Metabolomics and Data-Driven Bioinformatics Revealed Key Maternal Metabolites Related to Fetal Lethality via Di(2-ethylhexyl)phthalate Exposure in Pregnant Mice

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ABSTRACT: We performed serum metabolome analysis of di(2-ethylhexyl)phthalate (DEHP)-exposed and control pregnant mice. Pregnant mice (n = 5) were fed a DEHP-containing diet (0.1% or 0.2% DEHP) or a normal diet (control) from gestational days 0−18. After maternal exposure to 0.2% DEHP there were no surviving fetuses, indicating its strong fetal lethality. There were no significant differences in the numbers of fetuses and placentas between the 0.1% DEHP and control groups, although fetal viability differed significantly between them, suggesting that maternal exposure to 0.1% DEHP could inhibit fetal growth. Metabolomics successfully detected 169 metabolites in serum. Principal component analysis (PCA) demonstrated that the three groups were clearly separated on PCA score plots. The biological interpretation of PC1 was fetal lethality, whereas PC2 meant metabolic alteration of pregnant mice via DEHP exposure without fetal lethality. In particular, the first component was significantly correlated with fetal viability, demonstrating that maternal metabolome changes via DEHP exposure were strongly related to fetal lethality. Levels of some amino acids were significantly increased in the DEHP-exposed groups, whereas those of some fatty acids, nicotinic acid, and 1,5-anhydroglucitol were significantly decreased in the DEHP groups. DEHP-induced increases in glycine levels could cause fetal neurological disorders, and decreases in nicotinic acid could inhibit fetal growth. In addition, a machine-learning Random forest could determine 16 potential biomarkers of DEHP exposure, and data-driven network analysis revealed that nicotinic acid was the most influential hub metabolite in the metabolic network. These findings will be useful for understanding the effects of DEHP on the maternal metabolome in pregnancy and their relationship to fetal lethality.

INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) is generally used as a plasticizer in products made from polyvinyl chloride plastic such as feeding tubes and dishes, and it is easily eluted from such products under high temperature or upon exposure to alcohols.1−3 DEHP is sometimes detected in dairy products such as milk and cheese4−6 and, thus, can be unwittingly ingested. In particular, fetuses and infants can be affected by DEHP because it can be transferred not only into breast milk (owing to its high lipophilicity) but also into fetuses because of its strong placental transportability.7,8 Prenatal and postnatal toxicities to offspring have been proved by animal experiments.9−13 Moreover, epidemiological studies have revealed that DEHP can cause biological changes in humans,14 especially in pregnant women.15

DEHP exposure in utero induces neonatal undernutrition, resulting in obesity and hypothyroidism in mature adults;16 this is an example of the concept of DOHaD (developmental origins of health and disease).17−19 Studies of DOHaD involving DEHP have revealed a significant increase in food intake in the mature offspring of mice exposed to DEHP from prepregnancy to the weaning period.20 Also, triglyceride and fatty acid levels are significantly decreased in pregnant mice exposed to DEHP.21,22 Under the same conditions, hypoglycemia has been observed in infant mice on postnatal day 2.23 Moreover, Xu et al. have reported that lipid profiles in the fetal rat brain are altered by maternal DEHP exposure.24

Zhou et al. applied metabolome analysis to the plasma and urine of pregnant women exposed to phthalates under everyday conditions, including DEHP.15 They found that low- and high-
molecular-weight phthalates were related to metabolome alteration, although the effects of DEHP on the endogenous metabolome were limited. In that study, however, most of the pregnant women were overweight (mean BMI 26.4 ± 4.9 kg/m²) before pregnancy; this could have suppressed the metabolic effects of DEHP because DEHP is highly lipophilic and its pharmacokinetics could differ between lean and obese pregnant women. Therefore, it is essential to validate the effects of DEHP on endogenous metabolites by using animal models. As far as we know, however, there has been no reported animal experiment elucidating maternal metabolic changes under exposure to DEHP in pregnancy. To date, our group has reported various studies applying mass spectrometry (MS) based metabolome analysis to elucidate the pathophysiological profiles of different mouse models. Moreover, we have developed a new analytical platform based on ambient ionization MS and now have abundant experience in MS-based metabolome analysis. Therefore, here, we aimed to explore comprehensively the effects of DEHP on endogenous metabolites by applying MS-based metabolome analysis to DEHP-exposed pregnant mice.

