

**Association of adverse fetal outcomes with placental inflammation
after oral gestational exposure to hexafluoropropylene oxide dimer
acid (GenX) in Sprague-Dawley rats**

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Abstract

Hexafluoropropylene oxide dimer acid (HFPO-DA), known as “GenX” for its trade name, is gradually taking the place of Perfluorooctanoic acid (PFOA). However, there is a poor understanding of the developmental effects of GenX. This study aims to explore whether GenX produces adverse effects on offspring development in SD rats and the underlying mechanisms. Pregnant rats were orally administered with GenX (0, 1, 10 and 100 mg/kg/day) from gestational 0.5 to 19.5 days. Experimental data showed that the exposure to GenX resulted in increased rats’ gestational weight gain, whereas both body weight and body length of their fetuses born naturally were significantly reduced. This could contribute to the developmental delays of fetal body weight, body length and tail length from postnatal 1 to 21 days. Histopathological evaluation of placenta indicated that GenX exposure led to neutrophil infiltration in decidual zone and congestion in labyrinth zone. Moreover, placental proteomics showed changes at the expression levels of the inflammation-related proteins in the Rap1 signaling pathway. In conclusion, gestational exposure to GenX induced fetal intrauterine and extrauterine development retardation in SD rats. Placental inflammation may play a key role in this process through the Rap1 signaling pathway.

Keywords: HFPO-DA (GenX), Developmental toxicity, Placenta, Rap1 signaling pathway

1. Introduction

Perfluorooctanoic acid (PFOA) is one of the most common Poly- and perfluoroalkyl substances (PFASs) (Giesy and Kannan 2002). Due to its harmful effects on the environment and humans, the emissions and production of PFOA has been gradually restricted worldwide (U.S. EPA). Hexafluoropropylene oxide dimer acid (HFPO-DA), the trade name “GenX”, is a novel alternative to PFOA. Since 2010, extensive applications have been found in industrial and commercial applications (Mullin et al. 2019), while its production was officially estimated to reach approximately 10-100 tons a year in Europe in 2013 (ECHA, 2013). The estimated total riverine discharge of GenX in China exceeded 2.6 tons in 2018 (Pan et al. 2018).

GenX has been widely detected in various environmental matrices globally, such as groundwater, drinking water, indoor dust, sediment and soil (Feng et al. 2021; Galloway et al. 2020; Gebbink and van Leeuwen 2020; Heydebreck et al. 2015; McCord et al. 2019; Xu et al. 2021a). It is notable that GenX has a long half-life in water lasting for more than 1 year, and is difficult to remove by conventional adsorbents, requiring almost 4 times¹

Abbreviations: HFPO-DA (GenX): Hexafluoropropylene oxide dimer acid; PFOA: Perfluorooctanoic acid; PFASs: Poly- and perfluoroalkyl substances; SD rats: Sprague-Dawley; GD: Gestation day; PND: postnatal day; SPF: specific pathogen free; PFA: paraformaldehyde solution; DEPs: Differentially expressed proteins; FC: Fold Change; SEM: Standard error of mean; GWG: Gestational weight gain; AC: Average concentrations; SIPA1L2: Signal-induced proliferation-associated 1-like protein 2; MAPK14: Mitogen-activated protein kinase 14; TSP1: Thrombospondin 1.

the treatment cost of PFOA (Heidari et al. 2021). Therefore, this is a huge challenge to the safety of drinking water. Additionally, GenX has also been detected in various foods, such as agricultural crops, vegetables, fruits (Brandsma et al. 2019; Feng et al. 2021) and marine animals (Wang et al. 2021; Zhang et al. 2022). In particular, some foods (lettuce or cucumber) have higher levels of GenX than PFOA (Gu et al. 2023). These results suggested that humans are exposed to GenX mainly through drinking water and food consumption. This raised wide concern among people all over the world. In June 2019, the European Chemicals Agency identified GenX as a Substance of Very High Concern (SVHC) (ECHA, 2019). To date, GenX has been detected in blood samples from highly exposed populations (Petriello et al. 2022; Yao et al. 2020) and in urine samples (Calafat et al. 2019) from the general population, while the detection and concentration rates were low than those of PFOA. This can be explained that GenX has an obviously shorter half-life (1 month) in humans (Russell et al. 2013) than PFOA (4.4 years) (Olsen et al. 2007). These findings seem to mean that GenX is less toxic and less harmful to health.

However, in the laboratory, GenX has been shown to be associated with thyroid toxicity (Coperchini et al. 2020; Zhang et al. 2021), metabolic toxicity (Conley et al. 2019, Conley et al. 2021, Conley et al. 2022), cardiotoxicity (Xu et al. 2021b), gut toxicity (Xie et al. 2021) and even more severe hepatotoxicity than PFOA (Gomis et al. 2018). The

developmental toxicity of PFASs has always been a hot topic in the public health field. Many epidemiologic studies have suggested significant inverse associations of maternal PFOA concentrations with birth weight, birth length, and abdominal circumference in the general population (Fei et al. 2007; Midasch et al. 2007). In animal experiments, maternal exposure to PFOA during pregnancy had adverse effects on the development of offspring, such as increased full litter resorptions, decreased fetal survival rate and birth weight, and delayed the time of eye opening and vaginal opening times (Lau et al. 2006; Maconet al. 2011). To the best of our knowledge, no epidemiological studies have attempted to explore associations between maternal GenX exposure and birth weight outcomes. Reports on the developmental effects of GenX in animals are limited and controversial, and the evidence of mechanisms are even scarcer.

The placenta is a critical organ that mediates the transport of nutrients, oxygen, waste, and xenobiotics between mother and fetus, and the integrity of placental structure and function is critical to the fetal development (Hutcheon et al. 2012; Risnes et al. 2009). Previous studies confirmed that PFASs can cross the placental barrier and affect the expression of placental steroidogenic enzymes (Wang et al. 2019; Yao et al. 2019). Other studies have also found that PFOA could cause placental pathological lesion including interstitial edema, vessel shrinkage, uNK cell reduction and apoptosis (Jiang et al. 2020), and PFOA might inhibit the placental

function by downregulating of PRL-family gene expression (Suh et al. 2011). These changes could affect the rate of nutrient exchange between mother and fetus, ultimately causing fetal growth and development restriction (Suh et al. 2011). To date, there are only two studies on the effect of GenX on the placenta, and the possible mechanisms of GenX have not been explicitly explored (Bangma et al. 2020; Blake et al. 2020). In this study, we were interested in identifying whether in utero exposure to GenX causes intrauterine growth retardation of rat's fetuses; if so, does this result relate to placental damage? In addition, we used proteomics to analyze the potential mechanism of GenX in placental damage. We also evaluated the development data of fetal rats until postnatal day (PND) 21 (age at weaning) to examine on whether GenX has a lasting effect on inducing extrauterine growth retardation.

2. Methods

2.1 Animals

Ten-week-old male and female specific pathogen free (SPF) SD rats were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Shandong, China). SD rats were housed in polypropylene cages with sterilized bedding. The animal housing facility was maintained under a controlled temperature (23 ± 1 °C ambient temperature) and a 12 h light/dark cycle (lights off 18:00). The animals were fed with standard chow (LabDiet, 5001 Rodents Diet) and distilled water (in polypropylene

bottles) ad libitum. All animal experiments were carried out in compliance with and approved by the Committee on the “Use of Human and Animal Subjects” of the Qingdao Municipal Hospital.

