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Original Article

Extracellular vesicle small RNAs secreted from mouse amniotic fluid induced by repeated oral administration of VPA to pregnant mice

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ABSTRACT — Extracellular vesicles (EVs) are particles released not only from blood cells but also from various organs. EVs, which are lipid bilayer vesicles, contain proteins, DNAs, and RNAs. The RNA and proteins within EVs display cell-specific characteristics. EVs derived from tumor cells are identified as biomarkers with diagnostic accuracy exceeding 90% for early cancer detection. Furthermore, EV RNA in serum has serves as a biomarker for toxicity. EVs have been found in various body fluids, including saliva, tears, urine, and amniotic fluid. In this study, we aimed to investigate the potential use of EV RNA in amniotic fluid as an indicator of developmental toxicity. Pregnant mice were exposed to valproic acid (VPA), a developmental toxicant, at concentrations of 0, 300, or 600 mg/kg/day on gestational days (GDs) 9–11. The study involved measuring VPA concentration in maternal plasma and fetuses on GD11, fetal weight on GD15 and 18, and assessing external morphological abnormalities on GDs11, 15 and 18. Additionally, EVs were collected from fetal amniotic fluid, and a comprehensive gene expression analysis of EV RNA was conducted on GD15. As a result, the concentration of VPA in the fetuses was not associated with the implantation location. Additionally, the VPA-treated group exhibited intrauterine growth retardation and teratogenic effects, including neural tube defects and digit malformations. EV RNA analysis identified differentially expressed EV small RNAs, both suppressed and induced, in the VPA-treated group compared with the control (vehicle, 0.5% Methylcellulose) group. These findings suggest that EV RNA in amniotic fluid serve as an indicator of developmental toxicity.

Key words: Extracellular Vesicle (EV), Exosome, Valproic acid (VPA), Amniotic Fluid, Mouse, Teratogenicity

INTRODUCTION

Extracellular vesicles (EVs) represent a diverse category of membrane vesicles, commonly categorized into exosomes and microvesicles based on their origin. Exosomes, with a diameter ranging from 40 to 150 nm, are derived from endosomal membranes, while microvesicles, ranging from 150 to 1000 nm in diameter, are

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derived from the plasma membrane (Jeppesen *et al*., 2019; Thery *et al*., 2018). These EVs contain proteins, phospholipids, DNA, mRNA, noncoding RNA, and small RNAs, playing crucial roles as mediators in intercellular communication and facilitating horizontal gene transfer (Kawamura *et al*., 2019; Ono *et al*., 2019; Thery, 2011).

Recent findings indicate that circulating EVs in bodily fluids may serve as diagnostic biomarkers for various cancers (Choi *et al*., 2011; Gerstman and Levene, 1974; Logozzi *et al*., 2009; Lu *et al*., 2009; Rabinowits *et al*., 2009). Additionally, we have established a novel method for assessing toxicity, using EV RNA in the bloodstream as an indicator of liver toxicity induced by the oral administration of carbon tetrachloride (CCl4) (Ono *et al*., 2020).

EVs have been documented not only in in the bloodstream but also in various bodily fluids (Admyre *et al*., 2007; Keller *et al*., 2007; Klingeborn *et al*., 2017; Kosaka *et al*., 2010; Moon *et al*., 2011; Nakatani *et al*., 2006).

Prenatal detection of chromosomal and genetic abnormalities through amniocentesis, a procedure known for nearly 100% accuracy (Nizard, 2010), serves as a definitive diagnosis following maternal serum marker tests and advanced prenatal screenings (Nishiyama *et al*., 2015; Kang *et al*., 2023; Nizard, 2010; Wilmot *et al*., 2023). The identification of EVs in amniotic fluid, derived from fetal cells, demonstrates their potential as biomarkers for fetal toxicity (Choi *et al*., 2011; Gerstman and Levene, 1974; Logozzi *et al*., 2009; Lu *et al*., 2009; Rabinowits *et al*., 2009). Therefore, our study aims to investigate the detection of fetal toxicity using EV RNA in fetal amniotic fluid as a biomarker, with VPA, a recognized developmental toxicant, used as the model substance (Alsdorf and Wyszynski, 2005; Ardinger *et al*., 1988; Nau, 1994). Despite VPA's widespread use as an antiepileptic drug, it is associated with teratogenic effects, specifically neural tube closure disorders such as spina bifida and anencephaly (Alsdorf and Wyszynski, 2005; Koren *et al*., 2006; Nau, 1994; Rodriguez-Pinilla *et al*., 2000). According to the National Institute for Health and Care Excellence (NICE) guidelines, the total odds ratio for congenital abnormalities in humans is 4.07 (95% confidence interval [2.41, 6.88]). Furthermore, a prospective study endorsed by NICE revealed a significant association with neural tube defects, exhibiting an odds ratio of 10.41 (95% confidence interval [3.82, 28.14]).

Pregnant mice were orally administered VPA at concentrations of 0, 300, and 600 mg/kg/day on gestational days (GDs) 9–11. Initially, we determined the exposure status of the test substance in pregnant animals by measuring VPA concentrations in maternal plasma and fetal tissues post-administration. Additionally, we evaluated fetal development and external morphological abnormalities, collecting amniotic fluid for analysis. Utilizing a comparative approach to examine the transcriptome of EV RNA in amniotic fluid from the control (vehicle, 0.5% Methylcellulose)-treated, low-dose VPA-exposed, and high-dose VPA-exposed groups, we investigated the potential of EV RNA in amniotic fluid as a biomarker for VPA administration.

MATERIALS AND METHODS

Sample preparations

Test substance analyte was Sodium valproate (VPA) (Sigma-Aldrich Japan, Tokyo, Japan). Vehicle was 0.5 w/v% Methyl Cellulose 400 Solution, Sterilized (Fujifilm Wako Pure Chemical Corporation, Tokyo, Japan).

Administration route, method, duration, frequency, and rationale for selection

The selected administration route was oral, reflecting a clinically relevant approach. The administration period spanned 3 days, specifically from GDs 9–11, corresponding to the critical period for VPA's teratogenic effects. The chosen frequency of administration was once daily, aligning with common practice in repeated dosing studies, totaling three doses. The administration method involved a widely used forced oral administration method for rodents. Given VPA's known solubility in water for injection at a concentration of 5%, the administration volume of the test solution was standardized at 16 mL/kg. This solution was forcibly administered into the stomach using a flexible gastric tube, typically between 8:00 and 11:00. The volume of the administration solution for each animal (expressed in units of 0.01 mL) was calculated based on the body weight on the day of administration.

Dose and group composition for the EV analyses

Three doses—0 (vehicle, 0.5% Methylcellulose), 300, and 600 mg/kg/day—were selected, leading to the formation of nine groups, considering the timing of sample collection. Specifically, there were pregnancy examination groups for each dose on GD11, GD15, and GD18. The number of pregnant animals in each examination group was standardized at four animals. The group composition is detailed in the table below.