We applied MS-based metabolome analysis to the sera of maternal mice to which DEHP was administered at different doses (0%, 0.1%, or 0.2%) from days 0 to 18 days of pregnancy. In addition, R-based bioinformatics, including multivariate and network analyses, was used to reveal specific changes in the serum metabolomes of the mice.

## METHODS

### Materials

DEHP, methoxyamine hydrochloride, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), and L-glutamic acid-15N1, 13C5 (98 atom % 13C, 98 atom % 15N) were purchased from Sigma-Aldrich (St. Louis, MO), GL Sciences (Tokyo, Japan), and Taiyo Nippon Sanso Corp. (Tokyo, Japan), respectively. Pyridine was purchased from Fujifilm Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were from Fujifilm Wako Pure Chemical Industries and Tokyo Chemical Industry Co., Ltd., and these were of analytical grade or better quality. Animal feedstuff (solid form, CE-2) was obtained from CLEA Japan (Tokyo, Japan). The appropriate amount of DEHP was diluted with ethanol, and the DEHP ethanol solution was poured into the feedstuff, where the DEHP concentration was adjusted to 0.1 or 0.2 wt %, respectively. After the DEHP-exposed feedstuff had been shaken well, the ethanol was completely evaporated off at room temperature overnight. For the control treatment, the same amount of ethanol without DEHP was poured into the same animal feedstuff, and the DEHP concentration was adjusted to 0.1 or 0.2 wt %, respectively. After the DEHP-exposed feedstuff had been shaken well, the ethanol was completely evaporated off at room temperature overnight. For the control treatment, the same amount of ethanol without DEHP was poured into the same animal feedstuff, and the DEHP concentration was adjusted to 0.1 or 0.2 wt %, respectively. After the DEHP-exposed feedstuff had been shaken well, the ethanol was completely evaporated off at room temperature overnight.

### Animal Experiments

The animal experiments were approved by the Animal Experiment Committee of Nagoya University Graduate School of Medicine (approval no. 20033). Brooks and Johanson reported the statistical power of Tukey’s test for three group comparisons, and they demonstrated that total 99 samples were required for 3 group comparisons to obtain 0.8 statistical power of Tukey’s test (i.e., 33 samples/group) although it is too hard to set such a large cohort as animal experiments because of bioethical reasons. In addition, we reported animal studies for metabolomics, where we ordinarily set n = 5–6 because of bioethical reasons.

In this study, therefore, we set n = 5 for each group. After 129/Sv female mice (10 weeks old) had been mated with mice of the same strain and age, their pregnancies were confirmed by checking for plugs; the day on which a vaginal plug was visually recognized in each mouse was determined to be gestational day (GD) 0. The pregnant mice were randomly allocated to three groups (control, 0.1% DEHP, and 0.2% DEHP, n = 5 each). Each pregnant mouse was fed the relevant diet ad libitum from GD 0 to GD 18. At GD 18, the pregnant mice were fasted for 12 h before sacrifice to avoid any direct effects of feeding on the metabolome. After each pregnant mouse had been weighed, it was dissected under anesthesia and a blood sample was collected. The number of fetuses and placentas in each dam were counted, and the survival rates of the fetuses were calculated. Each blood sample was kept on ice for 1 h after dissection, and serum samples were prepared by centrifugation of the blood at 3000 rpm for 10 min at 4 °C. Serum samples were then immediately frozen and stored at −80 °C until analysis.

### Sample Preparation of Sera for Metabolome Analysis

Serum samples were prepared for MS-based metabolome analysis in accordance with our previous reports. Detailed information is given in the Supporting Information. Briefly, the frozen serum was thawed on an ice bath. An aqueous internal standard (L-glutamic acid, solution and a chloroform–methanol solvent mixture (1:1, v/v) were added to the samples, which were then vortexed. After centrifugation at 16,000 g for 3 min at 4 °C, distilled water was added to the supernatant and the samples were further vortexed, followed by centrifugation at 16,000 g for 10 min at 4 °C. The supernatant was concentrated by using a centrifugal evaporator (CC-105, TOMY, Tokyo, Japan). After being freeze-dried (FDU-2200, EYELA, Tokyo Rikakiki Co., Tokyo, Japan) overnight, the residue was derivatized by methoximation with methoxyamine hydrochloride at 30 °C for 90 min followed by trimethylsilylation with MSTFA 37 °C for 30 min. Finally, the derivatized samples were randomly analyzed by gas chromatography (GC)/MS/MS to avoid systematic bias.