2.2 Dosing Solutions

Dosing solutions were prepared using deionized water which was provided by the Central Laboratory of Qingdao Municipal Hospital. GenX ammonium salt (CAS:13252-13-6; Lot:C12467627) was purchased from Shanghai Macklin Biochemical Co., Ltd. GenX purity was more than 97% as determined by the supplier via perchloric acid titration. GenX dosing solutions were prepared with deionized water and administered to rats once daily via oral gavage. Maternal weight was monitored daily throughout gestation. Daily doses were administered between 12:00 a.m. and 2:00 p.m. and adjusted to the body weight of the rats based on the current weight at a volume of 10 mL/kg body weight. We reviewed the published toxicology studies of GenX. Firstly, Conley et al.’s study proved that when the dose was below 30 mg/kg, no effect was observed on fetal birth weight after pregnant rats exposure to GenX from gestation day (GD) 8 to PND 2 (Conley et al. 2019). When the exposure dose was 110.7 mg kg⁻¹, half of fetal died at birth (ED₅₀ = 110.7 mg kg⁻¹). Secondly, we conducted a pre-experiment and observed meaningful results. Based on these two points above, we chose the highest exposure dose of 100 mg kg⁻¹.

2.3 Experimental Design

The SD rats were acclimated for 1 week after arrival at the SPF Animal Laboratory. Female rats were bred with male rats at a ratio of 1:1 overnight, and those with spermatozoa in a vaginal smear and/or with a copulatory plug were on GD 0.5. Pregnant rats were exposed daily via oral gavage to 0 (deionized water only), 1, 10 and 100 mg/kg GenX (n=12 for each group) from GD 0.5 to GD 19.5. Pregnant rats of each dose group were randomly divided into two groups: one group underwent caesarean section with 10% chloral hydrate 2 hours after dosing on GD 19.5 for maternal and fetal examinations (n=6); and one group (n=6) were allowed to naturally deliver. To make sure we could collect placenta, we chose a suitably earlier caesarean section on GD 19.5. In the caesarean section group: on GD 19.5, maternal trunk blood from each rat was collected by heart blood collection and centrifuged at 2500 rpm for 15 minutes at 4 °C to produce a serum sample, which was stored at -80 °C for subsequent analysis of GenX. The gravid uterus was then removed and examined; and the number of the live or dead fetuses, malformation and resorptions were recorded. The individual live fetus and its placenta were weighed (by electronic balance) and photos were taken with a digital camera (Cannon EOS R6). Then, placentas were fixed in a 4% paraformaldehyde solution (4% PFA, Sigma). ImageJ software (NIH, USA) was used to measure the length and tail length of the fetuses and the diameter of the placenta (Figure S1). In the natural delivery group, after GD 19.5, pregnant rats were no longer

exposed to GenX. Rats were monitored at frequent intervals until parturition. The date and time of birth and the number of live and dead fetuses were recorded. Eighteen fetuses (nine male and nine female) were selected randomly at PND 1 from each dose group. All selected fetuses were carefully observed daily for any morphological change, growth and development related indicators, including body weights (by electronic balance), length, tail length and anogenital distance (by flexible rule) on PND 1, 2, 3, 5, 7, 9, 14 and 21. The time of the righting reflex was monitored on PND 3, 5, 7, 9 and 14. The time of eye opening was monitored daily beginning on PND 10.

2.4 Fetal Sex Identification

The sex of fetuses born by caesarean delivery at GD 19.5 was determined by polymerase chain reaction amplification of the Sry gene (forwards, 5'-GCTTCAGTAATCTCAGCACCTAGAA-3', and reverse, 3'-CACATTGGCATGATAGCTCCAAATT-5') using the fetal head (Supplementary Figure 2). The sex of the fetuses born by natural delivery was confirmed by anogenital distance.

2.5 Analytical Chemical Determination of GenX Concentrations

Maternal serum samples were centrifuged at 10,000 rpm for 30 seconds, and 50 μ L of serum was mixed with 3 μ L of internal standard ($^{13}\text{C}_4$ -PFOS, Wellington, Canada). Then these mixed samples were denatured using 50 μ L 0.1 M formic acid followed by a 250 μ L pre-cooled acetonitrile protein

crash. After 15 minutes of incubation on ice, the samples were centrifuged at 15,000 rpm for 10 minutes at 4 °C. The resulting supernatant was carefully aspirated and filtered through a 0.22 µm filter membrane to the injection vial. All unknowns, replicates, methods, and quality control samples were prepared in this fashion. The GenX standard was formulated into 6 concentrations of 10, 20, 100, 200, 1000 and 2000 µg/L. The internal standard was added to the standard working solution so that the final concentration of the internal standard solution was 10 µg/L. For each analysis, according to the 6 standard concentrations, a calibration curve was obtained and was used for quantification. The coefficient of determination of the calibration curve was high, with $R^2 \geq 0.99$ for all compounds.

Sample analysis was performed on a Thermo Vanquish ultrahigh-performance liquid chromatograph coupled to an LTQ-MS (Thermo Fisher Scientific Inc, Waltham, MA). The GenX in the sample was separated on a 1.7 µm C18 column (2.1 mm i.d. × 50 mm length, Waters, Ireland), and the column temperature was set at 35°C. Detailed parameters of the LTQ-MS are shown in the following Supplementary Table 1a and Table 1b. 15 blank plasma samples were added with 10, 200, and 2000 µg/L of GenX, respectively, and then these samples are processed according to the pretreatment method. Another 15 blank plasma samples were determined under the same conditions. After pretreatment, 10, 200, and 2000 µg/L of

GenX were added to prepare solutions, respectively. The ratio of the two peak area above were to calculated the recovery rate of samples that were 89.5%-104.5%, and the relative standard deviations were 8.9%.

2.6. Hematoxylin-Eosin Staining

Placentas were fixed in a 4% PFA, dehydrated in ethanol, cleared in xylene, and impregnated in paraffin. Tissue sections of 5 μ m were prepared. The sections were dewaxed, rehydrated in graded ethanol, and then incubated in 0.05% Tween-20 in Phosphate Buffered Saline. Tissue sections were stained using hematoxylin and eosin.

2.7. Proteome sequencing and analysis

Total protein of the placental tissue was extracted by homogenization. Briefly, an appropriate amount of the placental tissue was placed into the lysis buffer containing 2% sodium dodecyl sulfate, 7 M urea, and 1 mg/mL protease inhibitor cocktail, and homogenized for 3 minutes at 4 °C. The mixture was centrifuged to collect supernatant at 15,000 rpm for 15 minutes at 4 °C. The protein concentration of the samples was measured using the bicinchoninic acid method.

The protein sample was mixed with 1 M DL-dithiothreitol and 10 mM triethylammonium bicarbonate for the cleavage of disulfide linkages and reductive alkylation. Then, the protein was digested by trypsin (Promega, Madison, WI) and the digested peptide mixture was labeled using iTRAQ/TMT tags. The labeled peptide mixture was subjected to high-pH

reversed phase separation, and then the separated peptide fractions were performed low-pH nano-HPLC-MS/MS analysis (Orbitrap Fusion) in data-dependent acquisition mode.

The raw data from mass spectra were transformed into Mascot Generic Format files using Proteome Discovery 1.2 (Thermo, Pittsburgh, PA, USA) and analyzed with the Mascot search engine (Matrix Science, London, UK; version 2.3.2) based on the *O. niloticus* database. The peptide threshold for protein identification was a 1.0% false discovery rate. Differentially expressed proteins (DEPs) were analyzed by t test ($p < 0.05$, $|FC|$ value > 1.2). To analyze the protein function, DEPs were annotated against GO and KEGG databases.