	Autopsy timing	Test Group Dose (mg/kg/day)	Concentration (mg/mL)	Administration Volume (mL/kg)	Number of Successfully Mated Female Animals	Animal Numbers
Control Group (vehicle)	GD11	0	0	16	4	1101–1104
	GD15				4	2101–2106
	GD18				4	3101–3105
Low-Dose Group	GD11	300	18.75	16	4	4101–4107
	GD15				4	5101–5106
	GD18				4	6101–6105
High-Dose Group	GD11	600	37.5	16	4	7101–7106
	GD15				4	8101–8106
	GD18				4	9101–9106

Dosage setting rationale

The clinical therapeutic dose for the test substance is in the range of 400–1200 mg/day. Assuming a standard weight of 50 kg, this translates to 8–24 mg/kg/day. Pregnant individuals taking VPA within this range during early pregnancy have reported cases of congenital anomalies such as spina bifida, ventricular septal defects, polydactyly, cleft palate, and hypospadias.

Downing *et al*. (2010) reported abnormalities in the spine, ribs, and fingers following a single intraperitoneal injection of VPA at doses of 800 or 400 mg/kg on GD9 (plug day = GD0) in mice.

In this experiment, low-dose VPA exposure (400 mg/kg) and high-dose VPA exposure (800 mg/kg) groups, exhibiting clear teratogenic effects, were initially established for analyzing EVs in amniotic fluid.

However, during actual administration, maternal animals died after the first administration at 800 mg/kg/day, making it challenging to obtain the required number of TK samples at this dose. Therefore, the dosage was adjusted to 600 and 300 mg/kg/day.

Animals

All animal studies adhered to the guidelines set by the animal care committees of the National Institutes of Health Sciences and BoZo Research Center. Female and male C57BL6/J mice, aged 10 and 11 weeks, respectively, were procured from Jackson Laboratory Japan (Yokohama, Japan). The mice were provided with a standard chow diet and water ad libitum and were housed in a pathogen-free barrier facility with a 12 L:12 D cycle.

Mating involved cohabiting one female over 11 weeks of age with one male over 12 weeks of age overnight. Females with confirmed vaginal plugs the following morning were identified as mated animals, designating that day as GD0. Grouping was based on GD0, and computer-assisted blocking was used to balance the weights of each group as much as possible.

On GD11, GD15, and GD18, blood was collected as extensively as possible from the abdominal aorta using an untreated syringe under isoflurane inhalation anesthesia. Subsequently, euthanasia was performed by severing the abdominal aorta and exsanguination, followed by a detailed examination of major organs/tissues in the external body, thoracic cavity, and abdominal cavity. Then, on GDs 15 and 18, each stage of the fetus and placenta were separated, and their individual weights were recorded. In the fetus on GD15, amniotic fluid was collected using a barrier chip and transferred to a polypropylene container (low protein-binding).

Serum biochemistry analysis

Maternal blood was transferred to polypropylene containers with low protein-binding properties, left at room temperature for 30 min or more, and serum was obtained by centrifugation (4°C, 6000 ×g, 2 min). The serum was then stored in a freezer at −80°C (with an allowable value below −70°C) until further measurement.

Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE), and urea nitrogen (BUN) in each serum sample were determined using the automatic blood chemistry analyzer Dry-Chem NX 500 V (Fuji Film, Tokyo, Japan).

Measurement of VPA concentrations in GD11 maternal plasma and fetus

Two groups were formed on GD11 with doses set at 300 and 600 mg/kg/day, each consisting of three successfully mated female animals. Out of the five mice that successfully mated, three were selected based on smaller Animal Numbers. The detailed group composition is provided in the table below.

Test Group	Dose (mg/kg/day)	Concentration (mg/mL)	Administration Volume (mL/kg)	Number of Successfully Mated Female Animals	Animal Numbers
Low-Dose Group	300	18.75	16	5	4108–4112
High-Dose Group	600	37.5	16	5	7107–7111

The analysis was outsourced to BoZo Research Center Tsukuba Laboratory, Inc.

Samples for measurement included TK samples obtained from three individuals from each group and time points of pregnant animals 1 hr post-dose on the final administration day. The fetus was homogenized using Shake Master NEO (Bio Medical Science, Japan). The plasma and fetus homogenate samples were deproteinized with methanol and the supernatant was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Instrumentation and Models:

- Tandem Quadrupole Mass Spectrometer (MS/MS): (Waters Corporation Japan, Tokyo, Japan)
- Data Processing Software: MassLynx 4.1(Waters Corporation Japan, Tokyo, Japan)
- High-performance liquid chromatography (HPLC): ACQUITY UPLC I-CLASS (Waters Corporation Japan, Tokyo, Japan) (HPLC column: Zorbax SB-C18, 3.5 µm, 2.1 mm x 100 mm, Agilent Tchnologies Japan, Tokyo, Japan)

The mobile phase consisted of acetonitrile-water (45:55) containing 0.1% acetic acid at a flow rate of 0.5 mL/min. The target substance was VPA, and the standard substance was VPA (Lot Number: WXBD4552V, Salt Conversion Factor: 0.8677 [=144.21/166.19]). The internal standard substance was diclofenac sodium salt (Lot Number: P7E3B).

The precursor-to-product ion transitions m/z 142.99>142.99 for VPA and m/z 249.97>214.00 for the internal standard at positive mode were used.

EV Analysis

After removing debris by centrifugation at 10,000 g for 10 min, the supernatant was filtered through a 0.8-mm filter, and EVs were collected using exoRNeasy (Qiagen Japan, Tokyo, Japan). Nanoparticle tracking analysis (NTA) of the EVs was conducted using a NanoSight system (NanoSight, Malvern, UK) on EVs diluted 1000-fold with PBS, as previously described (Yoshioka *et al*., 2014). Western blotting for CD9a surface antigens of exosomes was performed using an anti-CD9 antibody (Abcam Japan, Tokyo, Japan #82390 anti-rat CD9 monoclonal antibody) and Goat anti-rat IgG, HRP-linked (GE Healthcare Japan, Tokyo, Japan) as a secondary antibody. Subsequently, the chemiluminescent reaction was initiated using ImmunoStar LD (Wako, Japan), and detection was carried out using LAS300 (Fuji Film, Tokyo, Japan).

To extract EV-associated RNA, TRIzol was used to lyse EVs, and EV-associated RNAs were extracted using a miRNeasy microelution kit (Qiagen Japan, Tokyo, Japan) following the manufacturer's instructions. The assessment of EV-associated RNA involved the use of a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), an Agilent 2100 Bioanalyzer, and RNA pico chips (Agilent Technologies, Palo Alto, CA, USA).

cDNA synthesis from EV-associated RNAs was carried out using a SMARTer smRNA-Seq Kit for Illumina (Clontech/TAKARA, Kyoto, Japan) following the manufacturer's instructions.

The quality and quantity of size-selected cDNA libraries were verified using a KAPA Library Quantification Kit Illumina® Platforms (Nippon Genetics, Tokyo, Japan) or a Qubit dsDNA High Sensitivity Assay Kit in a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Japan, Tokyo, Japan). Each sample's cDNA library of 2.0 pM underwent sequencing on an Illumina Nextseq2000 platform, generating 75-bp reads (Illumina Japan, Tokyo, Japan).

The initial processing of raw data (raw reads) in FASTQ format was carried out using the BCL2-FASTQ program (Illumina Japan, Tokyo, Japan). Subsequent data analyses were conducted on the Galaxy platform (https://usegalaxy.org). The Filter FASTQ program was utilized for FASTQ quality filtering, and both 5′ and 3′ adapters were trimmed using the Trim FASTQ program. The processed sequence reads were aligned to the reference genome (mm10) through the TopHat program, resulting in BAM files. These BAM files underwent processing and normalization using the Cufflinks and Cuffnorm programs to generate gene expression data for each small RNA.

