Fetal Liver Bisphenol A Concentrations and Biotransformation Gene Expression Reveal Variable Exposure and Altered Capacity for Metabolism in Humans

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ABSTRACT: Widespread exposure to the endocrine active compound, bisphenol A (BPA), is well documented in humans. A growing body of literature suggests adverse health outcomes associated with varying ranges of exposure to BPA. In the current study, we measured the internal dose of free BPA and conjugated BPA and evaluated gene expression of biotransformation enzymes specific for BPA metabolism in 50 first- and second-trimester human fetal liver samples. Both free BPA and conjugated BPA concentrations varied widely, with free BPA exhibiting three times higher concentrations than conjugated BPA concentrations. As compared to gender-matched adult liver controls, UDP-glucuronyltransferase, sulfotransferase, and steroid sulfatase genes exhibited reduced expression whereas β-glucuronidase mRNA expression remained unchanged in the fetal tissues. This study provides evidence that there is considerable exposure to BPA during human pregnancy and that the capacity for BPA metabolism is altered in the human fetal liver.
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INTRODUCTION

Bisphenol A (BPA) is incorporated into polycarbonate plastic and epoxy resin for use in a variety of products for its desirable commercial properties involving transparency, malleability, and high impact strength [1]. BPA is commonly found in products such as dental sealants, water bottles, canned food items, and thermal receipt papers [2–5]. The leaching of BPA from consumer products presents many opportunities for human exposure via inhalation, ingestion, and dermal routes. Hence, human exposure to BPA is widespread, and several studies have reported detectable levels of total urinary BPA in a majority of individuals in populations around the world, including the United States, China, and Korea [6, 7]. While most human biomonitoring studies report total BPA levels in urine, free BPA and BPA metabolite measurements in tissue are necessary to assess the relationships between internal dose and adverse health outcomes.

Animal studies suggest that BPA exposures, even at low doses well below the established U.S. Environmental Protection Agency (EPA) reference dose of 50 μg/kg BW/day, are associated with breast and prostate cancer and reproductive and behavioral abnormalities [8, 9]. Among the limited studies focusing on BPA exposure on human health, increased BPA levels were correlated with cardiovascular disease, decreased semen quality, altered childhood behavior, and recurrent miscarriages [10–13]. BPA can mimic or antagonize endogenous hormones, and subsequently perturb endocrine function, by binding weakly to several steroid receptors including the estrogen receptors (ER α and β) and thyroid hormone receptor [14–16].

BPA has a strong binding affinity for the transmembrane ER, G protein-coupled receptor 30 (GPR30), and the orphan nuclear receptor, estrogen-related receptor gamma (ERRγ) [17,18]. BPA can also activate transcription factors, such as peroxisome x receptor (PXR) and the aryl hydrocarbon receptor (AhR), which are often involved in cross-talk with steroid receptors [19, 20]. Many of these receptors play an important role in gene regulation, suggesting that BPA may influence normal differentiation and maturation processes especially during embryonic and fetal development.

While the debate on human BPA exposure–disease relationships continues, the National Toxicology Program declared in 2008 that there is concern for BPA toxicity in sensitive populations such as pregnant women and children. In adults, human pharmacokinetic data suggest rapid metabolism of estrogenic free BPA into the nonestrogenic BPA glucuronide and BPA sulfate metabolites, via UDP-glucuronyltransferase (*UGT2B15*) and sulfotransferase (*SULT1A1*) enzymes, followed by urinary elimination [21,22]. In human and rodent fetuses and neonates, however, there is generally reduced or altered capacity for chemical detoxification [23, 24], and thus, metabolism may not ensure negligible risk in these vulnerable populations. Furthermore, β-glucuronidase (*GUSB*) and steroid sulfatase (*STS*), enzymes that catalyze the breakdown of inactive BPA metabolites to free BPA, have been detected in mammalian placenta [25, 26], and a number of studies report detectable levels of active BPA in the human placenta and amniotic fluid [27–29], indicating the potential for dysregulation of important biological processes necessary for fetal development and disease progression later in life.