2.8. Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by least-significant difference as an estimation of the experimental error rate between two groups. All data are expressed as means $\pm$ standard error of the means (SEM). Asterisks (*) denote significant differences between the control and treated groups ($p < 0.05$).

3. Results

3.1 Maternal and fetal effects of gestational exposure to GenX in SD rats

Compared to GD 0.5, the gestational weight gain (GWG) of rats on GD 19.5 was significantly higher in the 10 and 100 mg/kg dose groups (Table 1). In Figure 1, the line chart shows that the pregnant rats weight gain

increase markedly from GD 14.5 to GD 19.5 in the 100 mg/kg dose group relative to control, while that increase from GD 18.5 to GD 19.5 in the 10 mg/kg dose group. Compared to the control group, we observed lower gestational weight gain in 1 mg/kg and 10 mg/kg dose groups prior to GD 17.5, but this difference was not statistically significant.

A few resorption fetuses and no nonviable fetuses were found in our study. We evaluated indicators of fetal development and the changes of placentas (Table 1 and Supplementary Table 2). There were no obvious differences in both litter size and fetus sex ratio among all the fetuses. In fetuses born by naturally, compared to the control group, significantly lower fetal body weight and shorter length were observed in the 10 and 100 mg/kg dose groups, and the fetal tail length in the 100 mg/kg dose group significantly was shorter. In fetuses born by caesarean section, compared to the control group, we observed fetal body length shortened obviously in the 10 and 100 mg/kg dose group, and the shorter tail length was observed in the 100 mg/kg dose group, while there was no difference in fetal weigh. As for the placenta, the placental weight significantly higher in the 10 and 100 mg/kg dose groups, and the fetal: placental weight ratios were significantly reduced in the 100 mg/kg dose group (Table 1).

Table 1. Toxicity parameters of pregnant rats and their fetuses after pregnant rats exposed to GenX for 19 days during gestation.

Parameter	Dose of GenX (mg kg ⁻¹)			
	0	1	10	100

Pregnant rats

Rats body weight at GD 0.5 (g)	266.88±7.74	266.91±8.48	266.46±4.18	267.87±7.72
Rats gestational weight gain ^a	107.40±6.30	110.44±6.84	124.76±4.40*	135.11±2.25*
Fetuses (born by natural delivery)				
Number of fetuses per rats	14.90±1.00	15.10±1.06	14.70±1.20	14.35±1.70
Females/males ratio of fetuses	1.10±0.20	1.00±0.40	1.12±0.42	1.03±0.30
Fetal weight (g)	6.34±0.52	6.56±0.75	5.82±0.56*	5.43±0.45*
Fetal length (mm)	53.77±1.80	53.29±2.28	49.88±2.74*	47.50±2.09*
Fetal tail length (mm)	16.38±1.28	16.58±2.34	15.50±0.70	14.38±0.97*
Fetuses (born by cesarean delivery)				
Number of fetuses per rats	15.00±1.41	15.00±1.26	14.40±1.64	14.16±1.83
Females/males ratio of fetuses	1.09±0.30	1.01±0.20	1.15±0.59	1.06±0.50
Fetal weight (g)	2.90±0.29	3.01±0.47	3.13±0.16	2.91±0.58
Fetal length (mm)	34.23±2.57	32.88±2.86	31.61±1.27*	29.31±1.35*
Fetal tail length (mm)	11.57±1.20	11.75±1.56	11.64±1.10	10.41±1.10*
Placental weight (g)	0.35±0.07	0.34±0.03	0.39±0.04*	0.46±0.04*
Placental diameter (mm)	13.36±1.46	13.04±1.08	12.97±1.93	12.44±1.33
Fetal /placental weight	8.29±1.55	8.87±1.45	8.05±1.00	6.33±0.65*

Values are mean ± SEM, GD = gestational day, ^a: compared to GD 0.5, the gestational weight gain of rats in GD 19.5, *: denotes significant differences between control and treated groups (p < 0.05).

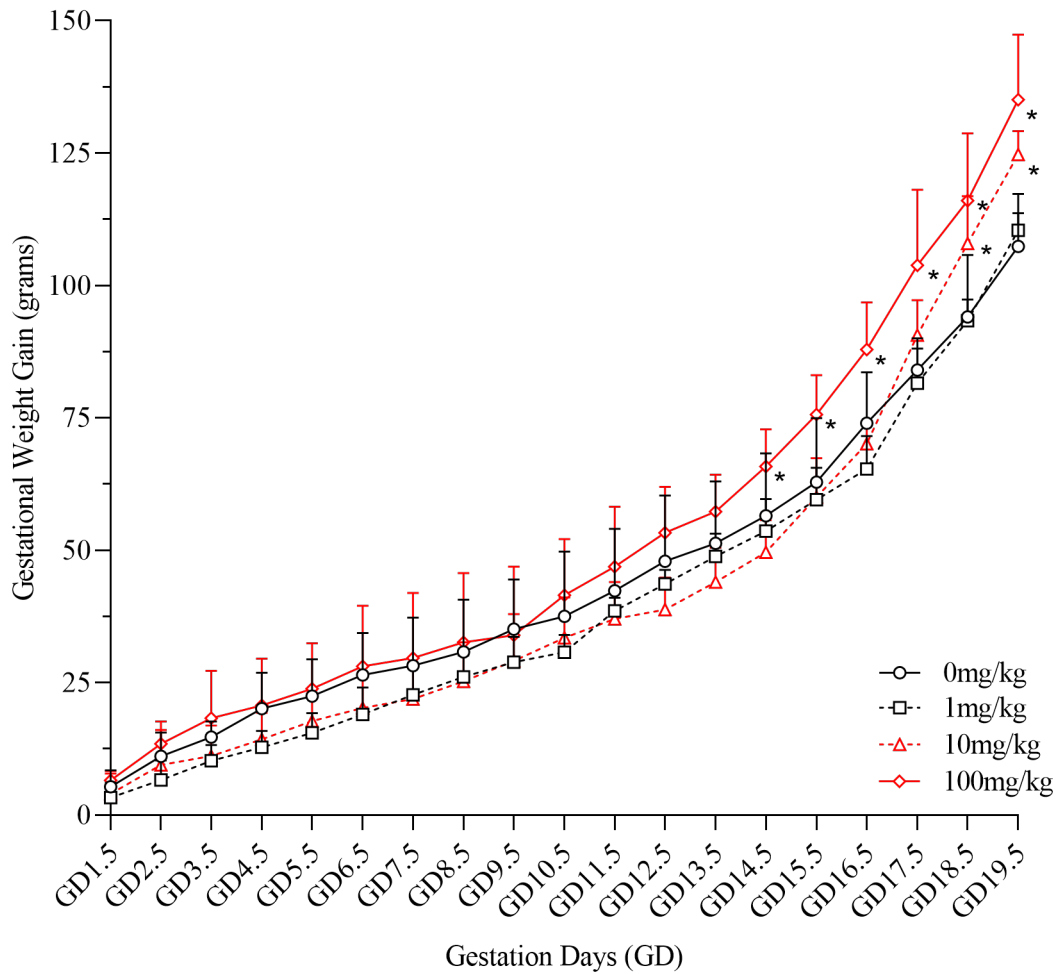


Figure 1. The trend of pregnant rats' gestational weight gain from gestation day (GD) 0.5 to GD 19.5. *: denotes significant differences between control and treated groups ($p < 0.05$).