Statistical Analysis

Presentation of all data is in the form of means ± standard deviation (SD), and comparisons were conducted using Welch one-way analysis of variance (ANOVA). A probability of 0.01 was considered statistically significant. Statistical analysis was performed using R (version 3.4.3) and the EZR40 software package (Kanda, 2013).

Table 1. Effects of VPA on serum biochemistry parameters.

	GD11-0mg	GD11-300mg	GD11-600mg	GD15-0mg	GD15-300mg	GD15-600mg	GD18-0mg	GD18-300mg	GD18-600mg
AST (U/L)	60 ± 3.56	81 ± 14.65	62.75 ± 6.95	41.25 ± 3.86	45 ± 13.89	46 ± 5.72	51.5 ± 1.73	45 ± 3.37	41.25 ± 0.96
ALT (U/L)	33.25 ± 4.79	31.5 ± 3.51	28.25 ± 2.99	25.25 ± 2.75	24 ± 6.52	21.25 ± 2.75	31.75 ± 2.87	27.5 ± 3.11	25.25 ± 1.26
BUN (mg/dL)	21.825 ± 3.55	31.85 ± 12.61	28.3 ± 9.66	23.45 ± 3.62	37.64 ± 8.48	35.35 ± 6.73	19.55 ± 2.81	24.6 ± 6.06	27.375 ± 6.39
CRE (mg/dL)	0.18 ± 0.03	0.2425 ± 0.07	0.2125 ± 0.05	0.1975 ± 0.02	0.34 ± 0.07	0.2325 ± 0.03	0.21 ± 0.01	0.215 ± 0.02	0.2375 ± 0.04

Summary of biochemical test values in the maternal serum of both the solvent-treated group and the VPA-treated group (300 mg/kg/day, 600 mg/kg/day) following repeated administration to pregnant mice (GDs 9–11). Mean and standard deviation (MEAN ± SD) are provided with a sample size of N = 4 for AST, ALT, BUN, and CRE.

RESULTS AND DISCUSSION

In this study, VPA was administered daily to pregnant mice from GDs 9–11, a crucial period for the manifestation of its teratogenic effects. This resulted in adverse effects on the development of fetuses. Samples were collected for a comprehensive gene expression analysis of EV in fetal amniotic fluid on GD15.

VPA, dissolved in a vehicle (0.5 w/v% methylcellulose), was orally administered to pregnant mice (C57BL/6J, 12–14 weeks old at the start of administration) at doses of 0, 300, and 600 mg/kg/day for 3 days, spanning from GD9 to GD11, once a day. Blood collection was conducted from all maternal animals on GD11 (the final administration day), followed by cesarean section to collect fetuses and placenta. Additionally, maternal serum, fetuses, amniotic fluid, and placenta were collected on GD15, and maternal serum, fetuses, fetal plasma, and placenta were collected on GD18. The weights of fetuses and placentas, excluding those on GD11, were measured. Due to low amniotic fluid volume, amniotic fluid samples were not collected on GDs 11 and 18. External morphological observations were performed on all fetuses.

Blood biochemical tests

VPA is a widely used medication for epilepsy and bipolar disorder, with hepatotoxicity being a significant complication (Ezhilarasan and Mani, 2022). Therefore, maternal animals repeatedly administered VPA (0, 300, and 600 mg/kg/day groups) from GD9 to GD11 underwent blood biochemistry tests on GDs 11, 15, and 18. No statistically significant differences ($P < 0.01$) were observed between the vehicle-treated and VPA-treated groups in AST levels (Table 1). This demonstrates that the administration of VPA to maternal animals did not induce hepatic or renal toxicity under the present experimental conditions.

Table 2. VPA concentrations in maternal plasma at GD11 exposed to VPA to dams on GDs 9–11 orally.

Dose level (mg/kg)	Animal No.	Concentration (μg/mL)
300	4108	179
	4109	231
	4110	263
	Mean	224
	SD	42
600	7107	529
	7108	363
	7109	603
	Mean	498
	SD	123

During GDs 9–11, repeated oral administration of VPA at doses of 300 and 600 mg/kg/day was conducted. Blood was collected 1 h post-dose on the final administration day (GD11), and the plasma concentration of VPA was measured.

VPA concentrations in maternal plasma and the fetus

The concentration of VPA in maternal plasma was measured 1 hr post-dose on the final administration day following VPA administration at doses of 300 and 600 mg/kg/day from GDs 9–11. The group mean value of VPA concentration in maternal animal plasma was 224.3 μg/mL in the 300 mg/kg/day group, while in the 600 mg/kg/day group, it was 498.3 μg/mL, indicating a 2.22 times increase with a doubling of the dose, demonstrating a dose-dependent increase (Table 2). However, individual values for maternal plasma exhibited considerable variability, and it was observed that this variability significantly influenced the VPA concentration in the fetuses. Furthermore, it became evident that the VPA concentration in the fetuses was unrelated to the implantation location. Examination on GD11, GD15, and GD18 revealed that no significant differences were observed in the number of fetuses per litter (Table 3).

Table 3. VPA concentrations in GD11 fetuses exposed to VPA to dams on GDs 9–11 orally.

Dose level (mg/kg)	Animal No.	Location	Concentration (μg/g)
300	4108	*R-1	2.60
		R-2	64.4
		R-3	70.2
		R-4	81.6
		R-5	64.5
		L-1	73.2
		L-2	67.1
		L-3	73.1
		L-4	39.2
		Mean	59.5
		SD	24.3
	4109	R-1	76.6
		R-3	86.5
		R-4	99.4
		R-5	106
		R-6	87.3
		R-7	78.4
		L-1	79.3
		L-2	70.0
		L-3	84.1
		Mean	85.3
		SD	11.3
	4110	R-1	113
		R-2	119
		R-3	109
		R-4	96.2
		R-5	101
		L-1	105
		L-2	102
		L-3	106
		L-4	106
		L-5	107
		Mean	106
		SD	6
600	7107	R-1	230
		R-2	335
		R-3	246
		L-1	160
		L-2	306
		L-3	135
		L-4	274
		L-6	270
		Mean	245
		SD	68
	7108	R-1	182
		R-2	183
		R-3	128
		R-4	173
		R-5	154
		R-6	172
		R-7	145
		R-8	157
		L-1	138
		Mean	159
		SD	20
	7109	R-1	317
		R-2	298
		R-3	357
		R-4	288
		L-1	297
		L-2	310
		L-3	344
		L-4	286
		Mean	312
		SD	26

Repeated oral administration of VPA at doses of 300 and 600 mg/kg/day during GDs 9–11. Fetus was collected 1 hr post-dose on the final administration day (GD11), and the fetal concentration of VPA was measured. "Location" refers to the implantation position, indicating left (L) or right (R) side within the uterus, along with the numerical order from the ovarian side. Note that the fetus with an asterisk (animal number 1102, R1) was whitish and small.

Teratogenic effects following VPA administration at 600 mg/kg/day from GDs 9–11

In mice, teratogenic effects of VPA have been documented. Using the C57BL/6J strain, a single intraperitoneal administration of VPA at 800 mg/kg or 400 mg/kg on GD9 (vaginal plug = GD0) resulted in 94% or 39% of fetuses displaying fusion or defects in vertebral bodies or neural arches, 55% or 9% exhibiting fusion, branching, or wavy ribs, and 27% or 0% presenting with finger defects or fusion, respectively (Downing *et al*., 2010).

