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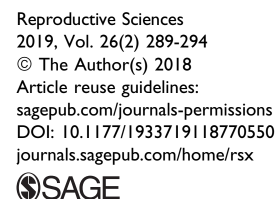

Maternal Phthalate and Personal Care Products Exposure Alters Extracellular Placental miRNA Profile in Twin Pregnancies

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Abstract

Prenatal exposure to endocrine-disrupting chemicals (EDCs) exerts both short- and long-term adverse effects on the developing fetus. However, the mechanisms underlying these effects have yet to be uncovered. Maternal–fetal signaling is mediated in part by signaling molecules (eg, microRNAs [miRNAs]) contained in extracellular vesicles (EVs) that are released by the placenta into the maternal circulation. We investigated whether maternal exposure to the EDCs phthalates and personal care products alters the miRNA profile of placental-derived EVs circulating in maternal blood. Blood and urine samples from pregnant women with uncomplicated term dichorionic, diamniotic twin pregnancies were analyzed as part of a larger study investigating correlations between exposure of phthalate and personal care products and epigenetic alterations in twin pregnancies. We explored correlations between maternal urinary levels of 13 phthalate and 12 personal care products metabolites and the miRNA profile of placental EVs (EV-miRNAs) circulating in maternal blood. The expression of miR-518e was highest among women with high urinary levels of monobenzyl phthalate and methyl paraben. miR-373-3p was the least expressed in women exposed to high levels of methyl paraben, and miR-543 was significantly downregulated in women exposed to high levels of paraben metabolites, dichlorophenol metabolites, and triclosan. In conclusion, this pilot study reveals that prenatal exposure to EDCs is associated with altered profile of circulating placenta-derived EV-miRNAs. Further studies are needed to generalize these results to singleton pregnancies and to assess whether these alterations are associated with pregnancy complications.

Keywords

phthalates, phenols, endocrine disruptor chemicals, placental extracellular vesicles, miRNAs

Introduction

Increasing concern regarding the adverse effects of endocrine-disrupting chemical (EDC) exposure, especially among pregnant women, has driven the investigation of potential mechanisms of action and the physiological consequences of EDC exposure. Two classes of EDCs, phthalates and phenols, including parabens, are widely used in cosmetics, personal care products, and food and beverage can linings.[1-3] Exposure to these compounds is ubiquitous and almost all pregnant women in Western countries have measurable concentrations of these chemicals or their metabolites in their urine.[4-8] These chemicals can penetrate the placenta,[9,10] and animal models, along with limited human studies, have shown that in utero exposure to these compounds may negatively affect the vulnerable fetus and even impair health later in life.[1-3,11-13] However, the mechanism(s) through which these chemicals affect fetal development remain poorly understood.

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A consistent body of literature has shown that the placenta actively releases extracellular vesicles (EVs) into the maternal circulation. Extracellular vesicles are membrane-bound and nanosized (0.05-1 μm) and contain signaling molecules such as microRNAs (miRNAs). MicroRNAs are small, highly conserved RNA molecules containing approximately 22 nucleotides that are excreted under both normal and pathological conditions.[14] MicroRNAs regulate gene expression patterns that drive major cellular processes such as cell differentiation, proliferation, and cell death and play key roles in both physiologic and pathologic processes. Placenta-derived EV-miRNAs are released from the placenta into the maternal circulation throughout pregnancy and have been shown to operate novel endocrine-like mediators to facilitate pregnancy and fetal growth.[15] Although phthalates and parabens can modify a large variety of endocrine pathways, whether they affect circulating EV-miRNAs has never been investigated.

The aim of this pilot study was to explore the correlation between maternal exposure to EDCs and a large profile of placenta-derived EV-miRNAs circulating in maternal blood.

Material and Methods

The study was approved by the institutional review board of Sheba Hospital (1717-14).