3.2 Postnatal developmental landmarks

In the 100 mg/kg dose group, significant decreased body weight, shorted body length and tail length were observed from PND 1 to PND 21 (Figure 2 A, B, C). Compared to the control group, males' anogenital distance significantly decreased in the 100 mg/kg dose group just in PND 21 (Figure 2 D). The time of eye opening of all fetuses was around PND 14-15. There was no difference in the time of righting reflex at PND 3, 5, 7, 9 and 14 among fetuses (Supplementary Table 3).

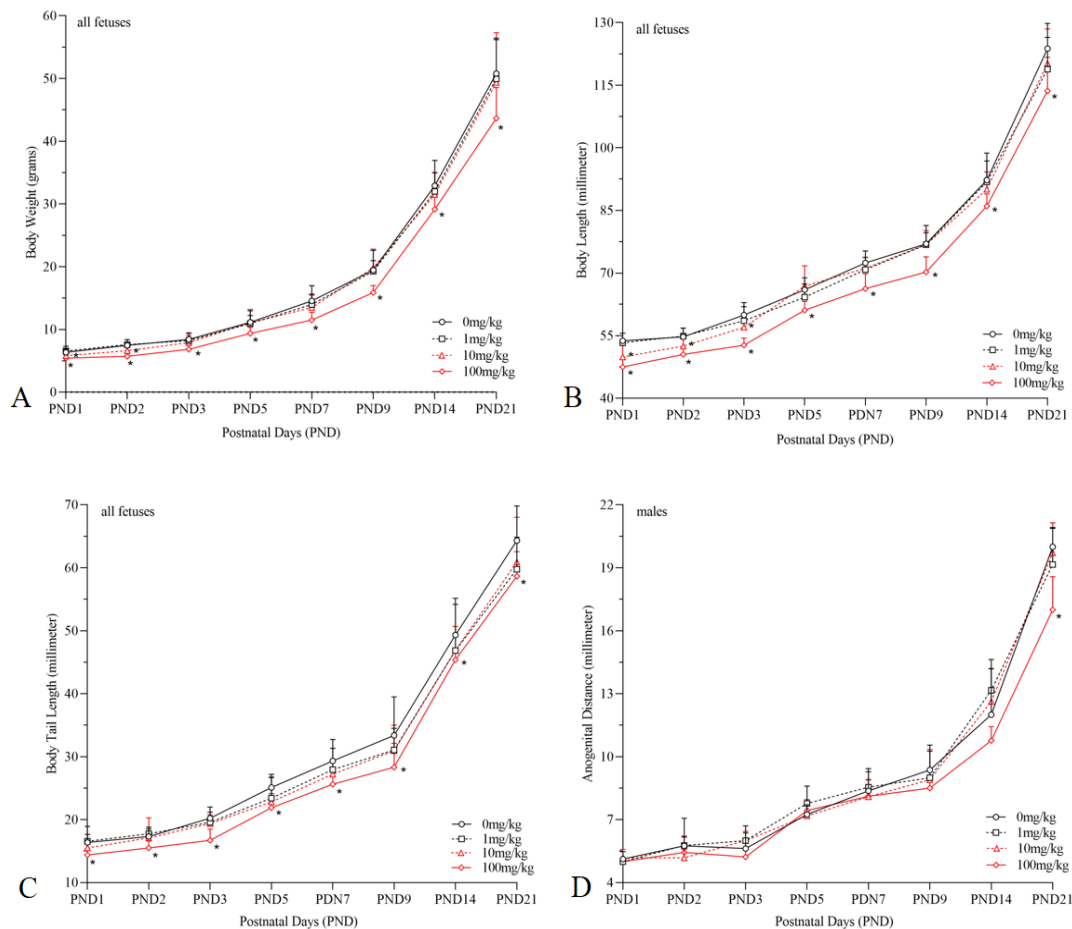


Figure 2. Effects of GenX exposure during gestation on fetal development. (A) Body weight, (B) body length and (C) body tail length in all fetuses, (D) anogenital distance in males from postnatal day (PND) 1 to PND 21. Data points represent means $\pm$ SEM. ANOVA indicated a significant treatment effect. *: denotes significant differences between the control and the treated groups ($p < 0.05$).

3.3 Serum concentrations of GenX

Maternal serum was collected on GD 19.5 approximately 2 h after the final oral dose. Following oral GenX dosing from GD 0.5 to GD 19.5, the concentration of GenX in serum increased in a dose-dependent manner with increasing exposed doses, but it was nonlinear. In the 1 mg/kg dose group, the maternal serum average concentration (AC) of GenX was 0.15 $\mu\text{g/mL}$. The serum levels of maternal exposure to GenX in the 100 mg/kg

dose group (AC: 3.92 $\mu\text{g/mL}$) were 6.8-fold greater than those in the 10 mg/kg dose group (AC: 0.57 $\mu\text{g/mL}$) (Figure 3).

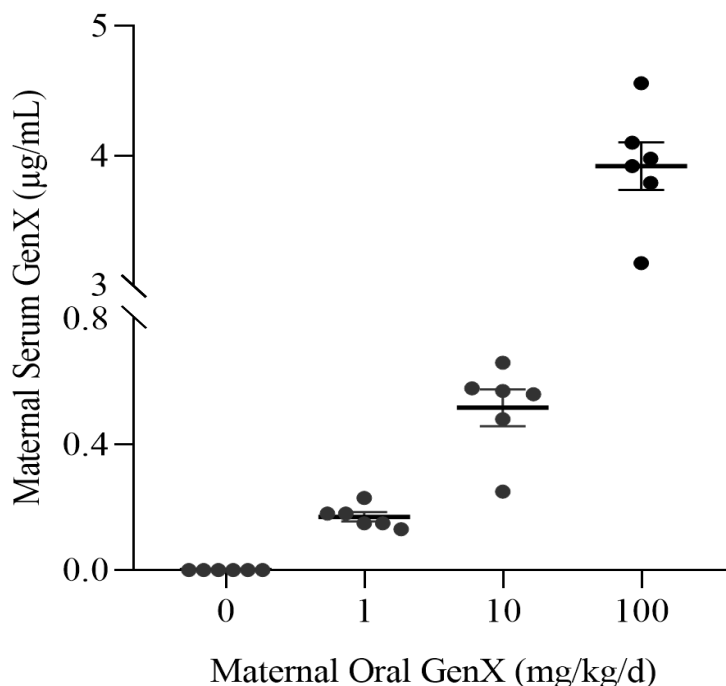


Figure 3. Concentrations of GenX in maternal serum following oral GenX dosing from gestation day (GD) 0.5 to GD 19.5. Data points represent individual replicates (n=6), bars and whiskers represent means $\pm$ SEM.

3.4 Histopathological Analysis of Placenta

Light microscopic examination of the placenta in the control and 1 mg/kg GenX groups showed normal histiocytic morphology. In the 10 and 100 mg/kg dose groups, these microscopic structural changes in the placenta included neutrophils, cellular debris and cell metamorphosis in the decidual zone in a dose-dependent manner (Figure 4C, D). In addition, we also observed significant dose-dependent congestion in the labyrinth zone in the 10 and 100 mg/kg dose groups (Figure 5C, D).