### Instrumental Analysis

Metabolome analysis was performed by a GCMS-TQ8040 gas chromatograph–tandem mass spectrometer (Shimadzu, Kyoto, Japan). The analytical conditions of the GC/MS/MS were the same as used in our previous reports. Selected reaction monitoring mode was used for relative quantification, and the selected reaction monitoring transitions were set in accordance with the GC/MS Metabolite Database (Ver. 2.0, Shimadzu). Annotation of metabolites and peak area integration were performed by GCMS Solution software (Ver. 4.20, Shimadzu) and the GC/MS Metabolite Database (Ver. 2.0, Shimadzu). Peak areas were normalized against the internal standard.

### Data Analysis

In accordance with our previous reports, the PiTaMap data pipeline by R software (version 3.6.3) was used for the statistical, multivariate (PCA and orthogonal projections to latent structures-discriminant analysis (OPLS-DA)), and network analyses. The R packages for multivariate analyses used in this study were described in our previous report. We also performed permutational multivariate analysis of variance (PERMANOVA) with first and second PCs based on Euclidean distances and 999 permutations using adonis function in R packages vegan. Permutational analysis of multivariate dispersions (PERMDISP) was performed to test for homogeneity of dispersions among groups based on Euclidean distances and 999 permutations using permutest and betadisper functions in R. We executed a machine learning Random forest using ranger packages in R and calculated area under the receiver operating characteristic curve (AUC) and the prediction values of the Random forest models using multROC and ranger.
Figure 1. Total number of fetuses, numbers of viable fetuses and placentas, and percentage fetal viability. **: \( p < 0.01 \), ***: \( p < 0.001 \) (Tukey’s test).

Table 1. Identified Metabolites in Serum of Pregnant Mice

| Metabolite                           | Control | 0.1 DEHP | 0.2 DEHP | Group                  |
|--------------------------------------|---------|----------|----------|------------------------|
| 1,5-anhydroglucitol                  |         |          |          | 0.1 DEHP               |
| 1-hexadecanol                        |         |          |          | 0.2 DEHP               |
| 2-aminoacetic acid                   |         |          |          | Control                |
| 2-aminoadipic acid                   |         |          |          | 0.1 DEHP               |
| 2-aminoisobutyric acid               |         |          |          | 0.2 DEHP               |
| 2-aminovaleric acid                  |         |          |          | Control                |
| 2-aminoxydride                       |         |          |          | 0.1 DEHP               |
| 2-deoxy-glucose                      |         |          |          | 0.2 DEHP               |
| 2-deoxyribose                        |         |          |          | Control                |
| 2-hydroxybutyric acid                |         |          |          | 0.1 DEHP               |
| 2-hydroxyglutaric acid               |         |          |          | 0.2 DEHP               |
| 2-hydroxyisobutyric acid             |         |          |          | Control                |
| 2-hydroxyisovaleric acid             |         |          |          | 0.1 DEHP               |
| 2-ketoglutaric acid                  |         |          |          | 0.2 DEHP               |
| 2-ketoisovaleric acid                |         |          |          | Control                |
| 2-methyl-3-hydroxybutyric acid       |         |          |          | 0.1 DEHP               |
| 2-methylmalonic acid                 |         |          |          | 0.2 DEHP               |
| 2-methylpropanoic acid               |         |          |          | Control                |
| 2-methylvaleric acid                 |         |          |          | 0.1 DEHP               |
| 2-methylnicotinic acid               |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
| 2-methylvaleric acid                 |         |          |          | 0.2 DEHP               |
| 2-methylphenylacetic acid            |         |          |          | Control                |
| 2-methylpropionic acid               |         |          |          | 0.1 DEHP               |
packages in R. Significant differences were determined by Tukey’s test. To control for multiple comparisons of metabolome data, the P-values obtained by Tukey’s test were adjusted by the false discovery rate (FDR) procedure proposed by Benjamini and Hochberg and the adjusted P-values were described as q-values.\(^3^3\) Spearman’s correlation coefficient was calculated by using the R software. We also performed a correlation-based network analysis, whereby the criterion was set at \(R > 0.75\) and the size of each node (circle) represented the corresponding betweenness centrality (BC) value. Network analysis was performed by using the igraph package\(^3^4\) for R software.

