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Environmental Pollutant Induced Cellular Injury is Reflected in Exosomes from Placental Explants

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Abstract

INTRODUCTION—Exosomes are intercellular signaling vesicles whose cargo reflects the physiological status of the cell of their origin and can regulate gene expression in other tissues. Polybrominated diphenyl ethers (PBDEs) and bisphenols (A [BPA], Tetrabromobisphenol A [TBBPA], and 2,4,6-Tribromophenol [TBP]) are common environmental pollutants known to increase the risk for spontaneous preterm birth (PTB). We hypothesized that placental exposure to these environmental pollutants causes exosome cargo changes that reflect exposure associated placental response.

METHODS—Full-term, C-section placenta explants were treated with PBDE congeners (47, 100, 153, 209), TBBPA, TBP or BPA for 24 hours. Exosomes were isolated from media by sequential ultracentrifugation and purified by size exclusion chromatography. Exosomes were characterized by electron microscopy, nanoparticle tracking analysis and western blot. Proteomics identified differentially expressed exosomal proteins and Ingenuity pathway analysis (IPA) determined biological functions and pathways represented by identified proteins.

RESULTS—Regardless of treatment, placental expressed exosomes markers (PLAP, CD9, CD63, 81 and ALIX), had a size distribution between 50–175 nm and were present in the conditioned medium at $5-8 \times 10^{11}$ exosomes/mL. Proteomic analysis identified 2598 proteins which demonstrated that specific pollutants caused differential expression of specific proteins, including alarmin, High Mobility Group Box 1 (HMGB1), MAPK14 (p38 MAPK) and GSK3 β . IPA

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revealed an inhibition of pathways associated with cell survival, tissue repair and proliferation, as well as activation of cell death pathways (e.g. necrosis).

CONCLUSION—Environmental exposure of placental explants did not change the quantity of exosomes or their characteristics. However, exosome cargo composition exposed to some environment pollutants may be involved in placental nuclear and cellular injury and inflammation.

Keywords

Extracellular vesicles; Flame retardants; Persistent organic pollutants; Preterm birth; Inflammation

INTRODUCTION

Spontaneous preterm birth (PTB; birth < 37 weeks gestation) is a major pregnancy complication that has proven to be difficult to prevent due a limited understanding about the factors that cause it, their biological mechanisms, and a lack of peripheral biomarkers that can be used to evaluate patients' risk for the [1–4]. Hence, the rates of PTB have not declined in the past several decades.

Recent awareness about the universal exposure of women to environmental pollution has led to epidemiologic studies linking environmental pollutants to PTB [5–15]. Ecological studies have demonstrated that residence within 250 meters of a major roadway increases the risk for PTB, as does proximity to pollution-monitoring stations that detect high levels of carbon monoxide and 10 µm particulate matter [10, 11, 16]. There is also increasing evidence that persistent organic pollutants (POPs) that are widely used in industry or agriculture may also increase the risk of PTB [12, 17, 18]. The greatest risk to women, however, may come from chemicals used in consumer products that are widely used by pregnant women that include flame retardants such as Polybrominated Diphenyl Ethers (PBDEs), 2,4,6-tribromophenol (TBP), and tetrabromosiphenol A and phthalates used to construct plastic containers such as, Bisphenol A (BPA) and its replacement Bisphenol S. These pollutants enhance placental inflammation and may increase the risk of preterm birth although the precise mechanisms are unknown [19–22].