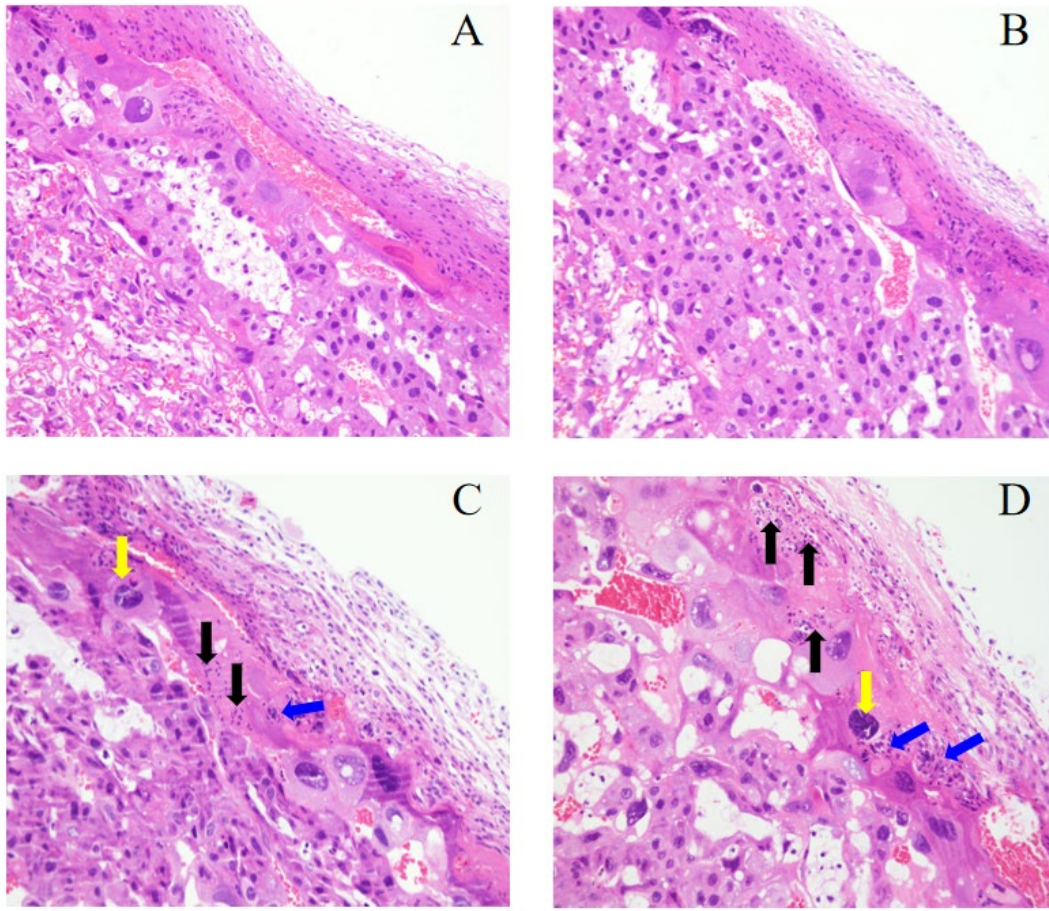


Figure 4. Histopathological changes of placenta in the decidua zone. (A) Control group rats and 1 mg/kg dose group (B) exhibited normal placental tissue structure. The placenta in the 10 mg/kg dose group (C) showed that small number of neutrophils (black arrow), cellular debris (blue arrow) and cell degeneration (yellow arrow). The placenta dose group (D) showed that a larger number of neutrophils (black arrows), cellular debris (blue arrows) and cell metamorphosis (yellow arrow). H&E stained, $\times 200$.

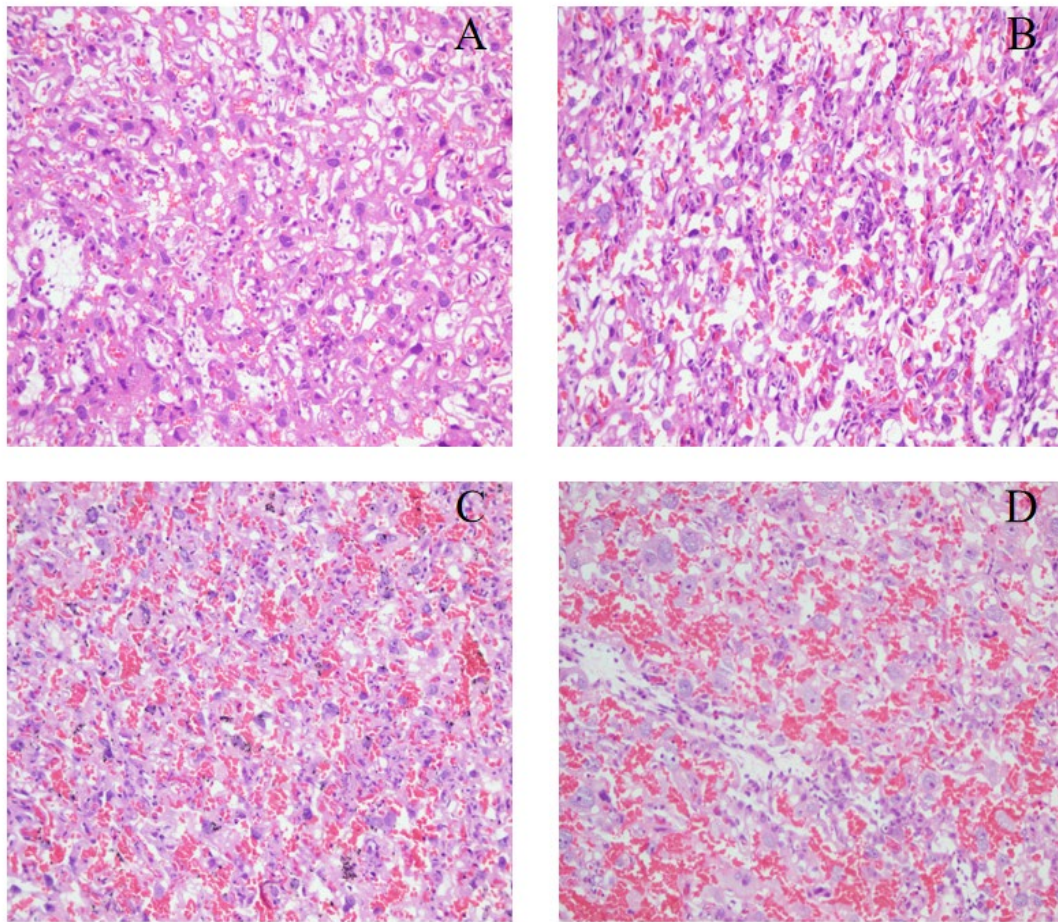


Figure 5. Histopathological changes of placenta in the labyrinth zone. Control group rats (A) and 1 mg/kg dose group (B) exhibited normal placental tissue structure. The placenta in the 10 mg/kg dose group (C) showed congestion. The placenta in the 100 mg/kg dose group (D) showed serious congestion. H&E stained, $\times 200$.

3.5 Proteome sequencing and analysis of differentially expressed proteins (DEPs)

We obtained 46369 high quality peptides through filtering of raw reads in the placenta between the control group (Ctrl) and 100 mg/kg dose group (High) of GenX. According to alignment against the reference sequence, 3983 proteins were identified, and the number of known proteins was 3923 (98.49%). The principal component analysis based on proteome sequencing data indicated distinct groups between the Ctrl group and High dose groups of GenX (Figure 6 A). After 19 days of pregnancy exposure

to the 100 mg/kg dose group, the proteome of 230 proteins in placenta was altered dramatically, including 169 upregulated proteins and 61 downregulated proteins (Figure 6B, C).