In this study, first- and second-trimester human liver specimens were analyzed using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) for endogenous BPA concentrations to assess in utero exposure. To assess the ability of the fetus to detoxify BPA, tissue-specific BPA concentrations were compared to expression of biotransformation enzyme isoforms specific to BPA metabolism. Furthermore, we compared BPA dose and mRNA expression between fetal and adult liver specimens.

MATERIALS AND METHODS

Tissue Samples

Human fetal liver samples, ranging from gestational day 74–120, were obtained from the NIH-funded University of Washington Birth Defects Laboratory fetal tissue bank (2R24 HD000836-47). These healthy tissue specimens were collected from volunteers undergoing elective pregnancy termination during the first or second trimester of pregnancy. After surgery and proper consent for donation, organs were flash-frozen and subsequently stored in polycarbonate-free polypropylene tubing at –80°C, prior to shipment to the University of Michigan (UM) on dry ice. From the date of receipt at UM to BPA processing, only one sample was stored at –80°C for 2 years (>75% stored for <1 year). BPA in urine samples has previously been assessed for degradation in –80°C storage [30] and thus is not expected to be a considerable limitation in our study. No significant correlation was observed between time in –80°C storage and free BPA levels ($R^2 < 0.03$).

Other than gestational age, and occasionally sex and race, no identifying information about the clinical samples was provided. Samples with missing sex information ($N = 10$) were assayed for the Y-chromosome specific *SRY* and the X-chromosome specific *ATL1* genes using a nested PCR analysis as described previously, with slight modifications [31]. As controls, healthy frozen adult liver samples were procured from Asterand (Detroit, MI). These normal adult liver specimens, belonging to one male and one female 50-year-old Caucasian subjects, were recovered from routine autopsies, stored in polycarbonate-free polypropylene tubing, and immediately frozen for storage. The liver specimens did not exhibit any pathological characteristics.

BPA Analysis and Quality Control

Frozen liver samples were processed for quantification of free BPA and conjugated BPA metabolites. An average of 630 mg (between 400 and 970 mg) of excised frozen tissue was pulverized into fine powder using a stainless steel mortar and ceramic pestle above liquid nitrogen and stored in a 2-mL polypropylene eppendorf tube. The homogenized tissue was shipped to the Wadsworth Center (New York State Department of Health, Albany, NY) overnight on dry ice, where samples were processed. Homogenized liver samples were spiked with 5 ng of the internal standard, $^{13}C_{12}$-BPA, and further homogenized in 5 mL of acetonitrile. The method for the analysis of free BPA and conjugated BPA has been previously described [32]. Briefly, tissue samples were extracted several times with acetonitrile and reconstituted with 10% dichloromethane in hexane. Sample extract was then loaded onto a Strata NH_2 cartridge (200 mg/3 cc; Phenomenex, Torrance, CA) preconditioned with 5 mL of 80% methanol in acetone and 5 mL of hexane. The cartridge was washed with 5 mL of hexane and eluted with 5 mL of 80% methanol

in acetone. The eluate was concentrated to 0.5 mL under a gentle stream of nitrogen. The resulting extract represents free BPA, whereas the residual pellet was further processed for conjugated BPA quantification. The pellet, after addition of 5 ng of the internal standard and 1 mL of water, was digested in 1 mL of 1 M ammonium acetate buffer containing 2 μL/mL β-glucuronidase (with 0.6% sulfatase activity; from *Helix pomatia*, 145,700 unit/mL; Sigma, St Louis, MO) at 37°C for 12 h. This conjugated BPA fraction was extracted thrice with ethyl acetate. The sample extract was concentrated to near dryness, reconstituted with 10% dichloromethane in hexane, and purified by passing through the Strata NH_2 cartridge as described above. The final eluate was concentrated to 0.5 mL.