Our work has been focused on understanding the mechanisms of Bisphenol A and PBDEs due to the widespread exposure to the chemicals among pregnant women via consumer products. Approximately 6 billion pounds of BPA is used in the manufacturing of polycarbonate plastics and epoxy resins per year [23, 24]. These plastics and resins are then used by the food industry to make containers and coat cans to package food [23]. PBDEs have been widely used as flame retardants in consumer products over the past 40 years and they have been detected globally in household air, dryer lint, household dust, food commodities, soil, sludge, outdoor air, birds, fish and animal tissues [25, 26]. Both PBDE and BPA are associated with PTB and BPA accumulation in the amniotic fluid is associated with preterm premature rupture of the membranes [27–29]. Interestingly, BPA in maternal plasma was associated with PTB with intact membranes, suggestive of their pathogenic effects depending on the site of accumulation. Specific congeners of PBDE have shown to have pathologic effects on human fetal membranes and placenta besides their well-reported endocrine disruption function [19, 21, 30]. PBDE congener-99 causes fetal membrane

senescence and senescence-associated sterile inflammation mediated via p38 mitogen-activated kinase activation [31]. Comorbidities of PBDE and infection have been found to exaggerate the inflammatory response from the placenta. Conversely, BPA is not associated with any inflammatory changes in the fetoplacental unit. Thus, environmental pollutants can produce distinct pathologic events in feto-maternal tissues and their inflammatory response is not homogeneous making it difficult to understand the pathogenic mechanisms stimulated by these agents.

Knowledge gained from epidemiologic and mechanistic studies have not yet translated into a suitable biomarker that can be used to predict exposure associated changes in the mother or fetus or indicate high-risk status [32–34]. Redundancy and overlapping pathways leading to PTB hinder the use of many of the tested biomarkers, specifically inflammatory mediators as indicators of environmental exposure [30, 35]. Recently extracellular vesicles, specifically exosomes, have been used as biomarkers of various disease conditions including pregnancy. Exosomes are bioactive, spherical, cell-derived vesicles (30–150 nm) secreted during the process of exocytosis. They increase in number as pregnancy progresses [36] and contain molecular constituents of their cell of origin, including proteins, lipids, and nucleic acids that can be cell-specific and reflect the physiological state of the cell source [37–41]. We have shown that exosomes can travel from the fetal to the maternal compartment in pregnant animals [42, 43] and fetal-derived exosomes can cause functional changes in maternal tissues. Recently, we have characterized exosomes from amniotic fluid and noted unique proteomic profiles at term and preterm gestations [44]. Placental-specific exosomes isolated from maternal plasma and their cargo analysis have indicated the biomarker potential of exosomes in determining various pregnancy risks as well as informed placental functional status [45, 46]. Thus exosomes can provide a functional blueprint of the placenta in response to specific exposures.

The effects of environmental pollutants (PBDE, TBBPA, TBP and BPA) on placental exosome release and its composition; however, are unknown. Therefore, we studied the effects of exposure to PBDE congeners and TBBPA/TBP/BPA on the chemical and physical properties of released exosomes from the placenta. We found that environmental exposure of PBDE and TBBPA/TBP/BPA causes placental tissue injury and inflammation that is reflected in the exosomal cargo.

METHODS

Placental tissues from patients undergoing elective, singleton, Cesarean section without labor (n=5) were used for this project. All samples were collected under guidelines for using anonymous, discarded tissues for research at NYU-Winthrop Hospital with Institutional Review Board oversight. Investigators were blind and had no access to personal health information.

After release by the attending surgeon, placental tissues were transported to the laboratory and washed with sterile saline. Visible blood clots were removed by gentle blotting and the segments of villous tissue were isolated by sharp dissection that were subsequently washed with and chopped on a McIlwain Tissue chopper (Mickle Engineering Co, Surry, UK) set to

cut at 0.1 mm 3–5 times. Chopped tissues were washed in DMEM supplemented with 5% fetal bovine serum (v/v), Penicillin (100 U/mL) and Streptomycin (100 µg/ml). One gram of tissue was placed into a 10 cm Petri dish in 10 mL complete medium then supplemented with either PBDE 47, 100, 153, 209, TBBPAA, TBP and BPA or an equivalent volume of vehicle (DMEM) for a final concentration of 20 µM. This concentration was selected based on the previous *in vitro* studies [30, 47]. Final concentration of DMSO for all cultures was 0.1% (v/v). Cultures were incubated for 20 hours in a humidified incubator under room air supplemented with 5% CO₂ (v/v). Conditioned medium was harvested, and debris was removed by centrifugation and stored at –80 °C until use.

Exosome isolation from conditioned media

Exosomes are defined as extracellular vesicles (EVs) isolated using ultracentrifugation followed by size exclusion chromatography to isolate particles between 30 – 150 nm as described previously [40–42], with modifications. Briefly, samples were centrifuged at 300 g for 10 min and 2000 g for 20 min at 4°C. The supernatant was transferred to Amicon™ Ultra 4 filter tubes (Millipore, Waltham, MA, 10,000 MWCO) and centrifuged for 20 min or until the sample volume was reduced to 500 µL. The concentrate was filtered through a Nalgene™ Syringe Prefilter Plus filters (Thermo Fisher, Waltham, MA) then centrifuged at 10,000 g for 30 min. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 100,000 g for 2 hours. The pellet was resuspended in 100 µL of cold PBS and passed through an Exo-spin™ column (Cell Guidance Systems, St. Louis, MO) following the manufacturer's instructions. Samples were aliquoted and stored at –80°C until characterization.

Determination of exosome size and quantification

The size distribution and concentration of exosomes were determined using NTA was performed using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and its corresponding software (ZetaView 8.02.28). Frozen exosomes in 1× PBS were thawed on ice and diluted 1:500 in filtered distilled water. The number of particles/ml and size distribution were counted for each sample. The machine was cleaned between samples using filtered water. The results of the ZetaView were used to calculate the number of exosomes produced per amnion cell for the two treatment types (control or OS). Results are reported as mean ± standard error of the mean (SEM).

Western blot analysis for exosome markers

Exosomes were lysed by the addition of 10× radioimmunoprecipitation assay (RIPA) lysis buffer (0.5 M Tris, pH 8.0; 1.50 M NaCl; 10% (v/v) Triton X-100; 10 mM EDTA, pH 8.0; and 10% (w/v) Sodium dodecyl sulfate-SDS) supplemented with protease and phosphatase inhibitor cocktails to final concentration. Western blot samples were subsequently processed as described previously [40–42, 48] to determine the expression of exosome marker ALIX (1:1000; Abcam, Cambridge, United Kingdom).

Determination of exosome shape using cryo-electron microscopy (EM)

Cryo-EM was used to determine the shape of exosomes in culture media as described previously. Briefly, 3 μ L of prepared exosome suspension was pipetted onto a copper grid with quantifoil support film (QUANTIFOIL, Großlobichau, Germany). The grid was vitrified to preserve exosomes in their native state and placed in a Gatan 626 specimen holder (Gatan, Pleasanton, CA), within a JEOL 2100 microscope for TEM (JEOL, Osaka, Japan). The samples were imaged at 15,000–30,000 magnification with a 200 kV electron beam from a LaB6 emission source, and images were recorded on a Gatan US4000 CCD camera.

Proteomic analysis of placenta-derived exosomes by mass spectrometry

Exosome protein clean-up and digestion—The protein profile of exosomes was established by liquid chromatography (LC) and mass spectrometry (MS). Exosomes in PBS were lysed in 10% (w/v) SDS in 0.1 M triethylammonium bicarbonate (TEAB) by sonication on ice for 10 min. 0.25 M Tris(2-carboxyethyl) phosphine (TCEP) was added to each tube to attain a final concentration of 0.01 M, and samples were incubated at 55°C for 1 hour. Samples were allowed to cool before 20 mM iodoacetamide was added to each tube and the samples were incubated for 45 min in the dark. The samples were subsequently transferred to tubes containing 8 M dry urea and vortexed. 12% phosphoric acid was added followed by the addition of S-Trap buffer (90% MeOH, 100 mM TEAB, pH 7.5). The lysate was added to the S-Trap microcolumn (Protifi, Huntington, NY) and centrifuged at 4000 $\times$ g for 2 min. The flow-through was discarded and the column was washed with S-Trap buffer before being centrifuged at 4000 $\times$ g for 2 min. Columns were transferred to new tubes and incubated with 16 ng/mL of trypsin for 2 hours at 47°C. Proteins were eluted by centrifugation at 4000 $\times$ g for 2 min with 50 mM TEAB, 0.2 % formic acid (FA), 50% acetonitrile (ACN)/0.2% formic acid, and finally 80% ACN/0.1% FA. After the elutions, the final eluate was dried and stored at –20°C until LC-MS/MS.

LC-MS/MS analysis of exosomes—Nano-LC-MS/MS data acquisition was performed on the Orbitrap Fusion mass spectrometer system (Thermo Fisher Scientific, San Jose, CA) coupled to a Dionex Ultimat 3000 nano-HPLC with a 40-well standard tray autosampler. The sample (5 μ L) was injected onto a trap column (300 μ m i.d. $\times$ 5 mm, C18 PepMap 100) and then a C18 reverse-phase nano-LC column (100 75 μ m $\times$ 25 cm, Acclaim PepMap) at 50°C. The loading pump flow rate was set to 8 μ L/min. The nano pump flow rate was set to 400 nL/min with a 130-min LC gradient, where the mobile phases were A (99.9% water and 0.1% FA) and B (99.9% ACN and 0.1% FA). The gradient was 0–5 min at 2% of B, 6–100 min at 4%–32% of B, an increase to 50% at 108 min, 5 min of 90% B wash, and 15 min equilibrium. Mass spectrometer parameters included tip spray voltage at +2.2 kV, and an ion-transfer tube temperature of 275°C. FTMS mode for the MS full scan from 350–1500 Da was used for the acquisition of precursor ions (resolution 120,000), ITMS top-speed (3 s) mode was used for MS/MS, and MS/MS was accomplished via HCD with a collision energy of 32%.

All MS/MS samples were analyzed using Proteome Discoverer 1.4.1.14, which was set up to search UniProt-human.FASTA (downloaded March 2018 from <https://www.uniprot.org>).

Proteome Discoverer was searched using a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Peptide charges considered were +2, +3, and +4. Scaffold (version 4.8.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >99.0% probability to achieve a false discovery rate (FDR) <1.0% by the Peptide Prophet algorithm [48, 49] with Scaffold delta-mass correction if they could be established at >99.0% probability and contained at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins were normalized to control samples (median area under the curve) and considered significantly different when $P < 0.01$ and the fold change was ± 1.5 . Bar graphs comparing identified proteins used for analyses were created in Prism (GraphPad, San Diego, CA) using the normalized total spectral counts for each replicate.

Ingenuity pathway analysis (IPA) of identified proteins

Hierarchical clustering (Cytoscape, www.cytoscape.org/) was used to demonstrate the expression patterns of the differentially expressed proteins. Pathway enrichment analyses were performed with IPA (Qiagen, Hilden, Germany) on proteins with a fold change ± 1.5 ($\log_2\text{FC} = \pm 0.6$) to identify canonical pathways and biological functions based on fold change and Z-scores. Canonical pathways and biological functions heatmaps are based on Z-scores from comparison analysis in IPA. Significantly enriched pathways for the proteins and scenarios were identified using $P < 0.01$.

Statistics

Exosome size and concentration ($n=5/\text{treatment}$) was analyzed using a One-way ANOVA and a $P < 0.05$ was considered significant. For proteomic spectral counts ($n=5/\text{treatment}$), data was analyzed using the Kruskal-Wallis test and a $P < 0.05$ was considered significant. All MS data and supplemental materials/tables can be accessed at <ftp://massive.ucsd.edu/MSV000084274/>.

Results

Placenta exosomes exhibit classic exosome characteristics regardless of treatment

Placental tissue derived exosomes had a size between 50–175 nm (173.4 ± 5.91 nm) (Figure 1A, Table 1), were present at 2.70×10^{10} to 3.85×10^{10} exosomes/mL (Figure 1B, Table 1), exhibited two tetraspanin exosome markers (CD81 and CD63, Figure 1C) and as well as showed placental specific placental alkaline phosphatase (PLAP), CD9 and ALIX based on proteomic analysis (see below), regardless of treatment (Figure 1D–F). Additionally, exosomes from all treatments had spherical morphology (Figure 1F).

Control and environmental pollutant treated placental exosomes carry differentially expressed protein cargo

Proteomic analysis identified 2598 differentially expressed proteins with the PBDE treatments (Figure 2A, 2C, Supplemental Table 1) and 2433 differentially expressed proteins with the TBBPA/TBP/BPA treatments (Figure 2B, 2D, Supplemental Table 2). As shown in

the Venn diagram, unique protein cargo in exosomes from placental explants treated with PBDE (Figure 2C) and TBBPA/TBP/BPA (Figure 2D) were also identified. We identified 1519 common proteins in all PBDE treatments and control as well as congener specific unique proteins; PBDE 47 had 75, PBDE 100 had 106, PBDE 153 had 93 and PBDE 209 had 120 unique proteins. Exosomes released from TBBPA/TBP/BPA tissues had 1499 common proteins with 110 unique to TBBPA, 92 unique to TBP and 136 unique to BPA.

Differentially expressed protein cargo from pollutant treated placenta-derived exosomes are associated with canonical pathways and biological functions

Proteins with a log₂fold change of ± 0.6 (or fold change of ± 1.5) compared to the control were analyzed using IPA. Canonical pathway analysis of PBDE congener treatment (Figure 3A) showed down-regulation of multiple protective pathways associated with cellular homeostasis, cell growth and survival, including EIF2, PAK, PDGF and EGF signaling pathways.

Canonical pathways associated with the differentially expressed proteins from TBBPA, TBP and BPA treated exosomes were similar to the various PBDE congeners (Figure 3B). Like the PBDE results, TBBPA, TBP and BPA showed down-regulation of EIF2, PAK, PDGF and EGF signaling pathways; However, TBBPA, TBP and BPA also had downregulation of HMGB1 signaling.

Analysis of the biological functions associated with the differentially expressed proteins from PBDE-treated placenta exosomes showed cellular stress-related changes (Figure 4A). Many of the biological functions, including cellular migration and cell death were upregulated in exosomes from PBDE treatment, regardless of congener.

Although the canonical pathways were similar between TBBPA, TBP and BPA treatment, the biological functions associated with each treatment had differences (Figure 4B). Top biological functions associated with these treatments were predominantly related to cell survival and migration; receptor-mediated endocytosis was upregulated in BPA treatment but downregulated with TBBPA and TBP treatment.

Exosomes released by placenta tissue treated with environmental pollutants have packaging of DAMPs

IPA analysis of exosomal proteins indicated that cellular functions were altered suggestive of cellular necrosis or tissue death in placental explants treated with various PBDE congeners. We have previously reported p38 MAPK mediated senescence of fetal membranes by PBDE 99 and to a lesser extent by PBDE 47 [31]. Senescent cells release signals of cellular damage, called damage associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1). These findings and IPA analysis results associated with the downregulation of cell viability pathways led us to examine cellular and nuclear damage associated changes reflected in specific exosomal proteins. Of the differentially expressed proteins, one of the DAMPs HMGB1 (Figure 5A) was common in all treatment types; however, exosomes from control cultures also showed HMGB1 suggesting that this is not a treatment effect. Caspase 4 (Figure 5B), inflammatory caspase involved in inflammasome activation, was also common to all treatments except 246 TBP.

Our lab has previously shown that p38 MAPK is packaged into exosomes by amnion epithelial cells exposed to oxidative stress in vitro [50]. PBDE 150, PBDE 209, 246TBP and BPA showed MAPK14 expression (also known as p38 MAPK, Figure 5C) compared to controls and other environmental toxins, suggesting that MAPK14 packaging may be associated with cellular response to environmental pollutants and a potential biomarker for exposure. GSK3 β was constitutive in all exosomes including controls suggesting that GSK3 β levels are not changing in the cell (Figure 5D).

Discussion

This study determined the characteristics and contents of exosomes released from placental tissue treated with environmental pollutants PBDE, TBBPA, 246TBP and BPA. The major findings of this study are: 1) exosomes released from placental explants do not vary in size, morphology, concentration or markers irrespective of pollutants; 2) treatment with environmental pollutants resulted in changes in exosome composition that reflect placental cellular and nuclear injury and cell death. Thus, our findings suggest that fetal exosomes released from the placenta during pregnancy may function as indicators of placental functions in response to maternal-fetal exposure of pollutants indicating tissue injury.

PBDE congeners are environmental pollutants and reported as risk factors associated with PTB [28, 30]. Regardless of congener treatment, placental derived exosomes had similar canonical pathways and biological functions that are likely in response to the core diphenyl ether structure. Unlike fetal membrane senescence induced by PBDE congeners 47 and 99, placental senescence was not reflected in exosomal cargo in response to PBDE. However, cellular dysfunctions indicative of cell stress, tissue damage and/or necrosis were evident through IPA and by the packaging of p38 MAPK. Data from this study showed p38 MAPK expression in PBDE 153, PBDE 209, 246TBP and BPA compared to controls and other environmental toxins. p38 MAPK is a stress-response indicator and its increased packaging in exosomes may be a potential biomarker for exposure.

Environmental pollutants, such as those studied here, have been associated with increased risk of PTB [16, 28, 53] and this study adds to our previous work suggesting that they may do this in part by promoting cell damage and placental inflammation. However, it has been difficult to determine if placental exosomes do this *in vivo*. Placental exosomes can be identified as early as 8 weeks gestation and have been used extensively as a biomarker for various adverse pregnancy outcomes [36, 54–56]. Therefore, it may be possible to study the effects of PBDEs on the placenta *in vivo* through changes in exosomes that can be easily isolated from maternal plasma. Further studies on RNA (miRNA, lncRNA, etc.), lipidome or metabolome cargo in exosomes from PBDE-treated cultures may also be useful in this regard.

Strengths of our study include the use of a well-established culture system that maintains all of the major cell types and villous formation and uses tissues from the general obstetric population. Use of standardized protocols for proteomic and exosome analysis along with strict statistical criteria for identifying differentially expressed proteins and possible pathways are further advantages. Our findings are not without limitation, however. As with

most cell culture systems, we are limited to experiments over the course of a few days. Therefore, we exposed the tissues to a relatively high concentration of toxin (20 mM) over a short period of time. When pregnant women would normally be exposed to much lower levels of brominated flame retardants or bisphenol A by ingestion of in household dust over a period of weeks and months that contains wildly varying levels of the toxins from as low as a 100 ng/g (0.184 nM) (PMID: 27623734) for TBBPA to 184.6 ug/g (0.192 uM) for BPDE (PMID: 21522188). Although the doses of the toxin used in these experiments are consistent with other researchers (PMID: 25461914, 25305463), they poorly reflect real-world exposures in patients. We also do not know the amount of PBDEs, TBBPA, 246TBP or BPA in the donated tissue prior to treatment. Although this could influence the responsiveness of the explants to the environmental toxins, it would most likely bias results towards the null. We are also limited by possibility that the patients sampled in this study are atypical in exposure to and responsiveness to environmental toxins. However, we would have had similar problems if we used placental lines and we would only know about exosome responses of the transformed trophoblasts. Furthermore, dozens of women would need in order to have a sample large enough in order to understand the distribution of responses to PBDEs taking into account factors such as ethnicity, age, BMI, etc. and our findings would still be have the all limitations of an *in vitro* culture system. Therefore, we feel that the bulk of future work in this field should be done in cohort studies where samples from asymptomatic patients are used to determine the extent to which changes in biomarkers in the exosome correlate with exposures to environmental toxins and evaluate the consequences of these exosome changes on pregnancy outcome.

In summary, we found that PBDE, TBBPAA, TBP and BPA exposure changes the composition of the protein components of placental exosomes in a congener/toxicant-specific manner but there is a consistent enhancement of proteins indicating cell damage and inflammation. Further studies are underway to determine if women with high levels of exposure to these toxins have similar changes to placental exosomes in their plasma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exosomes from environmental pollutants exposed placenta had classic features.
- Exosome characteristics were similar in pollutant exposed and control placenta.
- Exosomes carry differentially expressed protein cargo reflective of exposure.
- PBDEs and BPA/TBBP/246TBP produced exosome cargo reflective of placental injury.

Figure 1: Characterization of placental exosomes.

A: Nanoparticle Tracking Analysis shows exosome size is not significantly different between treatments and control, with a size ranging between 30–200 nm. Data are represented as mean ± SEM.

B: Exosome concentration ranged from 2.70×10^{10} to 3.85×10^{10} exosomes/mL regardless of treatment. Data are represented as mean ± SEM.

C: Western blot analysis showed exosomes from control and treated explants were enriched in exosome markers CD81 and CD63.

D: Representative cryo-electron microscopy images of control, PBDE 99 and BPA treated exosomes show spherical vesicles under 150 nm. Scale bar is 50 nm.

E-G: Proteomic analysis identified placenta-specific placental alkaline phosphatase (PLAP), CD9 and ALIX were also present in all our exosomes regardless of treatment.

Figure 3: Ingenuity pathway analysis (IPA) of differentially regulated proteins depicts canonical pathways associated with each treatment.
A-B: Canonical pathways associated with PBDE, TBBPA, 246TBP and BPA treatments show downregulation of multiple pathways associated with tissue homeostasis. Green represents upregulation, red is downregulation compared to untreated control.

Figure 4: IPA identified biological functions associated with different environmental pollutant treatments.
A-B: Biological functions associated with cellular migration, inflammation, and cell death were upregulated in exosomes regardless of treatment.

Figure 5: Damage-Associated Molecular Pattern (DAMPs – Alarmins are differentially expressed in placental exosomes treated with environmental pollutants.

A: HMGB1 packaging in exosomes was expressed in exosomes regardless of treatment type.

B: Caspase 4 was expressed in exosomes from all treatments except 246 TBP.

C: MAPK14 was expressed in PBDE 153 and 209, as well as 246TBP and BPA.

D: GSK3β expression in exosomes was constant in all treatments except 246 TBP.

Table 1:

Mean exosome size and concentration by treatment as determined by NTA. Values are shown as mean $\pm$ SEM

	Mean Size (nm)	Mean Concentration (particles/mL)
Control	176 $\pm$ 13.6	2.70 $\times 10^{10}$ $\pm$ 9.81 $\times 10^9$
PBDE 47	173 $\pm$ 11.8	2.78 $\times 10^{10}$ $\pm$ 6.51 $\times 10^9$
PBDE 100	174 $\pm$ 12.7	2.78 $\times 10^{10}$ $\pm$ 5.21 $\times 10^9$
PBDE 153	173 $\pm$ 12.4	3.10 $\times 10^{10}$ $\pm$ 4.93 $\times 10^9$
PBDE 209	185 $\pm$ 17.7	3.42 $\times 10^{10}$ $\pm$ 1.19 $\times 10^{10}$
TBBP	183 $\pm$ 12.8	3.50 $\times 10^{10}$ $\pm$ 1.20 $\times 10^{10}$
246TBP	182 $\pm$ 19.0	3.52 $\times 10^{10}$ $\pm$ 1.93 $\times 10^{10}$
BPA	177 $\pm$ 13.7	3.85 $\times 10^{10}$ $\pm$ 1.14 $\times 10^{10}$