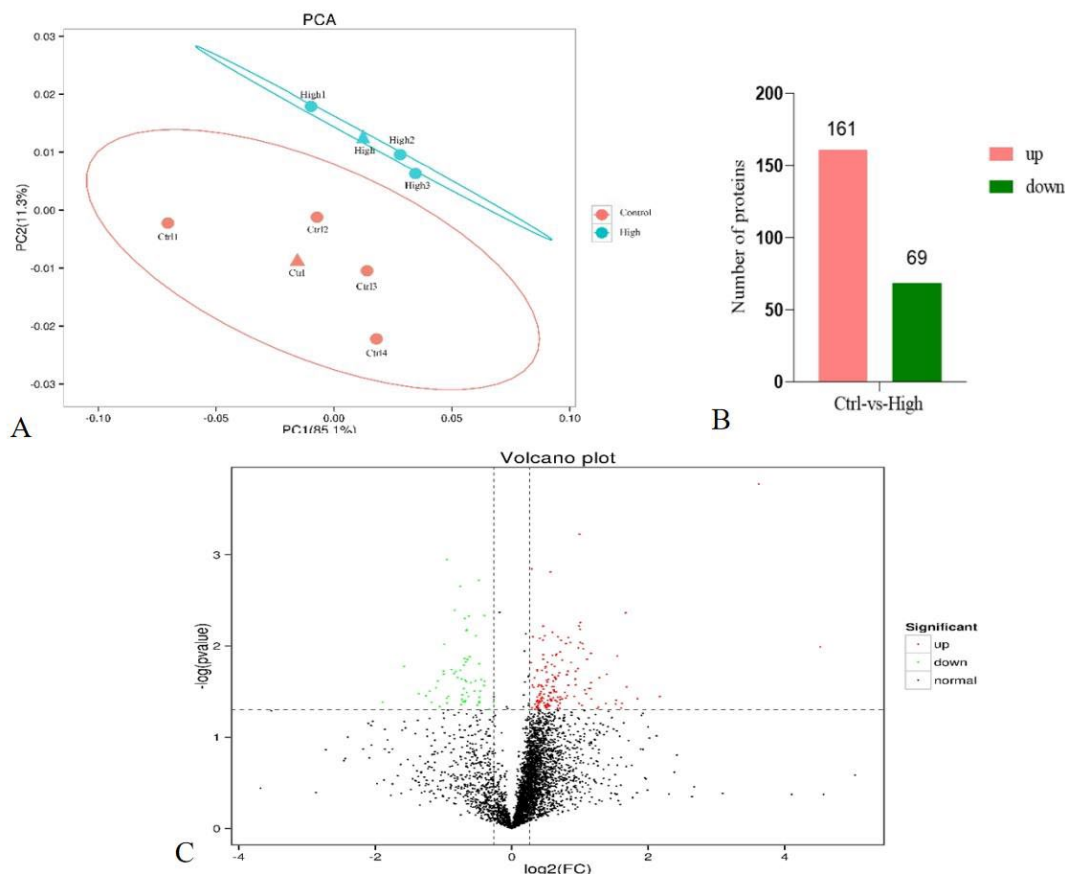
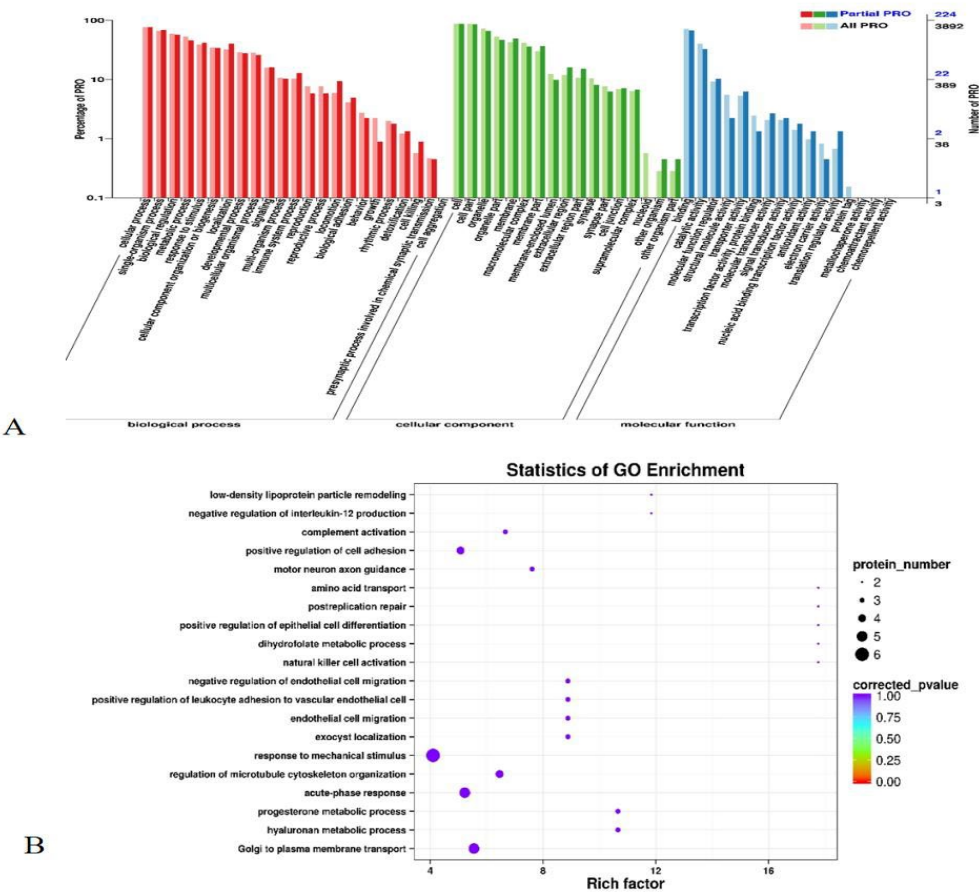


Figure 6. Differentially expressed proteins (DEPs) in proteome analysis between the control group and the 100 mg/kg dose group. Principal component analysis for the correlation among samples in the control (Ctrl) group and 100 mg/kg (High) dose groups (A). The number of significantly upregulated and downregulated proteins in placenta after the High dose group (B). Volcano plot of distribution trends for DEPs between the Ctrl group and the High dose group (C).

3.6 GO term enrichment analysis of DEPs

The GO enrichment analysis was performed to evaluate the biological function of the DEPs after High dose GenX exposure (Figure 7). Of all identified proteins, 3891 proteins were found in GO terms, and 224 proteins were also dramatically altered also in GO terms (Figure 7A). In

the biological process category, the DEPs were enriched mainly in response to mechanical stimulus, acute-phase response and Golgi to plasma membrane transport (Figure 7B). In the molecular function category, actin filament binding was the most abundant function. Specifically, the DEPs were involved in actin-remodeling protein, focal adhesion kinase 1, protein diaphanous homolog 1, filamin B, Gc-globulin and alpha-actinin-4 (Figure 7C). In the cellular component category, cell, cell part, and organelle contained the most DEPs, and integral component of membrane and extracellular space were two top GO terms (Figure 7D).