BPA and $^{13}C_{12}$-BPA detection from both extracts was quantified using a high-performance liquid chromatography (HPLC) coupled with API 2000 electrospray triple-quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Foster City, CA), and modified from a protocol published previously [33]. Ten microliters of each extract was injected onto an analytical column, set at 25°C and a flow rate of 300 μL/min with the mobile phase comprising of varying methanol and water mixtures (25–99% methanol gradient). Instrument parameters were as follows: MS/MS was set to electrospray negative mode, cone voltage set at –30 V, collision energy set at –25 V, capillary voltage set at –4.5 KV, and desolvation temperature set at 400°C. For detection, transitions were monitored at 227 > 212 for BPA and 239 > 224 for $^{13}C_{12}$-BPA using multiple reaction monitoring (MRM).

Several quality assurance and quality control measurements were taken to assure the validity of the analytical technique. Selected sample matrices were spiked with BPA standards and then passed through the entire analytical procedure. Results indicated an average recovery of 104% (90–120%) for spiked BPA and 85% (65–120%) for spiked $^{13}C_{12}$-BPA. An external calibration curve was prepared by injecting standards at varying concentrations (10 μL of 0.05–100 ng/mL), resulting in a calibration coefficient >0.99. In addition, a procedural blank (water) was analyzed between every 10 samples that were analyzed. To prevent contamination, only polypropylene or BPA-free supplies were used during sample processing. Furthermore, BPA-free water was stored at –80°C and then pulverized over the frozen mortar and pestle apparatus, similar to the process described above, to assess contamination introduced during homogenization. Free BPA and conjugated BPA concentrations were both below the limit of quantitation (LOQ) of 0.1 ng/g in controls and blanks. For data analysis, liver BPA concentrations below the LOQ were assigned a value of 0.071 ng/g, which was estimated by dividing the LOQ by the square root of 2.

RNA Extraction and cDNA Synthesis

Total RNA, in addition to genomic DNA and total protein, was purified from frozen liver tissue using the AllPrep DNA/RNA/Protein kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Before extraction, 10–20 mg of tissue was added to a 2-mL round bottom microcentrifuge tube containing 600 μL of Buffer RLT with 1% β-mercaptoethanol and a 5-mm stainless steel bead, and subsequently homogenized twice for 2 min at 20 Hz in the TissueLyser II (Qiagen). RNA quality and quantity was assessed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

To generate complementary DNA for each sample, approximately 1 μg of total RNA template was used with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol. The thermocycler settings for cDNA synthesis required incubation at 25°C for 5 min, 42°C for 60 min, and 90°C for 5 min.

Primer Design and Real-time Quantitative PCR

Primer pairs for real-time quantitative polymerase chain reaction (RT-qPCR) were designed for four genes related to BPA metabolism using GenScript Real-Time PCR (TaqMan) Primer Design Tool (GenScript, Piscataway, NJ). The final *UGT2B15* primer sequence was obtained from the Harvard Primer Bank. The primers for human liver reference genes, *GAPDH*, *UBC*, and *B2M* were similarly designed using GenScript. All RT-qPCR primers (Table 1) were verified using Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and chosen avoiding sequence alignment over polymorphisms or multiple binding sites. Most primers span introns or bridge exon–exon junctions, resulting in unique amplicons 50–200 base pairs long. All primer sets were tested for PCR efficiency using 10-fold serial dilutions of a standard template. Primers with the best standard curve slope and R^2 values (>0.97) were chosen as the final RT-qPCR assay.

Template amplification was set up in a 25-μL reaction for RT-qPCR using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each reaction, containing 2 μL of 10-fold diluted cDNA template and 100 nM primer concentration, was carried out in triplicate on a C100 Thermal Cycler with a CFX96 Real-Time head (Bio-Rad). Each 96-well plate contained no template controls for individual primer sets as well as plate control samples to adjust for interplate variability. RT-qPCR cycle parameters were set to 95°C for 3 min, and 40 cycles of

TABLE 1. RT-qPCR Primers for BPA Biotransformation Analysis

Gene Type	*Gene*	*Full Name*	*GenBank Number*	*Strand*	*Primers (5′ to 3′)*	T_m (°C)	*Amplicon Size*
Metabolism	*UGT2B15*[a]	UDP-glucuronosyl-transferase	NM_001076	Forward	CCAACCAATGAAGCCCCTG	60.7	94
				Reverse	GTTGTGAGCTGCGACTCGAA	62.7	
Metabolism	*SULT1A1*	Sulfotransferase	NM_001055	Forward	TCAAGGTGGTCTATGTTGCC	58.6	149
				Reverse	CCAGGATCCGTAGGACACTT	59.0	
Enterohepatic recirculation	*STS*	Steroid sulfatase	NM_000351	Forward	AGCACTGATAGGGAAATGGC	59.2	82
				Reverse	GAAGCCGTGATGTAAAGGGT	59.1	
Enterohepatic recirculation	*GUSB*	β-Glucuronidase	NM_000181	Forward	ATCGCCATCAACAACACACT	59.0	82
				Reverse	TGGGATACTTGGAGGTGTCA	58.9	
Reference	*GAPDH*	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046	Forward	CATCAATGGAAATCCCATCA	59.1	90
				Reverse	GACTCCACGACGTACTCAGC	58.5	
Reference	*UBC*	Ubiquitin C	NM_021009	Forward	GATCGCTGTGATCGTCACTT	58.8	112
				Reverse	TCTTTGCCTTGACATTCTCG	59.0	
Reference	*B2M*	Beta-2-microglobulin	NM_004048	Forward	TCGCTCCGTGGCCTTAGCTG	58.9	161
				Reverse	CAATGTCGGATGGATGAAACCCAG	56.1	

[a]Primer sequence from Harvard Primer Bank. All other primers were designed by GenScript.

95°C for 10 s and 55°C for 30 s, followed by 95°C for 10 s and 65°C for 5 s.

The average threshold cycle ($C(t)$) from triplicate runs was calculated for individual genes using the CFX Manager Version 1.6 software (Bio-Rad). An interrun calibration using the plate controls automatically adjusted data points between plates. For normalized expression, the software takes the $C(t)$ for each gene and then normalizes the values to the average $C(t)$ of the endogenous reference genes, *GAPDH*, *UBC*, and *B2M*. Results are reported as normalized fold expression change ($2^{-\Delta\Delta C(t)}$) with standard error of the mean. The final normalized fold change reported represents fetal expression relative to expression in a gender matched adult control, where fold change equals one.

Statistical Analysis

Distribution of BPA in liver tissue was examined and did not appear to be normal. Free BPA, conjugated BPA, and total BPA (ng/g wet weight) concentrations were presented as percentiles, geometric means, and variances. Bivariate nonparametric tests (Wilcoxon rank sum and Spearman correlation) were used to analyze free BPA and conjugated BPA concentrations with covariates such as sex and gestational age. Similar bivariate analyses were conducted separately with the primary predictors: mRNA expression of *UGT2B15, GUSB, SULT1A1*, and *STS*. Simple and multiple linear regression models, in which BPA concentration outcomes were log transformed, were tested to assess the relationship between BPA tissue concentrations and gene expression. Each of the four genes was individually analyzed with free BPA in a simple linear regression model. Regression models were not analyzed for conjugated BPA given that the exact BPA glucuronide and BPA sulfate metabolite composition was unknown. In the multiple linear regression models, gene expression and free BPA were analyzed after adjusting for gestational age, as a confounder, and sex, as an effect modifier. Regression coefficients (β) were analyzed and calculated as percentage change [100(e^β –1)] with p values <0.05 considered to be statistically significant. All statistical analyses were conducted using the stats and epicalc packages in R (version 2.14.2; The R Foundation for Statistical Computing 2012).

RESULTS

In the adult liver controls, the concentrations of conjugated BPA were both <LOQ, whereas the concentrations of free BPA were <LOQ and 0.38 ng/g wet weight (Supplementary Table 1 in the Supporting Information). Greater than 70% of the 50 fetal liver samples, however, contained detectable levels of free BPA and conjugated BPA, ranging from <LOQ to 50.5 ng/g (geometric mean: 2.26 ng/g) and <LOQ to 49.5 ng/g (geometric mean: 0.65 ng/g), respectively (Table 2 and Supplementary Table 1 in the Supporting Information). The majority (78%) of samples exhibited a free BPA to conjugated BPA ratio greater than 1, with a mean of 6.91 and median of 3.75, across all gestational age and sex (Supplementary Table 1 in the Supporting Information). No significant differences in free BPA (p value:

TABLE 2. Distribution of Free, Conjugated, and Total BPA (ng/g) in Human Fetal Liver Tissues (Gestational Age: day 74–120, $N = 50$)

BPA Species	Above LOQ[a] (%)	Percentile							Geometric Mean (GSD)
		Min	10th	25th	50th	75th	90th	Max	
Free	88	<LOQ	<LOQ	0.90	2.99	6.45	26.1	50.5	2.26 (6.26)
Conjugated	72	<LOQ	<LOQ	<LOQ	0.63	2.04	8.00	49.5	0.65 (6.15)
Total		<LOQ	<LOQ	1.18	3.44	12.1	35.7	96.8	2.92 (6.89)

[a] LOQ is 0.1 ng/g.

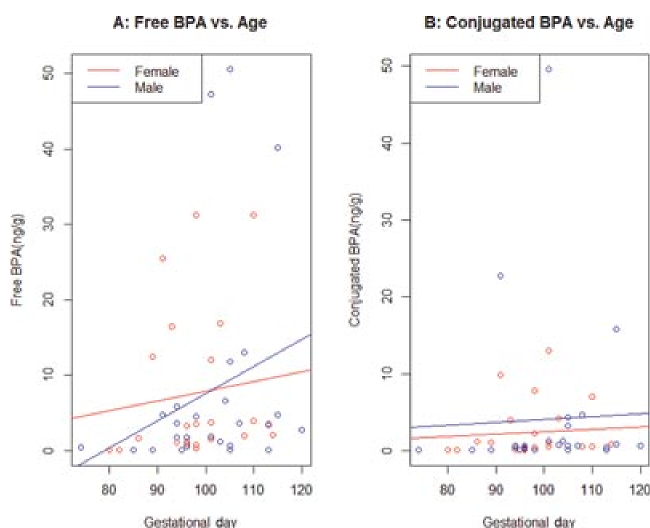

FIGURE 1. Liver tissue free BPA levels are associated with gestational age but not sex. (A) A significant positive correlation between free BPA levels and gestational age is observed (overall R^2: 0.316 and p value: 0.025). When the exposure-age correlation was stratified by sex, no significant differences were observed (female R^2: 0.333 and p value: 0.120; male R^2: 0.345 and p value: 0.078). (B) A marginally significant trend was found between conjugated BPA and gestational age (overall R^2: 0.269 and p value: 0.059; female R^2: 0.183 and p value: 0.404; male R^2: 0.062 and p value: 0.062).

0.675) or conjugated BPA (p value: 0.976) concentrations appeared between male and female specimens. After assessing BPA concentrations over developmental time span (Figure 1), gestational age was found to be significantly correlated with free BPA ($R^2 = 0.316$; p value: 0.025) but only marginally significant with conjugated BPA ($R^2 = 0.267$; p value: 0.059) across all samples.

In general, mRNA expression of *UGT2B15* and *SULT1A1* was significantly reduced (p values: 0.039 and 0.044, respectively), whereas *STS* expression was marginally reduced (p value: 0.054) in fetal liver compared to adult liver samples. Furthermore, normalized mRNA expression was significantly higher in male compared to female fetal livers, especially for *UGT2B15*, *SULT1A1*, and *STS* expression (p value: <0.005 for all three genes; Figure 2). *GUSB* displayed no significant difference in expression between fetal and adult liver samples (p value: 0.197). There were no significant correlations between expression of the four BPA biotransformation genes and gestational age (p value: >0.200 for all associations).

The association between fetal liver free BPA concentrations and expression of candidate genes related to BPA metabolism was assessed using linear regression. No significant associations were observed between free BPA concentration and expression levels in simple regression modeling (*UGT2B15* p value: 0.441, *SULT1A1* p value: 0.914, *GUSB* p value: 0.477, and *STS* p value: 0.753). The correlation between free BPA and many of the gene expression profiles, however, differed between male and female liver specimens, suggesting that sex may act as a potential effect modifier. Therefore, in the final model, an interaction term between sex and expression was added along with adjustment for gestational age. There were no significant relationships between free BPA tissue concentration and individual biotransformation gene expression values adjusting for gestational age, sex, and their interaction (p value: >0.500 for all associations).

DISCUSSION

Studies reporting internal dose of BPA in human tissues at vulnerable developmental stages are presently lacking. When we measured endogenous BPA concentrations in human fetal liver, detectable levels of free and conjugated species were found, ranging from <LOQ to 50 ng/g wet weight (parts per billion). Comparable concentrations of free BPA have been previously reported in 49 term placentas collected from Spanish women (<LOQ- 22.2 ng/g), 248 amniotic fluids from second-trimester Japanese women (<LOQ- 5.62 ng/mL), and 28 fetal livers from a Canadian population (<LOQ- 37.7 ng/g) [27,28,34]. Often, these studies only quantify free BPA or total BPA, and the LOQs are higher than 0.1 ng/g.

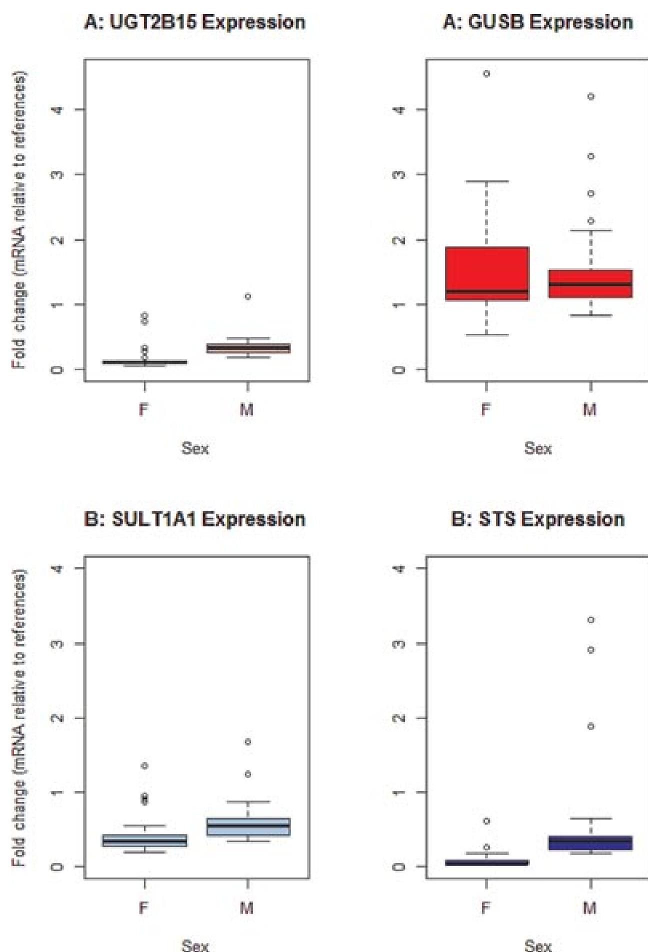

FIGURE 2. Biotransformation gene expression levels are associated with sex. Boxplots represent sex stratified biotransformation enzyme expression in fetal livers compared to adult livers, normalized to onefold change for each gene. (A) Fetal liver expression of *UGT2B15* is significantly reduced compared to adults (overall p value: 0.039). Here, normalized male expression is significantly higher than female expression (p value: < 0.001). *GUSB*, however, does not exhibit significant difference in expression between fetal and adult samples (overall p value: 0.197). Also, there is no significant difference in normalized expression between male and female (p value: 0.463). (B) Fetal liver expression of *SULT1A1* is also significantly reduced compared to adults (overall p value: 0.044). The difference in *STS* expression between fetal compared to adult samples is only marginally significant (overall p value: 0.054). Normalized male expression is significantly higher than female expression in both *SULT1A1* and *STS* (p value: 0.001 and p value: <0.001), respectively).