Study Participants

We included 10 women with uncomplicated term dichorionic, diamniotic twin pregnancies (37-39 weeks of gestation) at a tertiary university-affiliated medical center. Participants were enrolled as a part of a larger study testing associations between exposure to endocrine disruptor chemicals and epigenetic alterations in twin pregnancies. We excluded monochorionic, di-amniotic pregnancies and monochorionic, monoamniotic pregnancies as well as pregnancies complicated by preeclampsia or gestational diabetes mellitus. Signed informed consent was collected from all study participants before enrollment.

Sample Collection and Processing

Urine and blood samples were collected upon admission to the hospital for delivery before insertion of an intravenous line. To avoid contamination, patients were advised not to use any wipes before urine sample collection and only certified bisphenol A (BPA)/phthalate-free sterile polypropylene collection cups were used. Urine samples were aliquoted to 1 mL tubes and frozen at −80°C for storage before shipment for chemical analyses.

Ten milliliters of whole blood were collected from patients into K_2-EDTA vacutainer tubes. Tubes were centrifuged at 2000*g* for 10 minutes at room temperature, and plasma was aliquoted into 0.5-mL microcentrifuge tubes and frozen at −80°C.

Chemical Analysis

Urine samples were shipped to the Centers for Disease Control and Prevention, Atlanta, GA, for chemical analysis. The concentrations of 13 phthalate metabolites and 12 personal care products metabolites were quantified: monoisobutyl phthalate (MiBP), mono-3-carboxypropyl phthalate, mono(carboxy-isononyl) phthalate, mono(carboxy-isooctyl) phthalate (MCOP), mono-2-ethyl-5-carboxypentyl phthalate (MECCP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP), monobenzyl phthalate (MBzP), mono-n-butyl phthalate, mono-hydroxybutyl phthalate, mono-hydroxyisobutyl phthalate (MHiBP), mono-2-ethylhexyl phthalate (MEHP) and monoethyl phthalate (MEP), triclocarban, 2,4-dichlorophenol (24-DCP), 2,5-dichlorophenol (25-DCP), benzophenone-3 (BP-3), BPA, bisphenol F, bisphenol S, butyl paraben (B-PB), ethyl paraben (E-PB), methyl paraben (M-PB), propyl paraben (P-PB), and triclosan (TCS). The analytic approach, based on solid-phase extraction coupled with high-performance liquid chromatography–isotope dilution tandem mass spectrometry, followed standard quality assurance (QA)/quality control (QC) procedures.[16,17] The limit of detection (LOD) ranged from 0.2 to 0.6 μg/L for phthalates and 0.2 to 1.7 μg/L for phenols.

Isolation and Profiling of Placenta-Derived Circulating EV-miRNAs From Maternal Plasma

Fresh plasma samples were centrifuged 3 times at 1000*g*, 2000*g*, and 3000*g* at 4°C to remove any remaining cellular debris. To prepare EV pellets for miRNA extraction, 0.5 mL of plasma was transferred to a 10.4-mL polycarbonate ultracentrifuge tube (Beckman Coulter, CA, USA) filled with Phosphate buffered saline. Plasma was ultracentrifuged (Beckman Coulter Optima-MAX-XP) at 110 000*g* for 75 minutes at 4°C and decanted. MicroRNAs were isolated from the EV pellets using the miRNeasy Kit and RNeasy Cleanup Kit (Qiagen, Germany). Briefly, 700 μL of QIAzol Lysis Reagent was added to each ultracentrifuge tube to lyse membranized particles. MicroRNA extraction was performed according to the manufacturer's protocol. The final purified miRNA-enriched RNA was eluted in 20 μL of RNase-free water and stored at −80°C until further use. Extracellular vesicle-packaged miRNAs were prepared by standard reverse transcription (RT) and preamplification procedures, followed by real-time RT-PCR analysis with the QuantStudio 12K Flex Open Array Platform (QS12KFlex), as reported previously.[18] Briefly, RT was performed by using Megaplex RT Primers, Pool A v2.1 and Pool B v3.0, with the TaqMan MicroRNA Reverse Transcriptase Kit (Life Technologies, Foster City, California). Two distinct reactions were performed to cover RT of 754 target miRNAs (16 replicates of 4 internal controls: ath-miR159a, RNU48, RNU44, and U6). Each reaction included 0.75 μL of Megaplex RT Primers Pool A or Pool B, 0.15 μL of Deoxynucleotides (100 mM), 0.75 μL of 10× RT buffer, 0.90 μL of $MgCl_2$ (25 mM), 0.1 μL of RNase inhibitor (20 U/μL), 1.5 μL of