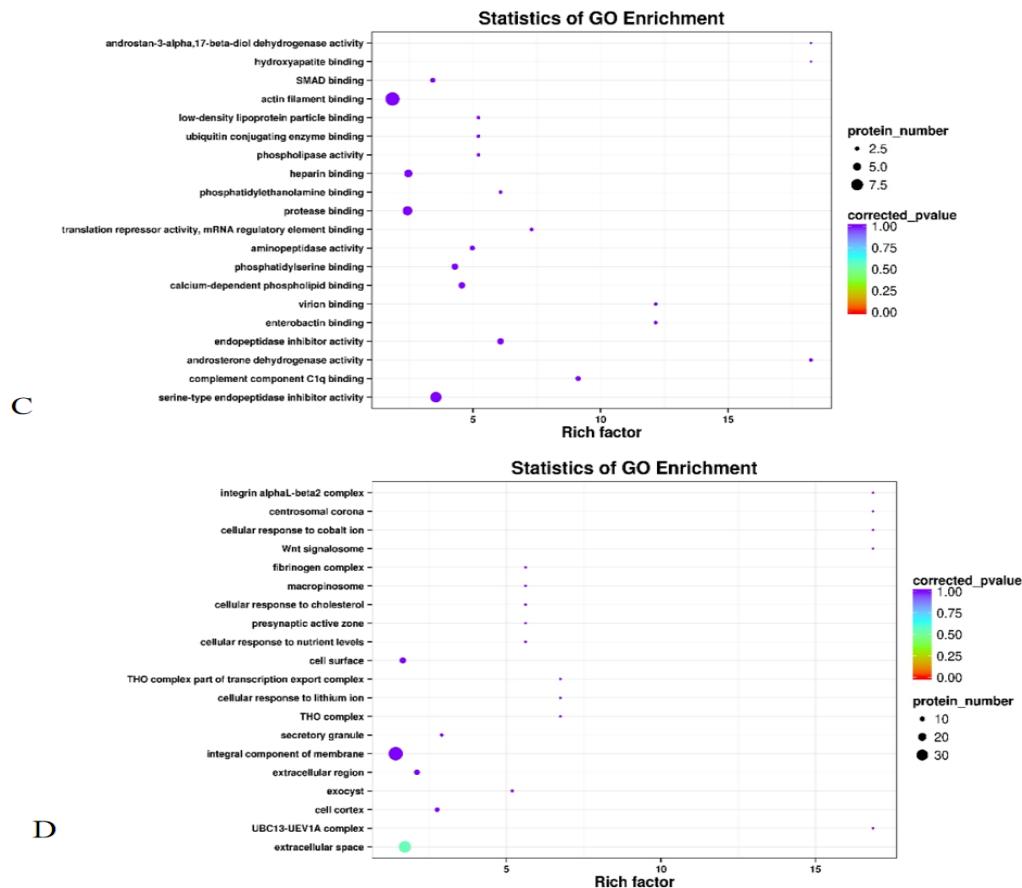


Figure 7. GO enrichment analysis of differentially expressed proteins (DEPs) in proteome data from placenta between the control group and 100 mg/kg dose group. All identified proteins and DEPs enriched in three categories: biological process, cellular component and molecular function (A). Top 20 GO terms in biological process category (B). Top 20 GO terms enrichment in molecular function category (C). Top 20 GO terms in cellular component category (D).

3.7 KEGG pathway enrichment analysis of DEPs

The DEPs were further annotated against the KEGG database to explore the key signaling pathways (Figure 8). A total of 230 significant DEPs were enriched in KEGG pathways involved in cellular processes (endocytosis and regulation of actin cytoskeleton), environmental information processing (Rap1 signaling pathway), genetic information processing (protein processing in endoplasmic reticulum and RNA transport), human diseases (proteoglycans in cancer, bacterial invasion of

epithelial cells, staphylococcus aureus infection, tuberculosis and amoebiasis), and organismal systems (complement and coagulation cascades and leukocyte transendothelial migration) (Figure 8A). The top 20 KEGG pathways with significant enrichment among DEPs (Figure 8B). These KEGG pathways included the inflammation-related Rap1 signaling pathway, which was consistent with our pathological findings.



Figure 8. KEGG enrichment analysis of differentially expressed proteins (DEPs) in proteome data from the placentas of pregnant rats in the control group and 100 mg/kg dose group. DEPs enrichment in KEGG pathways related to cellular processes, environmental information processing, genetic information processing, human diseases and organismal systems (A). The top 20 enriched KEGG pathways (B).

3.8 Changes in the Rap1 signaling pathway and inflammation-related protein expression

KEGG enrichment analysis showed that the Rap1 signaling pathway has changed pathway after 19 days of high dose GenX exposure during gestation in. In Rap1 signaling pathway, GenX exposure upregulated the expression of integrin β , R-Ras, signal-induced proliferation-associated 1-like protein 2 (SIPA1L2), mitogen-activated protein kinase 14 (MAPK14), transforming protein RhoA (also called RhoA), and catenin β 1, and downregulated the expression of thrombospondin 1(TSP1) ($p < 0.05$; Figure 9A, B).

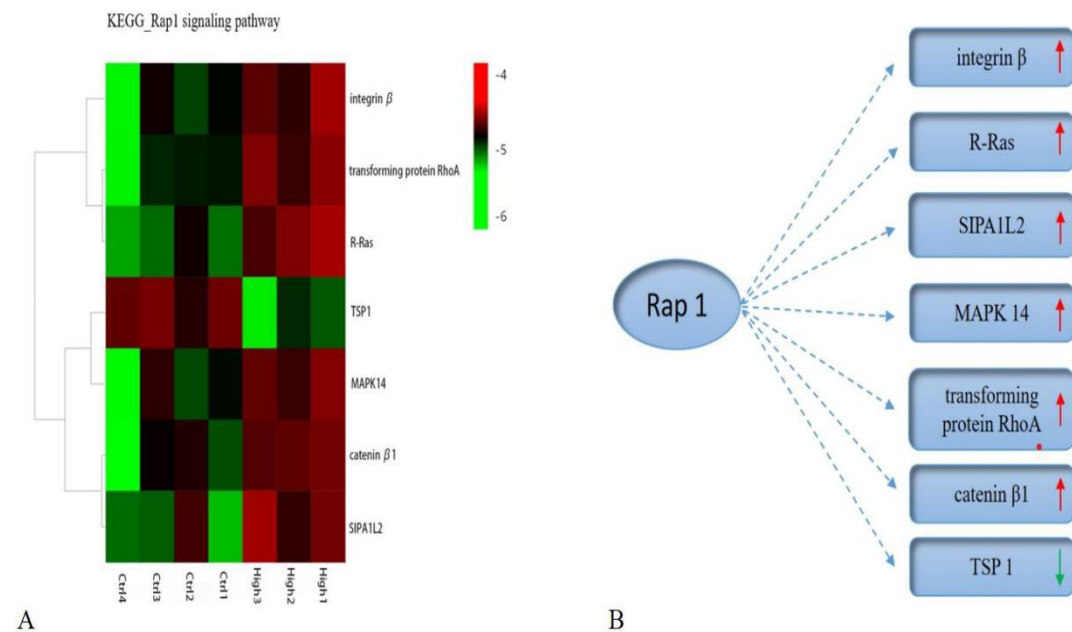


Figure 9. Potential influence on placenta metabolism after 19 days' 100 mg/kg GenX exposure. Differently expressed proteins (DEPs) in KEGG_Rap1 signaling pathway (A). DEPs' upregulated and downregulated in the Rap1 signaling pathway (B).

4. Discussion

Common PFASs toxicity studies of development have been widely reported in previous studies. However, to the best of our knowledge, limited studies have evaluated the developmental toxicities of GenX. The current study found that GenX retarded fetal intrauterine development. Even though the mother and offspring were no longer exposed to GenX after GD 19.5, fetal growth retardation can last up to PND 21. These changes can be attributed to placental inflammatory infiltration, which may be related to the up/down-regulation of proteins in the Rap1 signaling pathway in the placenta.