## RESULTS

### Effects of DEHP on Fetuses and Placentas

The total amount of food intake and absolute amount of DEHP intake are shown in the Supporting Information (Figure S1). We plotted the total number of fetuses, the numbers of placentas and viable fetuses, and the percentage fetal viability (Figure 1). Based on daily food consumption for each mouse, we converted DEHP doses at 0.1 and 0.2% DEHP in diet to 133 and 297 mg/kg/day (the mean values), respectively. In many previous studies, 500 and 750 mg/kg/day DEHP doses were ordinary used for pathological effects of DEHP exposure in animal experiments.\(^9,3^5−3^8\) Additionally, Lamb et al. reported developmental toxicity of DEHP in CD-1 mice, where 0.3% DEHP (i.e., 432 mg/kg/day) in the diet caused no survival fetuses, and No Observed Adverse Effect Level (NOAEL) was estimated at 0.01% DEHP (i.e., 14 mg/kg/day) in the diet.\(^3^9\) Thus, DEHP doses used in our study were set in the range from NOAEL (0.01%) to highest dose (0.3%) used in the study by Lamb et al. Unexpectedly, however, no fetuses survived in the 0.2% DEHP group, and almost no placentas were observed in this group. In contrast, there were no significant differences in the total number of fetuses or the numbers of placentas or viable fetuses between the 0.1% DEHP and control groups, although fetal viability was significantly greater in the control group (control group: 96.7%; 0.1% DEHP group: 65.3%; 0.2% DEHP group: 0%; \(P < 0.01\), Tukey’s test). We examined the body weights of the maternal mice on GD 18 (Table S1). Although there were no significant differences in body weights of the maternal mice between the 0.1% DEHP and control groups, the body weights of the maternal mice in the 0.2% DEHP group, in which there were no fetuses, were significantly lower than those in the other groups (\(P < 0.001\), Tukey’s test).

### Metabolome Analysis

We identified 169 metabolites in the serum of pregnant mice (Table 1). The raw data of the metabolome analysis and box-and-whisker plots for all metabolites are shown in the Supporting Information (Table S2 and Figure S2). Score and loading plots of the principal component analysis (PCA) were obtained by the PiTMaP data pipeline\(^3^0\) (Figure 2).
In the PCA score plots, the three groups were clearly separated. Additionally, PERMANOVA detected significant difference of PCs among groups (pseudo $F = 20.875$, $p = 0.001$) while no significant dispersion was detected among groups in PERMDISP ($F = 0.5676$, $p = 0.533$). Therefore, separation of the three groups in PCA score plots were statistically confirmed. Along the first principal component (PC1) axis, the 0.2% DEHP group was separated from the other groups. In contrast, the 0.1% and 0.2% DEHP groups were separated from the control group along the second principal component (PC2) axis. We calculated Spearman’s correlation coefficients between PC1 and fetal viability and between PC2 and fetal viability; this yielded a significant negative correlation between PC1 and fetal viability ($R = -0.876$, $P < 0.001$), although there was no significant correlation between PC2 and fetal viability ($R = 0.265$, $P = 0.33$) (Figure 3).

The important metabolites that contributed to the directions of the PC1 and PC2 axes were extracted by the loading of each, where the criterion was $>0.1$ in absolute loading value (Table S3). Under these criteria, 45 metabolites were determined as

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**Figure 3.** Spearman’s correlation coefficients (a) between PC1 and fetal viability and (b) between PC2 and fetal viability.

**Figure 4.** Network analysis of serum metabolome of DEHP exposed and control pregnant mice. Criterion was set at $R > 0.75$, and the size of each node (circle) was proposed to the corresponding betweenness centrality (BC) values. Each color means the clusters determined by the hierarchical cluster analysis.
PC1 related and 40 as PC2 related. These metabolites were also visually plotted in the loading plots (Figure 2), where PC1- or PC2-related metabolites were plotted in the upper/lower or right/left areas, respectively. Significance tests using Tukey’s test with FDR correction were applied to the metabolites contributing to PC1 (45 metabolites); the levels of 44 were significantly changed (q < 0.05) between the control and 0.2% DEHP groups and 39 were significantly changed between the 0.1% and 0.2% DEHP groups, whereas there were no significantly altered metabolite levels between the 0.1% and control groups (Table S4).