MultiScribe reverse transcriptase (50 U/μL), and 3.3 μL of miRNAs. After incubation on ice for 5 minutes, the mixture was subjected to the following thermal protocol in a C1000 Thermal Cycler (Bio-Rad, Hercules, California): 40 cycles of 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second, plus 1 cycle at 85°C for 5 minutes. The complementary DNA (cDNA) samples were stored at −20°C until use.

Each cDNA requiring preamplification was loaded onto a 96-well plate in accordance with the protocol provided by the manufacturer (Application Note 2011; Life Technologies). A total of 7.5 μL of each reverse-transcribed miRNA was combined with the following reaction mix: 20 μL of TaqMan PreAmp Master Mix (2×), 8.5 μL of nuclease-free water, and 4 μL of Megaplex PreAmp Primers Pool A or B (10×). Thermal conditions for the preamplification reaction were as follows: 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, 16 cycles of 95°C for 15 seconds and 60°C for 4 minutes, and 99.9°C for 10 minutes. Preamplified samples were stored at 4°C until expression analysis with the OpenArray system (Life Technologies, CA, USA). Each preamplified cDNA was diluted 1:20 with nuclease-free water. TaqMan OpenArray Real-Time PCR Master Mix (2×) was added in a 1:1 volume ratio. Then, 7 μL of the reaction RT-PCR mix was aliquoted with the MicroLab STAR Let instrument (Hamilton Robotics, Birmingham, United Kingdom) into 8 wells of a 384-well OpenArray plate. The reaction mix was loaded from the 384-well plate into a TaqMan (Life Technologies, CA, USA) Open Array Human miRNA Panel, with QuantStudio Accu-Fill System Robot (Life Technologies). The mixture was analyzed with the QuantStudio 12K Flex Real-Time PCR System with the Open Array Platform (QS12KFlex), according to the manufacturer's instructions. For each amplification curve, we obtained an AmpScore value, a quality measurement that indicates the low signal in the amplification curve linear phase (range: 0-2). MicroRNAs with Crt value >27 or AmpScore <1.1 or missing were considered unamplified and the Crt value was set to 28. Gene Expression Suite Software (Life Technologies) was used to process miRNA expression data from the miRNA panel. NormFinder[19] and geNorm[20] algorithms were used to select the best normalization strategy among global mean, RNU48, RNU44, and U6. Global mean was selected as the best normalization method. MicroRNA expression was determined using the relative quantification $2^{-\Delta Crt}$.[21] Briefly, for each sample, the global mean expression value was calculated as mean of Crt values of all the miRNAs amplified in at least one sample. For each sample, ΔCrt values for each miRNA are then calculated by subtracting the global mean Crt value from the Crt value of the considered miRNA. Relative quantification is calculated by raising 2 to the power of the negative ΔCrt value, since Crt values are related to the amount of miRNA logarithmically.

For our data analysis, we focused on placenta-specific EV-miRNAs identified a priori based on a literature search.[22-26] Specifically, several miRNAs from the chromosome 19 miRNA cluster (C19MC) and C14MC clusters were selected as candidates.[24]

Statistical Analysis

Urinary metabolite concentrations were adjusted for urinary dilution by multiplying the metabolite concentration by [(1.015 − 1)/(SG − 1)], where 1.015 is the mean SG level for all study urine samples and SG is the specific gravity of the participant's urine sample.[27]

We calculated Spearman correlations between prenatal phthalate metabolites and phenols and placenta-specific circulating extracellular miRNAs. Analyses were performed using R Statistical Computing Software (R Foundation for Statistical Computing, Vienna, Austria). $P < .05$ was considered statistically significant.

Results

The mean age (SD) of the 10 participants was 33.8 (7.8) years. Mean (SD) patients' body mass index was 26.4 (6.7) at the beginning of the pregnancy and 31.7 (5.5) at term. All women were Caucasian and delivered at term (gestational age was mean [SD]: 38 [0.4] weeks). Seven participants conceived following in vitro fertilization (2 from egg donation), 2 conceived spontaneously, and 1 following the use of clomiphene citrate. Nine participants were healthy; 1 had chronic hypertension. Of the twins, there were 3 pairs of female/female, 3 pairs of male/male, and 4 pairs of female/male. Circulating EV-miRNAs in maternal blood and urinary phthalate metabolites were measured in samples from all 10 women, and personal care product metabolites were measured in the urine from 9.

The average detection rate of 13 SG-adjusted urinary phthalate metabolites (n = 10) was 86.9% (range: 50%-100%). The MCOP, MEOHP, MEHHP, MECCP, MHiBP, MiBP, and MEP were detected in the samples from all 10 participants.

The average detection rate of 12 SG-adjusted urinary personal care product metabolites (n = 9) was 67.5% (range: 0%-100%). The 25-DCP, B-PB, BP-3, and BPA were detected in samples from all participants. Detection rates and concentrations were in line with the levels of the chemicals of the parent cohort (n = 50, Ronit Machtinger, 2017, unpublished data). In brief, M-PB was the metabolite with the highest concentration, with a mean of 89.8 ng/mL (range: <LOD to 475.5 ng/mL). Mean concentration and range of 24-DCP and 25-DCP were 0.9 ng/mL (<LOD to 2.8 ng/mL) and 17.7 ng/mL (0.2-79.9 ng/mL), respectively. Mean concentration and range of P-PB and E-PB were 0.8 ng/mL (<LOD to 39.5 ng/mL) and 1.2 ng/mL (0.2-23.6 ng/mL), respectively. Mean concentration and range of B-PB and TCS were 3.1 ng/mL (0.2-14.1 ng/mL) and 57.4 ng/mL (<LOD to 342.9 ng/mL), respectively. Mean concentration and range of MBzP were 2.5 ng/mL (<LOD to 7.7 ng/mL).

We tested possible correlations of 20 placenta-derived circulating EV-miRNAs in maternal plasma with phthalate and phenol metabolites: miR_15a, miR_127-3p, miR_142_3p, miR_185, miR_323a-3p, miR_372-3p, miR_373-3p, miR_376a-3p, miR_376c-3p, miR_409, miR_410-3p, miR_411, miR_495-3p, miR_517a, miR_518e, miR_519d, miR_522-3p, miR_525-5p, miR_526b-5p, and miR_543. In

Table 1. Significant Associations Between Phenols, Phthalates, and Placental EV miRNAs in Maternal Blood in the Third Trimester.

	miR_373_3p		miR_543		miR_518e	
Metabolites	Correlation Coefficient	*P*	Correlation Coefficient	*P*	Correlation Coefficient	*P*
24-DCP	−0.60	.07	−0.75	.01	0.41	.24
25-DCP	−0.60	.07	−0.63	.05	0.09	.80
B-PB	−0.25	.49	−0.71	.02	0.21	.56
E-PB	−0.16	.65	−0.70	.02	0.36	.31
M-PB	−0.70	.02	−0.77	.01	0.64	.05
P-PB	−0.18	.63	−0.71	.02	0.36	.31
TCS	−0.41	.24	−0.64	.04	0.50	.14
MBzP	−0.02	.95	−0.25	.49	0.69	.03