In our study, the body weight and body length of fetuses born naturally reduced significantly after utero exposure to 10 and 100 mg/kg doses of GenX for 19 days. Conley *et al.*'s results in SD rats were in line with ours, but in a shorter time (16 days) and higher concentration (≥ 30 mg/kg) of GenX exposure (Conley et al. 2021). At the same time, their study found that GenX's EC50 was 35.4 $\mu\text{g/mL}$ in maternal serum. When its serum concentration was <10 $\mu\text{g/mL}$, the fetal survival was almost 100%. In our study, the highest concentration of GenX in maternal serum was 3.92 $\mu\text{g/mL}$ and all the fetuses were alive. However, the weight of fetuses born by caesarean weren't reduced after exposure to GenX in utero even in the 100 mg/kg dose group. Blake et al.'s findings were consistent with our results in CD-1 mice born by caesarean section at GD 17.5 (Blake et al. 2020). These may due to the ignored effect of gestational days on fetuses

born by caesarean section. In zebrafish studies, Liu *et al.* found that exposure to GenX (2-20 mg/L) for 3 days, embryo hatchability was reduced, with ventricular edema, and tail curvature (Liu et al. 2023). Spinal deformities and edema were evident among embryos exposed to very high levels of GenX (1000-16000 mg/L) for 3 days in Gong *et al.*'s study (Gong et al. 2022). However, Gaballah *et al.* and Satbhai *et al.* did not find significant morphological deformities after zebrafish fertilized eggs were exposed to GenX for 5 days (Gaballah et al. 2020; Satbhai et al. 2022), even though Satbhai et al.'s exposure concentration reached 170 mg/L (Satbhai et al. 2022). In view of this, only the studies in rats and mice had basically consistent results, which proves that GenX has a certain degree of developmental toxicity. However, the results are limited and more reliable animal experiments as well as epidemiological studies are needed. With the deepening of research, the long-term toxicity effects of PFASs have attracted increasing attention. In epidemiological studies, Høyer *et al.* found that prenatal PFOA exposure may be associated with child obesity around the waist-to height ratio > 0.5 at 5-9 years of age (Hoyer et al. 2015). Similarly, Halldorsson *et al.* reported that a positive association between prenatal PFOA and overweight and central obesity (i.e., waist circumference) in young female adults (mean age, 20 years) (Halldorsson et al. 2012). Animal experiments on PFOA have shown that in utero exposure not only caused fetal body weight to decrease within 6.5 weeks

after birth, but also delayed in offspring eye-opening, mammary gland development and differentiation, and accelerated sexual maturation in male offspring (Lau et al. 2006; Macon et al. 2011). Our study showed that fetal postnatal development during PND 1-21 was significantly hampered, including body weight, body length and tail length. In previous studies in CD-1 mice, exposure to GenX from preconception through weaning reduced fetal weight at PND 1 that persisted through PND 21 (DuPont-18,405-1,037). Conly *et al.* 's another study in rats found that offspring body weight was significantly reduced at PND 2, PND 27 and puberty, but this finding was restricted to female rats (Coperchin et al. 2020). Rice *et al.*'s study on the toxicokinetics of GenX can supports Conly *et al.* 's review of the higher half-life of GenX in female rats than in male rats (Rice et al. 2021). However, no differences in development were found between male and female fetal with GenX in our study (Supplementary Table 3).

The placenta plays an important role in fetal development, but the mechanism of GenX's effect on the placenta is still unclear. In the 10 and 100 mg/kg dose groups, obvious neutrophils in the decidua zone and significant congestion in the labyrinth zone were observed. Therefore, we suspect that the increased placental weight is due to the placenta inflammatory state, which is not only related to neutrophil infiltration of the decidua zone but also to significant congestion in the labyrinth zone. Decidual and labyrinth zones play an important role in embryo

implantation, placental blood vessel growth and placental development. Its abnormalities can lead to pre-eclampsia, premature birth, or fetal developmental restriction (Gong et al. 2017; Tripathi et al. 2015). To date, only one study in CD-1 mice found that GenX caused placental weight to increase and placental pathological changes including labyrinth zone congestion, labyrinth atrophy and labyrinth necrosis (Blake et al. 2020). This found of pathological lesions in the labyrinthine zone by Blake et al. echoes our study, but they did not find in the decidual zone. What's more, the mechanism of placental lesions was not explored. Our placental proteomic analysis indicated that the toxicity mechanisms of GenX towards the placenta were related to the upregulation of integrin β , R-Ras, SIPA1L2, MAPK14 RhoA, and catenin β 1 and the downregulation of TSP1 in the Rap1 signaling pathway. R-Ras, RhoA, MAPK14, TSP1, integrin β and catenin β 1 are common proteins in the Rap1 signaling pathway (Supplementary Figure 3). R-Ras and RhoA are members of the Ras family, which are upregulated in endothelial cells in response to inflammation cytokine (Blazeby et al. 1995; Komatsu and Ruoslahti 2005). MAPK14 is an osmoregulatory protein kinases that plays a crucial role in inflammation-related diseases such as Crohn's disease and rheumatoid arthritis (Cuenda and Rousseau 2007; Denise Martin et al. 2012). TSP1 is a multifaceted protein and through different receptor interactions, TSP1 can exert proinflammatory or anti-inflammatory effects (Halper and Kjaer

2014; Punekar et al. 2008; Ramirez et al. 2018; Stein et al. 2016). Although integrin β and catenin $\beta 1$ have no obvious correlation with inflammatory factors, they are all related to cell adhesion, migration and polarity. Placental miRNA genes abnormally expressed in the Rap1 signaling pathway caused children's developmental abnormalities in a previous study (He et al. 2022). Thus, the Rap1 signaling pathway may be an important target for placental lesions. More studies are needed in the future to explore the specific proteins in the Rap1 pathway that are involved in placental function.

In this study, we have observed adverse effects of GenX exposure on both pregnant rats and their fetuses. It should be noted that the exposure levels of SD rats in our experiment were higher than that in human exposure in the environment. Previous studies showed that the GenX concentration in urine samples of the general population was 0.07-0.4 $\mu\text{g/L}$ (Calafat et al. 2019). It was 0.13-1.72 $\mu\text{g/L}$ in serum samples of the high exposure population (Pan et al. 2017). In our study, the concentrations of GenX in the serum of pregnant rats in the 1, 10 and 100 mg/kg dose groups were 0.15, 0.57 and 3.92 $\mu\text{g/mL}$, respectively. In terms of dose per unit of body weight, the equivalent dose for rats is 6.3-fold that for humans (Wei et al. 2017). The GenX concentration of 10 mg/kg dose group in pregnant rats which was the lowest observed effect concentration in our study, was 52.4-fold higher than that in human samples. However, considering that humans

are exposed to GenX for a longer time in the environment, it is not reasonable to ignore the exposure time and only compare the exposure dose. Therefore, more toxicological studies on long-term chronic exposure to GenX are needed.

5. Conclusions

This paper reported GenX's adverse effects against the maternal-embryo-placenta unit. GenX induced elevated GWG, fetal intrauterine retardation, and placental inflammatory infiltration. More importantly, we provided evidence that illustrates fetal extrauterine retardation during PND 1-21, even though the mothers and offspring were no longer exposed to GenX after GD 19.5. This demonstrates that GenX's persistent effects, especially on development, should not be ignored. Finally, we used proteomics to prove that placental inflammation may play a key role in the developmental toxicity of GenX through the Rap1 signaling pathway. This study suggests that medical professionals should take this chance to further explore the deep-seated molecular mechanism of GenX development toxicity in Rap1 signaling pathway. This can provide a reference for discussing the effects of GenX on human fetal growth and its future development.

Author contributions

Di Lv: Software, Formal analysis, Data analysis, Writing-Original draft preparation, Writing-Review & Editing. