane- 17.4 pg/g) and also on a lipid basis (amniotic fluid- 404 ng/ml; gestational membrane- 5.6 ng/g). Our understanding of the causes for this difference is limited because this is the first report of PBDE levels in either of these matrices. Further studies are needed to determine whether PBDEs are accumulating in the amniotic fluid over time or if they are mobilized and incorporated into the amniotic fluid as it is formed.

Although the gestational membrane and amniotic fluid PBDE concentrations reported here were both from samples collected at the University of Michigan Women's Hospital, limitations in sample collection prevented the direct comparison of gestational membrane and amniotic fluid levels using paired samples. The amniotic fluid samples were collected from clinical amniocentesis samples, and likely represent an older sample population. Because some PBDEs accumulate over time, an individual's age can influence the total tissue burden. Further, I did not collect demographic information from either sampling group, preventing the identification of other potential confounding factors such as body mass index, socioeconomic status, occupational or behavioral exposures, number of previous pregnancies and gestational age at time of sampling.

In addition to the greater total PBDE abundances observed in the amniotic fluid compared to gestational membranes, we also saw a strikingly different

congener profile between these compartments. The gestational membranes were dominated by small tetra- and penta-BDE congeners, accounting for >80% of the total PBDE loading, and had no hepta- through deca- congeners. In contrast, tetra- and penta- congeners accounted for only 18% of the total loading in amniotic fluid and octa- through deca- congeners contributed over half of the total PBDEs. These tissue compartment differences in PBDE profiles suggest preferential accumulation of specific congeners in the various matrices, possibly due to steric exclusion of larger PBDE congeners from the dense gestational membranes. Because these studies are from the same sampling population but not from the same women, future studies using paired samples and toxicokinetic modeling will be necessary to establish preferential accumulation of PBDEs in various reproductive compartments. Development of a toxicokinetic model of PBDE congener distributions in utero gains additional importance because lower brominated congeners with eight or fewer bromine atoms have been shown to have greater developmental toxicities than higher brominated congeners.[5]

Several studies have investigated PBDE concentrations in paired maternal and fetal sera samples.[17-19] Most such studies observed strikingly similar concentrations for maternal and fetal sera samples, suggesting that PBDEs pass freely from maternal to fetal circulation through the placenta. A recent study by Doucet et al. investigating the relationship of paired placenta and fetal liver samples further supports passage of PBDEs through the placenta to the fetus.[20] However, Doucet et al. found no correlation between placental PBDE levels and the much higher fetal liver levels taken from mid-gestation abortus material in

North America between 1998 and 2006. This latter finding suggests the fetal liver continuously accumulates PBDEs from the fetal circulation, whereas the placenta is allowing PBDEs to pass.

My research suggests that maternal-fetal blood transfer at the placenta may not be the only route of PBDE exposure for the fetus, but that amniotic fluid provides a second route of exposure for the fetus. Amniotic fluid accumulates within the confines of the gestational membranes primarily during the second trimester. The total volume of fluid increases from about 200 ml at 16 weeks gestation to 1000 ml at 28 weeks gestation.[21] This increase in fluid is almost entirely of maternal origin, and may contain toxicants mobilized from maternal body burdens. Throughout gestation, the fetus is continuously swallowing and "inhaling" amniotic fluid. Intake into the gastrointestinal and respiratory tracks along with dermal exposure provide direct routes of transport into the fetus for toxicants that enter the amniotic compartment. Because it surrounds the fetus during gestation, amniotic fluid is possibly highly correlated to the PBDE exposure to the fetus. The high amniotic fluid levels seen in this study provide evidence that this route of exposure is significant and warrants further investigation.

The present study shows that BDE-209 is present in 80% of human amniotic fluid samples at concentrations up to 1286 pg/ml fluid. Because BDE-209 has a short biological half-life (15 days),[22] the latter finding is an indication of continuous exposure to BDE-209. Furthermore, the presence of BDE-209 provides an explanation for the high levels of nona-BDE congeners observed in

amniotic fluid. Previous studies showed that BDE-209 is metabolized to nona-BDE congeners in fish and birds.[23-25] Because nona-BDEs are not commercially produced for use as flame retardants, it is plausible that the high levels of nona-BDEs seen in this study are a result of deca-BDE breakdown rather than direct environmental exposure to nona-BDEs.