In our study, external morphological observations revealed neural tube closure defects in two fetuses. One is in the GD11 group at 600 mg/kg/day (Fig. 1b, Tables 4–6, fetus ID: 712) and the other is in the GD15 group at 600 mg/kg/day (Fig. 1d, Tables 7–9, fetus ID: 819). Within the GD15 group at 600 mg/kg/day, 30% of the fetuses

Table 4. External malformations in the 0 mg/kg/day of VPA group on GD11.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetus ID	neural tube closure defect	digit malformation
0	1101	R-1	live	101	-	-
		R-2	live	102	-	-
		R-3	dead			
		R-4	live	103	-	-
		R-5	live	104	-	-
		R-6	live	105	-	-
		R-7	live	106	-	-
		R-8	live	107	-	-
		L-1	live	108	-	-
		Mean				
		SD				
	1102	R-1	live	109	-	-
		R-2	live	110	-	-
		R-3	live	111	-	-
		R-4	live	112	-	-
		R-5	live	113	-	-
		R-6	live	114	-	-
		L-1	live	115	-	-
		L-2	live	116	-	-
		L-3	live	117	-	-
		L-4	live	118	-	-
		Mean				
		SD				
	1103	R-1	live	119	-	-
		R-2	live	120	-	-
		R-3	dead			
		L-1	live	121	-	-
		L-2	live	122	-	-
		L-3	live	123	-	-
		L-4	live			
		5	live	124	-	-
		Mean				
		SD				
	1104	R-1	live	125	-	-
		R-2	live	126	-	-
		R-3	live	127	-	-
		R-4	live	128	-	-
		R-5	live	129	-	-
		L-1	live	130	-	-
		L-2	live	131	-	-
		L-3	live	132	-	-
		L-4	live	133	-	-
		L-5	live	134	-	-
		Mean				
		SD				

There are no external malformations in the control group. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

Table 5. External malformations in the 300 mg/kg/day of VPA group on GD11.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetus ID	neural tube closure defect	digit malformation
		R-1	live	401	-	-
		R-2	live	402	-	-
		L-1	live	403	-	-
		L-2	dead			
	4103	L-3	live	404	-	-
		L-4	dead			
		L-5	dead			
		Mean				
		SD				
		R-1	live	405	-	-
		R-2	live	406	-	-
		R-3	live	407	-	-
		R-4	live	408	-	-
		L-1	live	409	-	-
	4104	L-2	live	410	-	-
		L-3	live	411	-	-
		L-4	live	412	-	-
		L-5	live	413	-	-
		Mean				
		SD				
300		R-1	live	414	-	-
		R-2	live	415	-	-
		R-3	live	416	-	-
		R-4	live	417	-	-
		R-5	live	418	-	-
	4105	R-6	live	419	-	-
		R-7	live	420	-	-
		L-1	dead			
		L-2	live	421	-	-
		Mean	106			
		SD	6			
		R-1	live	422	-	-
		R-2	live	423	-	-
		R-3	live	424	-	-
		R-4	live	425	-	-
		R-5	live	426	-	-
	4106	L-1	dead			
		L-2	live	427	-	-
		L-3	dead			
		Mean				
		SD				

There are no external malformations in the 300 mg/kg/day group. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

Table 6. External malformations in the 600 mg/kg/day of VPA group on GD11.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetus ID	neural tube closure defect	digit malformation
600	7102	R-1	live	701	-	-
		R-2	live	702	-	-
		R-3	live	703	-	-
		R-4	live	704	-	-
		R-5	live	705	-	-
		R-6	live	706	-	-
		R-7	live	707	-	-
		L-1	live	708	-	-
		L-2	live	709	-	-
		L-3	live	710	-	-
		L-4	live	711	-	-
		Mean				
		SD				
	7103	R-1	live	712	exencephaly	-
		R-2	live	713	-	-
		R-3	live	714	-	-
		R-4	live	715	-	-
		R-5	live	716	-	-
		R-6	live	717	-	-
		R-7	live	718	-	-
		L-1	dead			
		L-2	live	719	-	-
		Mean				
		SD				
	7105	R-1	live	720	-	-
		R-2	live	721	-	-
		R-3	live	722	-	-
		R-4	live	723	-	-
		R-5	live	724	-	-
		L-1	live	725	-	-
		L-2	live	726	-	-
		L-3	live	727	-	-
		L-4	live	728	-	-
		Mean	106			
		SD	6			
	7106	R-1	live	729	-	-
		R-2	live	730	-	-
		R-3	live	731	-	-
		R-4	live	732	-	-
		L-1	live	733	-	-
		L-2	live	734	-	-
		L-3	live	735	-	-
		L-4	live	736	-	-
		L-5	live	737	-	-
		Mean				
		SD				

Fetus with exencephaly (fetus ID: 712)was observed in the 600 mg/kg/day group. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

Table 7. Fetal and placental weight and external malformations in the 0 mg/kg/day group on GD 15.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID	neural tube closure defect	digit malformation
0	2102	R-1	live	0.33	0.10	201	-	-
		R-2	live	0.23	0.08	202	-	-
		R-3	live	0.33	0.11	203	-	-
		L-1	live	0.32	0.10	204	-	-
		L-2	live	0.36	0.10	205	-	-
		L-3	live	0.37	0.11	206	-	-
		L-4	live	0.34	0.12	207	-	-
		L-5	live	0.34	0.10	208	-	-
		Mean		0.33	0.10			
		SD		0.04	0.01			
	2103	R-1	live	0.33	0.14	209	-	-
		R-2	live	0.33	0.12	210	-	-
		R-3	dead					
		R-4	live	0.29	0.11	211	-	-
		L-1	live	0.37	0.12	212	-	-
		L-2	live	0.34	0.11	213	-	-
		L-3	live	0.38	0.10	214	-	-
		L-4	live	0.35	0.18	215	-	-
		Mean		0.34	0.13			
		SD		0.03	0.03			
	2104	R-1		0.27	0.08	216	-	-
		R-2	dead					
		R-3	live	0.24	0.08	217	-	-
		R-4	live	0.32	0.08	218	-	-
		R-5	live	0.33	0.09	219	-	-
		R-6	live	0.27	0.08	220	-	-
		L-1	live	0.32	0.09	221	-	-
		L-2	live	0.33	0.09	222	-	-
		L-3	live	0.26	0.09	223	-	-
		Mean	106	0.29	0.09			
		SD	6	0.04	0.01			
	2105	R-1	live	0.34	0.07	224	-	-
		R-2	live	0.41	0.10	225	-	-
		R-3	live	0.38	0.09	226	-	-
		R-4	live	0.39	0.11	227	-	-
		R-5	live	0.35	0.10	228	-	-
		L-1	live	0.37	0.09	229	-	-
		L-2	dead					
		Mean		0.37	0.09			
		SD		0.12	0.03			

EVs were collected from the amniotic fluid (highlighted in red), and comprehensive gene expression analysis of EV RNA was conducted. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

Table 8. Fetal and placental weight and external malformations in the 300 mg/kg/day group on GD 15.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID	neural tube closure defect	digit malformation
300	5102	R-1	live	0.35	0.10	501	-	-
		R-2	dead					
		R-3	dead					
		R-4	live	0.38	0.12	502	-	-
		R-5	live	0.32	0.09	503	-	-
		L-1	live	0.36	0.12	504	-	-
		L-2	live	0.34	0.10	505	-	-
		L-3	dead					
		Mean		0.35	0.11			
		SD		0.02	0.01			
	5103	R-1	live	0.29	0.11	506	-	-
		R-2	live	0.36	0.11	507	-	-
		R-3	live	0.36	0.12	508	-	-
		R-4	live	0.31	0.07	509	-	-
		R-5	live	0.33	0.10	510	-	-
		L-1	live	0.36	0.09	511	-	-
		L-2	live	0.35	0.09	512	-	-
		L-3	live	0.30	0.08	513	-	-
		L-4	live	0.33	0.10	514	-	-
		Mean		0.33	0.10			
		SD		0.03	0.02			
	5104	R-1	dead					
		R-2	dead					
		R-3	live	0.37	0.12	515	-	-
		R-4	live	0.31	0.09	516	-	-
		L-1	live	0.37	0.09	517	-	-
		L-2	live	0.35	0.08	518	-	-
		L-3	live	0.31	0.08	519	-	-
		L-4	live	0.36	0.09	520	-	-
		Mean		0.35	0.09			
		SD		0.03	0.01			
	5105	R-1	live	0.25	0.10	521	-	-
		R-2	live	0.31	0.11	522	-	-
		R-3	live	0.33	0.11	523	-	-
		R-4	live	0.30	0.10	524	-	-
		R-5	live	0.29	0.09	525	-	-
		R-6	live	0.28	0.08	526	-	-
		R-7	live	0.29	0.08	527	-	-
		L-1	live	0.34	0.07	528	-	-
		L-2	live	0.35	0.09	529	-	-
		Mean		0.30	0.09			
		SD		0.03	0.01			

EVs were collected from the amniotic fluid (highlighted in red), and comprehensive gene expression analysis of EV RNA was conducted. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-"

Table 9. Fetal and placental weight and external malformations in the 600 mg/kg/day group on GD 15.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID	neural tube closure defect	digit malformation
600	8102	R-1	live	0.37	0.12	801	-	-
		R-2	live	0.34	0.10	802	-	-
		L-1	live	0.36	0.10	803	-	-
		L-2	live	0.34	0.10	804	-	-
		L-3	live	0.31	0.10	805	-	-
		L-4	live	0.36	0.09	806	-	-
		L-5	live	0.32	0.09	807	-	-
		L-6	live	0.32	0.09	808	-	-
		Mean		0.34	0.10			
		SD		0.02	0.01			
	8103	R-1	live	0.28	0.08	809	-	-
		R-2	live	0.32	0.09	810	-	-
		R-3	live	0.30	0.09	811	-	-
		R-4	live	0.28	0.09	812	-	-
		R-5	live	0.32	0.10	813	-	-
		R-6	live	0.28	0.08	814	-	-
		L-1	live	0.30	0.09	815	-	-
		L-2	live	0.29	0.09	816	-	-
		L-3	live	0.27	0.09	816	-	-
		Mean		0.29	0.09			
		SD		0.02	0.01			
	8104	R-1	live	0.17	0.06	818	-	Adactyly, forelimb, the 4th, Left Malpositioned digit, forelimb, the 5th, Left
		R-2	live	0.24	0.08	819	exencephaly	-
		R-3	live	0.27	0.07	820	-	Adactyly, forelimb, the 5th, Left Adactyly, forelimb, the 1st & 5th, Right Malpositioned digit, forelimb, the 5th, Right
		R-4	live	0.30	0.08	821	-	Adactyly, forelimb, the 5th, Left Adactyly, forelimb, the 5th, Right
		R-5	live	0.24	0.09	822	-	Adactyly, forelimb, the 5th, Left
		R-6	live	0.24	0.07	823	-	Adactyly, forelimb, the 5th, Right
		R-7	live	0.25	0.07	824	-	Pendulous digit, forelimb, the 5th, Right Malpositioned digit, hindlimb, the 1st, Right
		L-1	live	0.22	0.06	825	-	Adactyly, forelimb, the 5th, Right
		Mean		0.24	0.07			
		SD		0.04	0.01			
	8105	R-1	live	0.34	0.08	826	-	-
		R-2	dead					
		R-3	live	0.33	0.08	827	-	-
		R-4	dead					
		R-5	live	0.35	0.09	828	-	-
		L-1	live	0.31	0.08	829	-	-
		L-2	live	0.32	0.08	830	-	-
		Mean		0.33	0.08			
		SD		0.02	0.004			

Fetuses with external malformations were observed (fetus ID: 818-825). EVs were collected from the amniotic fluid (highlighted in red), and comprehensive gene expression analysis of EV RNA was conducted. Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

Table 10. Fetal and placental weight and external malformations in the 0 mg/kg/day group on GD18.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID	neural tube closure defect	digit malformation
	3101	R-1	live	0.95	0.10	301	-	-
		R-2	live	0.93	0.11	302	-	-
		R-3	live	0.96	0.08	303	-	-
		R-4	live	1.04	0.11	304	-	-
		R-5	live	1.05	0.13	305	-	-
		R-6	live	1.00	0.11	306	-	-
		L-1	live	0.96	0.10	307	-	-
		L-2	live	1.08	0.10	308	-	-
		Mean		1.00	0.11			
		SD		0.05	0.01			
	3102	R-1	live	1.09	0.11	309	-	-
		R-2	live	1.06	0.08	310	-	-
		R-3	live	1.06	0.10	311	-	-
		R-4	live	1.11	0.10	312	-	-
		R-5	live	1.06	0.10	313	-	-
		R-6	live	1.02	0.09	314	-	-
		L-1	live	1.12	0.10	315	-	-
		L-2	live	1.04	0.08	316	-	-
		Mean		1.07	0.10			
		SD		0.03	0.01			
0	3104	R-1	live	1.05	0.10	317	-	-
		R-2	live	1.04	0.09	318	-	-
		R-3	live	1.03	0.08	319	-	-
		L-1	live	1.06	0.08	320	-	-
		L-2	live	1.00	0.07	321	-	-
		L-3	live	1.00	0.06	322	-	-
		L-4	live	1.05	0.08	323	-	-
		L-5	live	1.03	0.08	324	-	-
		L-6	live	1.00	0.09	325	-	-
		Mean	106	1.03	0.08			
		SD	6	0.02	0.01			
	3105	R-1	live	1.10	0.11	326	-	-
		R-2	live	1.00	0.09	327	-	-
		L-1	live	1.10	0.10	328	-	-
		L-2	live	1.00	0.08	329	-	-
		L-3	live	1.12	0.09	330	-	-
		L-4	live	1.05	0.09	331	-	-
		L-5	live	1.07	0.10	332	-	-
		L-6	dead					
		Mean		1.06	0.09			
		SD		0.35	0.03			

Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as “-”.

Table 11. Fetal and placental weight and external malformations in the 300 mg/kg/day group on GD18.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID number	neural tube closure defect	digit malformation
300	6102	R-1	live	1.06	0.09	601	-	-
		R-2	live	1.06	0.10	602	-	-
		R-3	live	1.01	0.08	603	-	-
		L-1	live	1.08	0.11	604	-	-
		L-2	live	0.93	0.07	605	-	-
		L-3	live	1.03	0.08	606	-	-
		L-4	live	1.05	0.10	607	-	-
		Mean		1.03	0.09			
		SD		0.05	0.01			
	6103	R-1	live	0.93	0.09	608	-	-
		R-2	live	1.00	0.08	609	-	-
		R-3	live	0.95	0.08	610	-	-
		R-4	live	1.00	0.08	611	-	-
		L-1	live	1.00	0.07	612	-	-
		L-2	dead					
		L-3	live	1.10	0.09	613	-	-
		L-4	live	1.00	1.00	614	-	-
		Mean		1.00	0.21			
		SD		0.05	0.35			
	6104	R-1	live	1.00	0.09	615	-	-
		R-2	live	1.00	0.07	616	-	-
		L-1	live	1.04	0.08	617	-	-
		L-2	live	1.02	0.07	618	-	-
		L-3	live	1.00	0.09	619	-	-
		L-4	live	1.12	0.10	620	-	-
		L-5	live	1.08	0.07	621	-	-
		Mean		1.04	0.08			
		SD		0.05	0.01			
	6105	R-1	live	0.86	0.06	623	-	-
		R-2	live	0.85	0.08	624	-	-
		R-3	live	0.85	0.08	625	-	-
		R-4	live	0.79	0.07	626	-	-
		L-1	live	0.89	0.07	627	-	-
		L-2	live	0.87	0.09	628	-	-
		Mean		0.85	0.08			
		SD		0.30	0.02			

Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as “-”.

Table 12. Fetal and placental weight and external malformations in the 600 mg/kg/day group on GD18.

Dose level (mg/kg)	pregnant dam No.	in utero Location	fetus status live or dead	fetal weight (g)	placental weight (g)	fetus ID number	neural tube closure defect	digit malformation
600	9101	R-1	live	0.84	0.09	901	-	-
		R-2	live	0.67	0.07	902	-	Adactyly, hindlimb, the 1st, Left
		R-3	dead					
		R-4	live	0.89	0.09	903	-	-
		R-5	live	0.81	0.08	904	-	-
		R-6	live	0.68	0.07	905	-	Adactyly, hindlimb, the 1st, Left
		L-1	live	0.87	0.08	906	-	-
		L-2	dead					
		Mean		0.79	0.08			
		SD		0.10	0.01			
	9102	R-1	live	0.75	0.11	907	-	-
		R-2	live	0.87	0.08	908	-	-
		R-3	live	0.86	0.09	909	-	-
		R-4	live	0.94	0.12	910	-	-
		L-1	live	0.89	0.10	911	-	-
		L-2	live	0.77	0.07	912	-	-
		L-3	live	0.99	0.06	913	-	-
		L-4	live	0.87	0.09	914	-	-
		Mean		0.87	0.09			
		SD		0.08	0.02			
	9103	R-1	live	0.67	0.08	915	-	-
		R-2	live	0.80	0.08	916	-	-
		R-3	dead					
		L-1	live	0.79	0.08	917	-	-
		L-2	live	0.85	0.08	918	-	-
		L-3	live	0.79	0.07	919	-	-
		L-4	dead					
		L-5	live	0.86	0.09	920	-	-
		L-6	live	0.83	0.10	921	-	-
		Mean	106	0.80	0.08			
		SD	6	0.06	0.01			
	9104	R-1	live	0.92	0.09	922	-	-
		R-2	live	0.86	0.07	923	-	-
		R-3	live	0.90	0.09	924	-	-
		R-4	live	0.83	0.08	925	-	-
		L-1	live	0.90	0.08	926	-	-
		L-2	live	1.00	0.10	927	-	-
		L-3	live	0.73	0.08	928	-	-
		Mean		0.88	0.08			
		SD		0.28	0.03			

Fetuses with adactyly were observed (fetus ID: 902 and 905). Live fetus was defined as the presence of a detectable fetal heartbeat. Negative for external malformation was represented as "-".

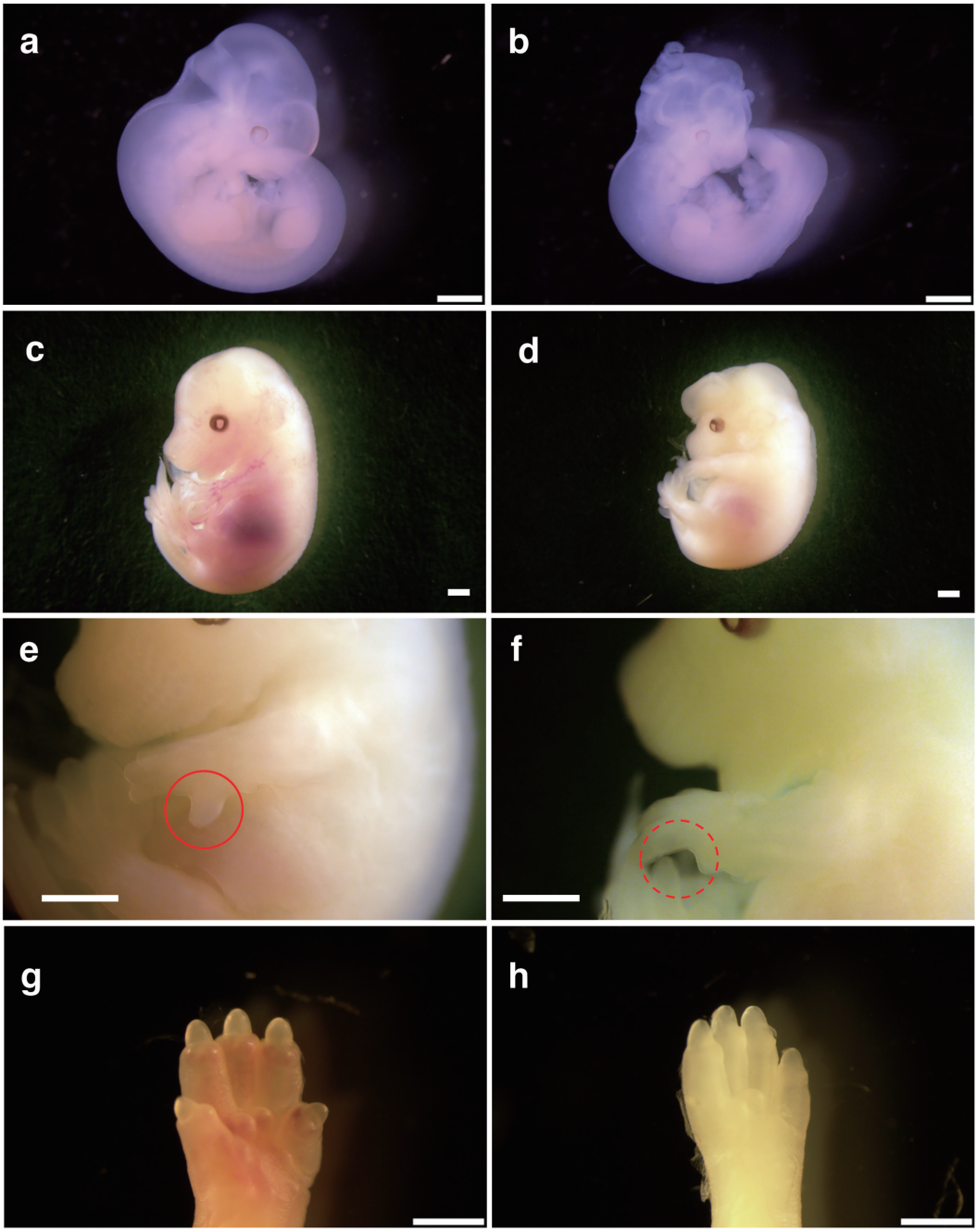

Fig. 1. Morphological changes in fetuses induced by VPA oral administration. Fetuses exhibited neural tube defects and digital malformations as a result of VPA administration at a dose of 600 mg/kg/day from GDs 9–11. Control fetus (vehicle) on GD11 (0 mg/kg/day: fetus ID: 110) (a). Fetus with exencephaly on GD11 (600 mg/kg/day: fetus ID: 712) (b). Control fetus (vehicle) on GD15 (0 mg/kg/day: fetus ID: 205) (c). Fetus with exencephaly on GD15 (600 mg/kg/day: fetus ID: 819) (d). Fetus with malpositioned fifth digit on the left forelimb on GD15 (600 mg/kg/day: fetus ID: 818: red circle) (e). Fetus with adactyly on the fifth digit on the left forelimb on GD15 (600mg/kg/day: fetus ID: 820: red dot circle) (f). Control fetus (vehicle) on GD18 with normal digit on the left hindlimb on GD18 (fetus ID: 301) (g). Fetus with adactyly on the first digit on the left hindlimb on GD18 (600 mg/kg/day: fetus ID: 905: red dot circle) (h). Scale bar = 1 mm

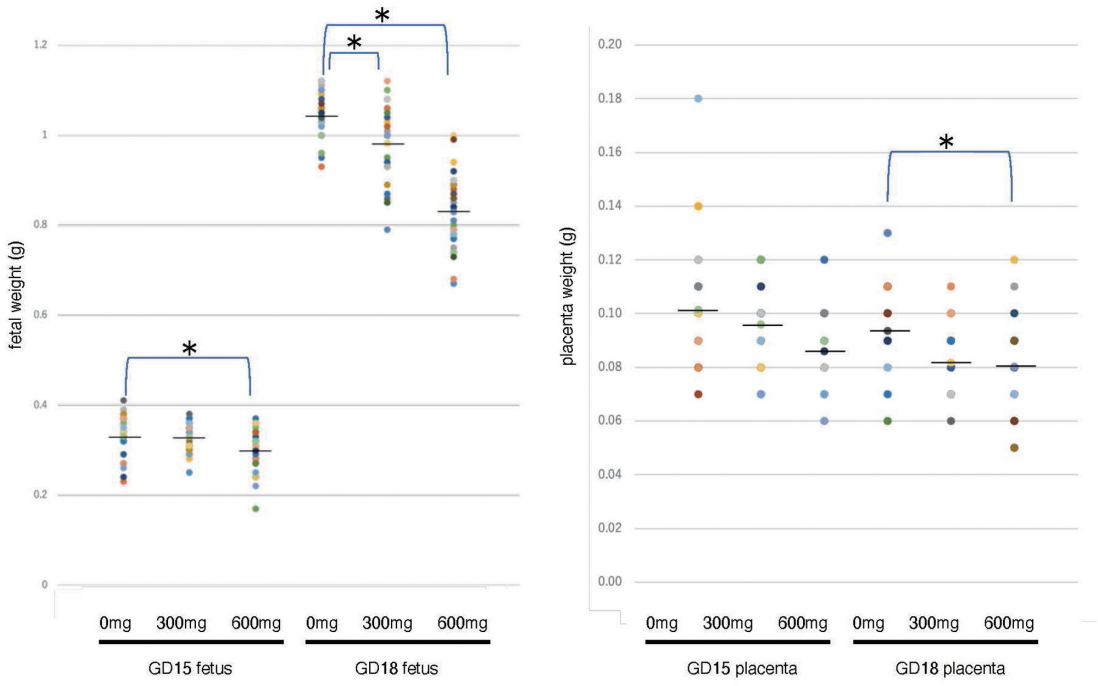

Fig. 2. Growth retardation observed in fetuses on GDs 15 and 18 as well as in the placenta on GD18 due to VPA administration. Each dot represents the individual weight of the fetuses and placenta, while the black bars represent the mean values. *P < 0.01, different from the control (the vehicle-treated group).

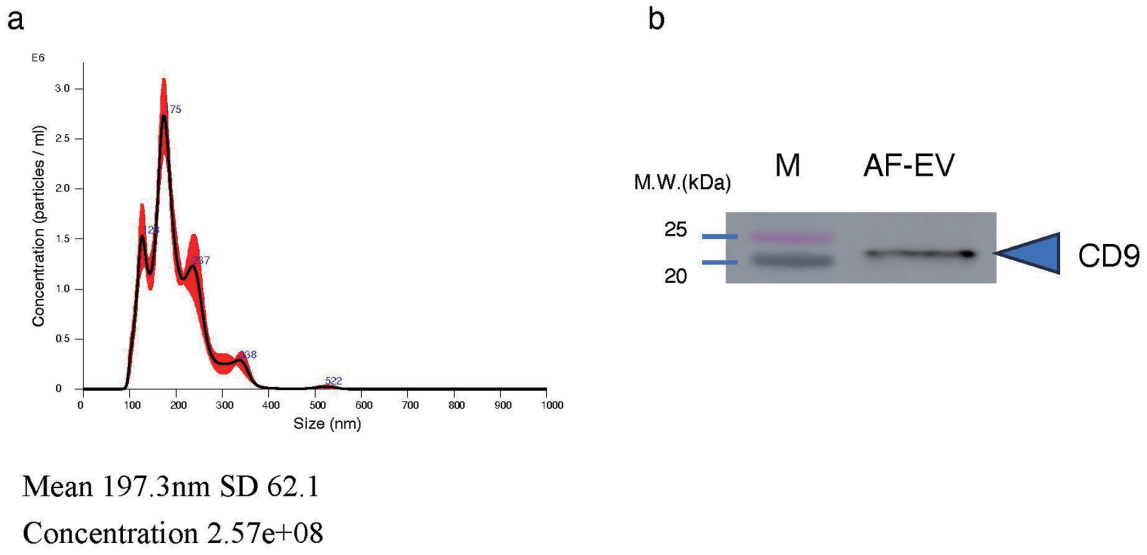

Fig. 3. Amniotic fluid–EV characterization. (a) Size analysis of amniotic fluid–EV using NanoSight, a Nanoparticle Tracking Analysis (NTA). (b) Representative Western blot analyses of CD9 expression in the lysate of amniotic fluid–EVs (mixture of three amniotic fluid (0 mg/kg/day group), fetus ID: X, Y, and Z). "M" represents the protein marker. "AF-EV" represents the amniotic fluid EV.

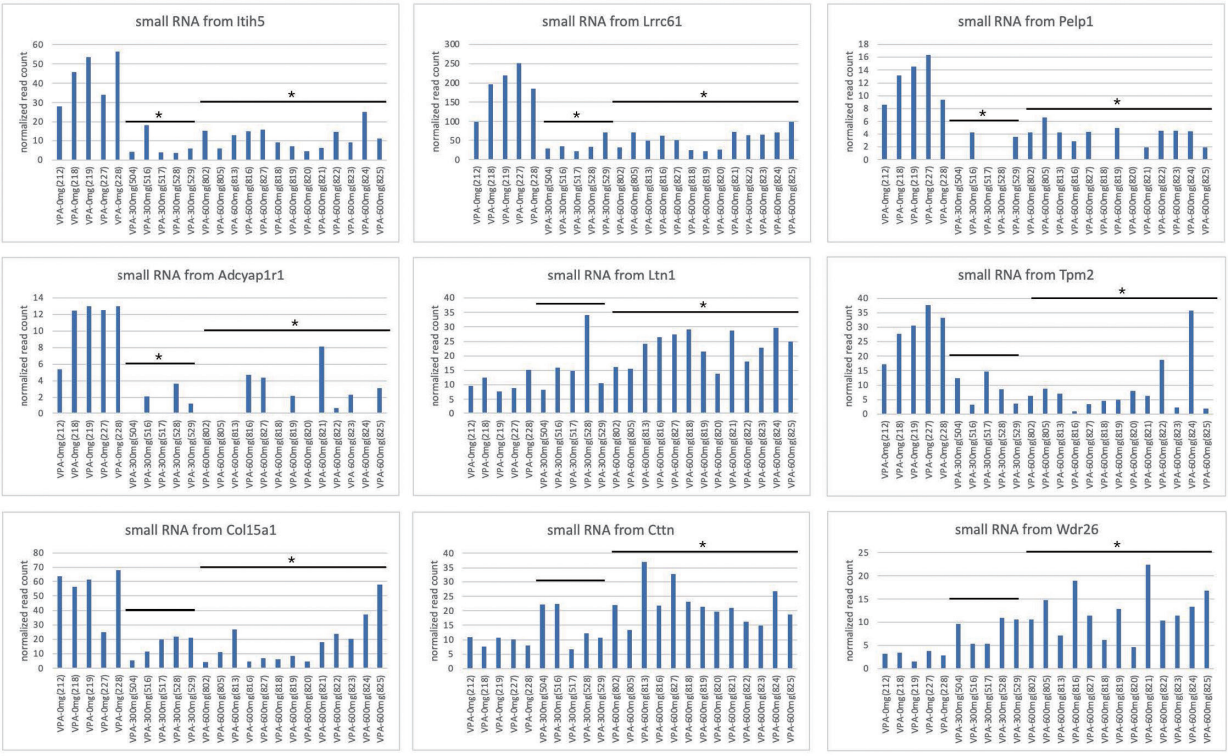

Fig. 4. Normalized read counts of small RNA in amniotic fluid–EV with differential expression between the vehicle-treated (0 mg/kg/day) group and the VPA-treated (300 mg/kg/day or 600 mg/kg/day) groups on GD15. *P < 0.01, different from the control (the vehicle-treated group).

Table 13. List of EV small RNAs significantly upregulated (more than twofold) and downregulated (less than half) on GD15 after repeated VPA oral administration at doses of 0, 300, and 600 mg/kg/day from GDs 9–11, ranked by P values (P < 0.01).

EV small RNA chromosomal region	Fold change (300 mg/kg)	P-value (300 mg/kg)	Fold change (600 mg/kg)	P-value (600 mg/kg)	Associated transcript
chr2:10251250-10252047	0.164675797	0.000356384	0.270070311	9.35E-07	Itih5
chr6:48569539-48570339	0.197762008	0.00045638	0.28556754	1.44202E-06	Lrrc61
chr11:70409748-70410245	0.125681067	0.000273181	0.277137259	2.31618E-06	Pelp1
chr6:55498300-55498660	0.123410302	0.000301313	0.173319734	8.22869E-06	Adcyap1r1
chr16:87415549-87416323	1.556451898	0.243775633	2.132070127	0.000277057	Ltn1
chr4:43522593-43522926	0.289661787	0.001011075	0.286706652	0.000410094	Tpm2
chr4:47312337-47313097	0.293802578	0.00163551	0.324220874	0.000499773	Col15a1
chr7:144461067-144461428	1.569234811	0.135849146	2.350385999	0.000777446	Cttn
chr1:181177217-181177400	2.814389481	0.00337985	4.153382538	0.000917419	Wdr26

displayed digital malformations, such as absence, small size, or malposition of the forelimb digits, absence or shortening of the fifth finger, and abnormal positioning of the fifth finger (Fig. 1e, f, Tables 7–9). Within GD18 group at 600 mg/kg/day, two out of 28 fetuses displayed hindlimb digital malformations, such as lack or shortening of the first toe (Fig. 1h, Tables 10–12).

Moreover, fetal weight exhibited a significant decrease of 9.7% ($P < 0.01$) on GD15 with 600 mg/kg/day administration and 5.8% and 20.2% ($P < 0.01$) on day 18 with 300 mg/kg/day and 600 mg/kg/day administration, respectively (Fig. 2). On day 18, placental weight decreased by 11.1% ($P < 0.01$) in the VPA-treated group (Fig. 2).

These findings indicate a concentration-dependent occurrence of teratogenic effects due to VPA. Furthermore, VPA administration from GDs 9–11 resulted in a reduced rate of weight gain, demonstrating a delayed effect with approximately 20% weight loss by GD18 (Fig. 2). It has also been reported that 71% of children exposed to VPA in utero exhibited growth retardation or neurological abnormalities (Ardinger *et al*., 1988).

Isolation and evaluation of EVs in the amniotic fluid

Several studies have reported the isolation of EVs from human and mouse amniotic fluid (Gebara *et al*., 2022; Hu *et al*., 2022; Jankovicova *et al*., 2020). In our study, we collected mouse amniotic fluid from fetuses on GD15, resulting in a limited volume of approximately 100 µL per individual. To address this limitation, we used the membrane affinity spin column exoEasy (Qiagen) for EV isolation (Lang *et al*., 2022). Subsequently, Nanoparticle Tracking Analysis (NTA) and Western blot analysis were performed on a subset of the 0 mg/kg/day group. NTA revealed the presence of approximately 2.57e+08 particles, with a median size of 197.3 nm (Fig. 3). The size distribution of these EVs closely resembled that of previously reported human amniotic fluid EVs (Gebara *et al*., 2022). Furthermore, to confirm the nature of these particles as EVs, Western blot analysis using the CD9 antibody, a marker protein for EVs, was conducted, confirming the presence of EVs containing CD9 (Fig. 3).

Subsequently, a comprehensive analysis of EV RNA expression in fetal amniotic fluid was conducted. In fetal amniotic fluid on GD15, it was revealed that there were small RNAs in EVs with significant differences between the vehicle-treated (0 mg/kg/day) group and the VPA-treated group (Table 13, Fig. 4). Variations in gene expression of these EV small RNAs are induced by transient histone modification changes caused by VPA, a histone deacetylase inhibitor (HDACi). These EV small RNAs not only serve as biomarkers for developmental toxicity but also contribute to the expression of various phenotypes induced by VPA administration, making them potential epigenetic disruptors.

Conclusion

In this study, with the aim of investigating the possibility of using EV RNA in amniotic fluid as an indicator of developmental toxicity, pregnant mice were exposed to concentrations of the developmental toxicant VPA (0, 300, or 600 mg/kg/day) on GDs 9-11. In the VPA-treated groups, EV RNA analysis identified differentially expressed EV small RNAs, both suppressed and induced, suggesting that EV RNA in amniotic fluid serve as an indicator of developmental toxicity.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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