Abbreviations: B-PB, butyl paraben; DCP, dichlorophenol; E-PB, ethyl paraben; EV, extracellular vesicle; MBzP, monobenzyl phthalate; miRNA, microRNA; M-PB, methyl paraben; P-PB, propyl paraben; TCS, triclosan.

order to overcome possible batch effect, all the RNAs were extracted at the same time. Retrotranscription was done with the same mix at the same time and all samples were run in the same day using the same batch of TaqMan OpenArray Human miRNA Panels. As the miRNAs are measured in maternal blood, we conducted exploratory analysis comparing miRNAs between gender-concordant babies and did not observe any significant difference.

Maternal Urinary Phthalate, Personal Care Products Metabolites, and Placental Extracellular miRNA in Maternal Circulation

Although MBzP was positively correlated with miR_518e ($R_{\text{Spearman}} = 0.69$; $P = .03$), no correlations between maternal urinary concentrations of other phthalates and placenta-derived EV-miRNAs in maternal plasma were identified. Bisphenol F was excluded from our analysis because the detection rate was very low in our population. Urinary concentrations of M-PB were positively correlated with miR_518e ($R_{\text{Spearman}} = .64$; $P = .04$), but negatively correlated with both miR_373-3p ($R_{\text{Spearman}} = -0.70$; $P = .03$) and miR_543 ($R_{\text{Spearman}} = -0.77$; $P = .01$). Urinary concentrations of E-PB and P-PB were also negatively correlated with miR_543 ($R_{\text{Spearman}} = -0.70$ and $R_{\text{Spearman}} = -0.71$, respectively, $P = .02$ for both). In addition, increased urinary concentrations of other phenols, 24-DCP, 25-DCP, and TCS, were associated with significant decreases in miR_543 expression ($R_{\text{Spearman}} = -0.75$, $P = .01$; $R_{\text{Spearman}} = -0.63$, $P = .05$; and $R_{\text{Spearman}} = -0.64$, $P = .04$, respectively). Table 1 shows the statistical significant correlations between maternal urinary personal care products and phthalate metabolites and placenta-derived EV-miRNAs detected in maternal plasma.

In an exploratory analysis, based on our data, the top correlated miRNA level did not differ by fetal gender. To investigate the functional significance of the placenta-specific miRNAs, we applied DIANA-miRPath (http://diana.imis.athena-innovation.gr/DianaTools/index.php), a collection of tools including target prediction algorithms and a software capable of identifying potentially altered molecular pathways by the expression of a single or multiple miRNAs (mirPath). We focused on the pathways representative of the 3 miRNA target intersection and then identified 3 enriched pathways (TGF-β signaling pathway, proteoglycans in cancer, and transcriptional misregulation in cancer).

Discussion

Our preliminary results reveal that maternal exposure to EDCs such as parabens, phenols, and phthalates is associated with altered profile of circulating placenta-derived EV-miRNAs. Specifically, the expression of miR-518e was higher among women with high urinary levels of MBzP and M-PB, miR-373-3p was downregulated in women exposed to high levels of M-PB, and miR-543 was significantly downregulated in women exposed to high levels of parabens (B-PB, E-PB, M-PB, P-PB, DCP, and TCS).

High maternal exposures to MBzP and M-PB were associated with increased expression of placental EV-miR-518e circulating in maternal blood. miR-518 is a member of the C19MC family that is restricted to the placenta and the reproductive system.[28] The expression of C19MC increases significantly from first to third trimester.[29] miR-518e has been reported to be highly expressed in the placentas of women with preeclampsia compared to placentas from uncomplicated pregnancies.[26,30] Interestingly, in the HOME study, urinary levels of MBzP concentrations in the 16th week of pregnancy were significantly associated with maternal diastolic blood pressure before 20 weeks' gestation and women in the top MBzP tertile at 16 weeks of gestation had a higher risk of pregnancy-induced hypertensive diseases later in pregnancy.[31] However, in the present study, only women with term pregnancies were enrolled and none were diagnosed with preeclampsia.

High maternal urinary levels of M-PB were correlated with the lower expression of miR-373. miR-373 belongs to the miR-371-3 cluster and miRNAs within this cluster are known to be augmented slightly during pregnancy.[29] Extracellular vesicles containing miR-371-3 cluster miRNAs confer viral resistance to recipient cells through autophagy and may help immunologically protect the developing fetus.[32] Therefore, the observed correlation of M-PB with lower miR-373 may have adverse

consequences for the regulation of immunity during pregnancy. Targets of miR-373 include genes such as LEFTY1 that play a role in TGF-β signaling, DKK1, which blocks Wnt signaling, and tumor suppressor and RECK that functions as a tumor suppressor and that are essential for mammalian development.[28,33] The expression levels of miR-373 are downregulated in several types of cancers, including cholangiocarcinoma, ovarian cancer, colon cancer, lung cancer, and pancreatic cancer.[34-36] MiR-543 is a member of the C14MC cluster of miRNAs. The expression of this cluster decreases as pregnancy progresses.[29]

A previous in vitro study showed that treatment with MEHP induces miR-16, leading to increased apoptosis in human trophoblast cell lines, in a time- and a dose-dependent manner.[37] Another study demonstrated that BPA significantly altered miRNAs in several placental cell lines.[38] Both studies tested miRNAs but not specifically at EV-miRNAs.

In this study, we focused on EV-encapsulated miRNAs that are expressed from the placenta and these EV-miRNAs should not be expected to necessarily show correlations with expression in placental tissues. It was recently hypothesized that EV-miRNAs may be enriched with miRNA marks indicative of their cell of origin, contain highly specific RNA biomarkers, and protect their RNA load from degradation in the bloodstream, and therefore, the analysis of miRNAs encapsulated in EV may be superior to miRNAs in whole plasma or serum.[39] Previous studies that measured placental tissue miRNAs and circulating miRNAs showed different types of relationships between the 2 measures, including positive, negative, and no correlations.[33,40-42] Although technical differences may help explain for these differences, these results generally demonstrate that there is no straightforward correlation between circulating and placental tissues miRNAs. Indeed, growing evidence indicates that miRNAs are actively sorted in placental cells and released in the circulation through a highly regulated process, which is expected to vary in the presence of physiological and disease conditions.

Our study has some limitations. First, our sample size is small, and therefore, we did not have sufficient statistical power to test correlations in multivariate regression. As for the small sample size, the analysis was not modified to consider important differences in miRNA levels or in correlation with chemicals, by fertility treatment or chronic hypertension or to consider differences attributed to high- or low-molecular-weight phthalates. Second, by design, our study included only twins. Therefore, our results might not be generalizable to singleton pregnancies. However, it is possible that a twin model enables us to detect higher rates of circulating placenta-derived EV-miRNAs compared to singletons, given their increased placental mass. None of our P value passed the Bonferroni threshold. However, this is a hypothesis-generating analysis, and the RNAs with small P value also had big magnitude of correlation (>0.6).

Important strength of our study is that we propose a feasible method that can be applied in larger studies of pregnant women, with confirmation at the miRNA, mRNA, protein, and even functional levels.

In conclusion, in this exploratory analysis, we showed that maternal exposure to EDCs might influence placenta-specific circulating extracellular miRNAs. Our findings indicate EV-miRNAs can be a novel endocrine-like pathway that may be sensitive to EDC disruption. Identifying the specific roles of EV-miRNAs may lead to novel paths for intervention; for instance, synthetic miRNAs could be in principle administered to pregnant women via EVs to protect them against adverse pregnancy outcomes.[43] Further studies are needed to find out if these results are applicable also to singleton pregnancies and to understand the mechanism through which intrauterine exposure to phthalates and phenols affects fetal health.

Declaration of Conflicting Interests

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