Biomonitoring studies often report biological loading of lipophillic contaminants in units of weight per gram lipid within a given tissue. This metric provides a standardized base for describing the chemical kinetics within an organism and a standard by which to compare between organisms or between geographic regions. Although this practice is useful for assessing chemical dynamics and accumulation patterns, from a toxicological standpoint it is more useful to examine tissue contamination data as weight per gram tissue. The measured concentration of a chemical within the tissue more directly reflects the toxicant dose within an organism or tissue. Therefore the results reported in this dissertation have been described primarily on a weight per gram tissue basis, with secondary reference to lipid content within the gestational compartments and concentrations on a lipid weight basis.

Although the PBDE biomonitoring data are reported on a weight per gram tissue basis to provide biological significance to the findings, it may also be of interest to consider these data on a mole weight per gram tissue basis when comparing between PBDE congeners. Because of the high atomic weight of bromine (a.w. = 79.904 gram/mol), PBDE congeners have widely variable molecular weights. For example the formula weights of the PBDEs measured in

these studies range from 406.90 g/mol for BDE-17 to 959.17 g/mol for BDE-209. Therefore, for a given weight per gram tissue concentration of PBDEs there would be more than twice as many molecules of BDE-17 then there would be of BDE-209. From a toxicological standpoint, the number of molecules available to interact with receptors or target proteins within a given tissue may be of more importance than the total weight of the molecules.

To identify the most appropriate model system for evaluating PBDE-stimulated cytokine release from the extra-placental gestational membranes, I conducted a comparative assessment of the two primary explant culture systems; free-floating biopsy punch explant cultures and transwell-mounted explant cultures. To make this comparison I used lipopolysaccharide (LPS) as a model stimulant because it has been well established as a modulator of cytokine secretion in the scientific literature of both culture systems. This study showed significantly increased effects for several cytokines using the biopsy punch explant culture system compared to the transwell explant culture system. The increased cytokine response in the punch cultures suggest an amplification of cytokine signaling through non-physiologic signaling between opposing faces of the membrane. Based on these findings, future investigations should select carefully the model system that most appropriately meets the needs of the research.

This work did not show a stimulatory effect for BDE-47 or BDE-153 using the biopsy punch explant system (Chapter 5: Appendix A). Furthermore, I was unable to show a significant increase in pro-inflammatory cytokine release in

response to BDE- 47, 100 or 153 exposure in isolated amniotic cells.

The absence of an effect seen in the PBDE treatment studies does not provide conclusive evidence that PBDEs do not induce a pro-inflammatory cytokine response in this tissue. These experiments evaluated only a select group of cytokines exposed to only two congeners, at a single time point. Being a lipophillic compound,[6] the concentration of PBDEs reaching the site of action is difficult to quantify in the in vitro culture systems due to adsorption to the culture wells.[26] In addition to the mechanistic and toxicological studies currently being undertaken in this laboratory and others, the broad range of sites for potential immunomodulation suggests a microarray screen of gene modulation is warranted for these chemicals.

It is also plausible that if PBDEs do not directly induce an increase in pro-inflammatory cytokines, they may act collaboratively with infectious agents to cause gestational membrane inflammation and adverse birth outcomes. Recently, Lundgren et al. showed that in vivo PBDE exposure resulted in marked decreases or complete absence of select pro-inflammatory cytokines using a murine model exposed to a coxsackie B3 infection.[27] Equally noteworthy, the inhibition of cytokines due to PBDE exposure was also seen in uninfected control animals. PBDE accumulation within the gestational membranes may provide a selective block in immune signaling pathways, allowing the proliferation of ascending vaginal bacteria within immunocompromized gestational membranes.

The research contained in this dissertation has addressed the questions of PBDE accumulation in the human gestational membranes and amniotic fluid. In

addition, it has compared in vitro model systems used to identify cytokine stimulation and used them to evaluate several PBDE congeners. But in the end it has generated more questions than it has answered. This work provides a basis for future investigations into the compartmental dynamics and toxicokinetics of PBDEs within the human gestational compartment. Based on the PBDE profiles in the gestational membranes and amniotic fluid it is likely that all PBDE congeners do not partition proportionately between tissues and fluids. This implies a yet-to-be-developed model for selective accumulation of more toxic congeners within a single tissue. This work also provides a basis for future investigations of PBDE interactions within human gestational membranes. By establishing that PBDEs accumulate within the human gestational membranes, I have placed them within a tissue that is known to mediate birth outcomes. Interactions with other persistent organic pollutants, infectious agents and signaling cascades still need to be addressed. Finally, my comparison of in vitro explant model systems for evaluating cytokine release has spawned questions regarding the physiologic relevance of the punch culture system. Identified differences between the punch and transwell culture systems may prevent the direct comparison of studies using these two culture methods for studying cytokine release.

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