Significance tests using Tukey’s test with FDR correction were also applied to the metabolites contributing to PC2 (40 metabolites); the levels of seven metabolites were significantly changed between the control and 0.1% DEHP groups and of 11 between the control and 0.2% DEHP groups, although there were no significantly altered metabolite levels between the 0.1% and 0.2% DEHP groups (Table S5). Although the phenotypes of control, 0.1% and 0.2% groups were strictly different, we supplementally performed OPLS-DA to (1) control and 0.1% vs 0.2% groups and (2) control vs 0.1% groups, and the results by OPLS-DA are shown in Figure S3. Here, almost all metabolites that contributed to group separation in the PCA score plots were overlapped, with the metabolites that were extracted by OPLS-DA (Tables S6 and S7). Additionally, we applied a machine learning Random forest to the metabolites that contributed to PC1 or PC2 for discriminating three groups and calculated the AUC and prediction values of each model, where we changed the number of metabolites and number of variables randomly sampled as candidates at each split (i.e., mtry value) (Figure S4).

On the basis of the highest AUC and prediction values for the Random forest models, 16 metabolites (Pantothenic acid, tyrosine, 2-ketoisocaproic acid, leucine, valine, phenylalanine, tryptophan, pyridoxal, ornithine, cysteine, 3-hydroxyphenylacetic acid, aspartic acid, fructose, methionine, glycine) were determined as the most important metabolites for the discriminating groups (AUC value = 1.0, prediction value = 75.7%). These metabolites are also asterisked in Table S3.

Network Analysis. We plotted the results of the network analysis, including the hub metabolites, by the BC values (Figure 4). Hierarchical cluster analysis also demonstrated that there were 27 clusters in the metabolome network (Figure S5), and these are shown in each color in Figure 4. Nicotinic acid had the highest BC value (1558) among the metabolites (average BC value = 139.4). BC values were also higher than 450 for catechol (905), glycine (797), uracil (627), phenylalanine (578), 2-ketoisocaproic acid (578), tryptophan (496), 4-hydroxyphenylacetic acid (492), tyrosine (489), mesoerythritol (484), arginine (478), fructose (465), and fumaric acid (451). Details of the BC data are listed in the Supporting Information (Table S8).

DISCUSSION

Biological Interpretation of PC1 and PC2 and Metabolic Alterations Caused by DEHP Exposure. Exposure of pregnant mice to 0.2% DEHP (i.e., 297 mg/kg/day) induced strong embryonic lethality because most of the placentas in the 0.2% DEHP group were eliminated or were not formed in pregnancy. In contrast, exposure of pregnant mice to 0.1% DEHP (i.e., 133 mg/kg/day) lethally inhibited the growth of about 30% of fetuses. As described above, Lamb et al. reported developmental toxicity of DEHP in CD-1 mice, where 0.3% DEHP (i.e., 432 mg/kg/day) in the diet caused no survival fetuses, and NOAEL was estimated at 0.01% DEHP (i.e., 14 mg/kg/day) in the diet. Zong et al. reported that maternal exposure to DEHP disrupts placental growth and development in pregnant CD-1 mice, although embryonic lethality in our study was somewhat stronger than that in their study, which was likely due to mouse strain differences or different routes of administration. The three groups were separated in the PCA score plots (see Figure 2), although the biological interpretations of PC1 and PC2 were different: the biological interpretation of PC1 was fetal lethality (i.e., loss of fetuses), whereas the biological interpretation of PC2 was metabolic alteration of pregnant mice via DEHP exposure without fetal lethality. Although a combination of other confounding factors can also contribute to group separation on PCA score plots, there was the significant negative correlation between PC1 and fetal viability (see Figure 3). Thus, the above-mentioned speculation was strongly supported by the significant negative correlation between PC1 and fetal viability. Among the metabolites with significantly changed levels that contributed to PC1 (i.e., fetal lethality), the levels of some amino acids (e.g., tyrosine, leucine, glycine, tryptophan, arginine, valine, methionine and phenylalanine) were significantly increased (see Figure S2), whereas those of some fatty acids such as decanoic and octanoic acids were significantly decreased. Di Giulio et al. reported that serum levels of the aforementioned amino acids, such as tryptophan and arginine, are significantly decreased in pregnant women; thus, the increased levels of tyrosine, leucine, glycine, tryptophan, arginine, valine, methionine, and phenylalanine that we observed here were likely due mainly to the loss of fetuses via 0.2% DEHP exposure in pregnancy. In addition, as Nakashima et al. have reported that fatty acid levels are significantly increased in pregnant mice, the decreases in levels of some fatty acids in the 0.2% DEHP group were also likely due to the loss of fetuses. Moreover, the fetal viabilities of three of the maternal mice in the 0.1% DEHP group (#1, #2, and #5) ranged from 40% to 67%, and these were plotted near the 0.2% DEHP group in the PCA score plots (Figure 2). However, the fetal viabilities of the other mice in the 0.1% DEHP group (#3 and #4) were 80%, and these were plotted far away from the 0.2% DEHP group in the score plots (Figure 2). Consequently, the biological interpretation of PC1 was strongly supported by these findings.

As is well-known, glycine shows neurotoxicity, and its accumulation in the fetal brain can cause neurological disorders (i.e., glycine encephalopathy). Therefore, xenobiotics that can increase glycine levels in the maternal blood (i.e., causing nonketotic hyperglycinemia) have high potential to accumulate glycine in fetuses. For instance, Ericsson et al. have shown that pirimic acid (WY-14643), a PPARz (peroxisome-proliferator-activated receptor α) agonist, increases plasma glycine levels. The metabolites of DEHP are also known to be PPARα agonists; thus, DEHP exposure of pregnant mice can potentially increase glycine levels in their blood. In fact, serum glycine levels in the 0.1% and 0.2% DEHP groups were significantly higher than that in the control group (see Figure S2), and this could have caused accumulation of glycine in fetuses, which may cause fetal lethality. In addition, Yang et al. reported that DEHP modulates glycine receptor function in Xenopus laevis oocytes, enhancing agonist-induced currents from glycine receptor. Therefore, DEHP could modulate glycine receptor in fetuses, which may also contribute to fetal glycine encephalopathy.
Among the metabolites with significantly changed levels that contributed to PC2, nicotinic acid, 1,5-anhydroglucitol (1,5-AG), tagatose, and glycolic acid commonly had decreased levels in the 0.1% and 0.2% DEHP groups (Table S5 and Figure S2). As mentioned above, Zhou et al.15 reported that the levels of some phthalate metabolites were correlated with those of nicotinic acid, suggesting that phthalate exposure affects the biosynthesis of nicotinic acid. In addition, Santhosh et al.50 reported an inhibitory effect of DEHP on nicotinamide adenine dinucleotide (NAD⁺) synthesis in erythrocytes. Because nicotinic acid is a precursor of NAD⁺,51 the significant decrease in nicotinic acid levels that we observed was likely due to DEHP exposure. Also, Fratta et al.52 demonstrated that niacin including nicotinic acid is essential for fetal growth; thus, the decrease in nicotinic acid levels upon DEHP exposure was related to fetal growth in our study. Fukuwatari et al.53 reported that DEHP exposure. These metabolites also showed significant differences (q < 0.05, see Table S4) between groups. These approaches were based on intergroup differences, although the hub metabolites in the metabolic network could not be determined. Interestingly, Yu et al. reported that they determined hub molecules and the bottlenecks in protein networks with a high BC obtained by network analysis.59 Determination of hub metabolites is also meaningful to understand maternal metabolome disruption by DEHP exposure because there is a possibility that hub metabolites are center of propagation in metabolic alteration. To investigate this issue further, we applied a correlation-based network analysis to the metabolome data in accordance with our previous report.7 The network analysis revealed that nicotinic acid was the most influential hub metabolite in the metabolome disruption caused by DEHP in pregnant mice. Also, catechol, glycine, uracil, phenylalanine, 2-ketoisocaproic acid, tryptophan, 4-hydroxyphenylacetic acid, tyrosine, meso erythritol, arginine, fructose, and fumaric acid had strong influences on the metabolic network as hub metabolites in DEHP exposure, although the roles of catechol, 2-ketoisocaproic acid, 4-hydroxyphenylacetic acid, tyrosine, meso erythritol, and fumaric acid were not elucidated in this study.

As mentioned above, nicotinic acid is essential for fetal growth because it strongly contributes to de novo synthesis of NAD⁺ via the bioconversion of tryptophan to nicotinic acid. NAD⁺ is required to supply protons for oxidative phosphorylation in mitochondria, and thus, the disruption of NAD⁺ synthesis could cause mitochondrial dysfunction, which is also induced by mono(2-ethylhexyl)phthalate (MEHP), inhibiting placental growth.51 Martinez-Razo et al. reported detailed review of mechanisms of action of DEHP in placental development and pregnancy disorder, and mitochondrial dysfunction via reactive oxygen species (ROS) production is one of the mechanisms of action of DEHP and MEHP.51 Network analysis could determine hub metabolites in the targeted network, and thus, nicotinic acid could be influential in the metabolome disruption caused by DEHP exposure, which might be due to mitochondrial dysfunction. To confirm the fetal effects in more detail, however, we will need to analyze the offspring of DEHP-exposed pregnant mice. Additionally, different DEHP doses may cause other biological effects, and thus, further experiments are underway.

### CONCLUSIONS

We applied MS-based metabolome analysis to serum samples of DEHP-exposed and control pregnant mice. In PCA score plots, the three groups were clearly separated although biological interpretations of the first and second component axes were different: PC1 was interpreted as fetal lethality (i.e., loss of fetuses), whereas PC2 was interpreted as the metabolic alteration of maternal mice via DEHP exposure without fetal lethality. The levels of some amino acids, in particular, were increased in the DEHP groups, possibly because of the loss of fetuses. In addition, nicotinic acid and 1,5-AG metabolism were altered by DEHP exposure. We also applied a machine learning Random forest to discriminate three groups and calculated AUC and prediction values. Based on the highest AUC and prediction values of the Random forest models, 16 metabolites (pantothenic.acid, tyrosine, 2-ketoisocaproic acid, leucine, valine, phenylalanine, tryptophan, pyridoxal, hypotaurine, ornithine, cystamine, 3-hydroxyphenylacetic acid, aspartic acid, fructose, methionine, glycine) were determined as potential biomarkers of DEHP exposure. These metabolites also showed significant differences (q < 0.05, see Table S4) between groups. These approaches were based on intergroup differences, although the hub metabolites in the metabolic network could not be determined.
revealed that nicotinic acid was the most influential hub metabolite in the sera of DEHP-exposed pregnant mice. These findings will be useful not only for understanding the effects of DEHP on the maternal metabolome in pregnancy but also for exploring potential biomarkers for DEHP exposure in humans.

**ASSOCIATED CONTENT**

 Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acsomega.2c02338.

Details of sample preparation of serum for metabolome analysis; (Table S1) number of total fetuses, viable fetuses and placentas, fetal viability, and body weights of maternal mice and Tukey’s test results; (Table S2) raw data of metabolome analysis; (Table S3) metabolites contributed to PC1 or PC2 (absolute loading value >0.1); (Table S4) q values of PC1-related metabolites by Tukey’s test with FDR correction; (Table S5) q values of PC2-related metabolites by Tukey’s test with FDR correction; (Table S6) comparison of the extracted metabolites by OPLS-DA (VIP > 1.0) for control/0.1% DEHP vs 0.2% DEHP groups, and PC1 in PCA; metabolites written in red means overlapped ones; (Table S7) comparison of the extracted metabolites by OPLS-DA (VIP > 1.0) for control vs 0.1% DEHP groups and PC2 in PCA; metabolites written in red means overlapped ones; (Table S8) betweeness centrality (BC) values; (Figure S1) total amount of food intake and absolute amount of DEHP intake; (Figure S2) box-and-whisker plots for all metabolites; (Figure S3) OPLS-DA score and loading plots for (a) control/0.1% DEHP vs 0.2% DEHP groups, and (b) control vs 0.1% DEHP groups; (Figure S4) AUC and prediction values of Random forest models; (Figure S5) dendrogram for hierarchical cluster analysis (PDF)

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**Author Contributions**

K. Z. conceived the idea and designed and supervised the experiments. K. Z. and A. I. wrote the manuscript. K. Z. performed experiments, and K. Z., J. C. and A. I. executed bioinformatics analyses. K. Z., T. A. and A. I. revised manuscript. K. Z., D. K., J. C., K. H., M. T., T. A. and A. I. discussed the results. D. K., H. K., M. T. and T. A. commented the manuscript.

**Notes**

The authors declare no competing financial interest.

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