A higher free BPA to conjugated BPA ratio in this sample population is especially notable, given that free BPA normally represents 10–30% of total BPA in adult urinary excretions [35, 36]. The discrepancy in our clinical samples may be attributed to varying metabolic potential across tissues and time points, or as a result of contamination during sample processing. Several experimental precautions were taken, including use of polycarbonate-free equipment and negative controls throughout processing. Any exogenous BPA introduced during tissue procurement likely affected all samples; however, the wide range of free BPA and <LOQ levels observed implies otherwise. Furthermore, a recent study examining BPA in second- and third-trimester amniotic fluid from American women found that free BPA accounted for more than 80% of total BPA levels [37].

The biotransformation system undergoes significant changes during hepatic maturation, resulting in differential response to xenobiotic chemicals. Hepatic mRNA and protein expression of 13 *UGT* genes, including *UGT2B15*, were not detectable in the fetus and were reduced in children compared to adults [38]. In our larger collection of samples, we also found significantly reduced mRNA expression of *UGT2B15* in fetal liver. While fetal *GUSB* did not show differential expression, *STS* exhibited significantly reduced expression compared to adult livers, as supported by other studies [39–41]. Hepatic *SULT* genes, especially *SULT1A1*, often exhibit protein expression levels that are equivalent to adult levels [42, 43]. Although we observed reduced mRNA expression of *SULT1A1* in fetal liver, there was wide variability in our samples. Future studies can be improved by increasing the number of adult liver controls to account for intersubject variability resulting from not only differential exposure but also genetic variation contributing to altered xenobiotic metabolism. Furthermore, integration of gene ontogeny with genetic variants involved in differential BPA metabolism, such as the *UGT2B15* D85Y substitution [44], among others, will be necessary for complete understanding of altered BPA metabolism in human fetal samples.

When the relationship between biotransformation gene expression and free BPA concentration was examined, no significant correlations were observed. In addition to small sample size, several reasons may help explain the lack of association, even after adjustment for gestational age and gender. First, the mRNA levels quantified in these first- and second-trimester liver samples may not be a good proxy for measuring functional activity of biotransformation enzymes at this particular window of development. Thus, experiments that assess protein activity of BPA biotransformation enzymes in fresh human tissue will be valuable. Second, there is also the possibility that, despite experimental precautions described above, free BPA was introduced exogenously during sample procurement and this contamination may have negated any true associations between expression and exposure. Therefore, the optimal regression model should evaluate biotransformation gene expression with their respective BPA species. Future analyses will require separate characterization of both BPA glucuronide and BPA sulfate in

tissue. Finally, the high free BPA to conjugated BPA ratio observed may also be a result of deconjugation in the placenta or differential exposure from physiological changes occurring in the mother during pregnancy [40, 45]. To assess BPA fate throughout human pregnancy, investigation of BPA metabolism and transport in the placenta, maternal liver, and fetal liver is required.

As is true in many human studies, bioethical issues and the paucity of appropriate samples limit our ability to fully comprehend the role of BPA biotransformation during this sensitive window of development. This study is one of the first of its kind to investigate both BPA conjugating and deconjugating enzyme gene expression in relation to endogenous BPA exposure in the developing human fetus. Characterizing biotransformation gene ontogeny with BPA exposure from early gestation to birth will enhance our understanding of BPA fate and subsequent health effects.

SUPPORTING INFORMATION

The supporting information is available from the corresponding author (ddolinoy@umich.edu) on request.

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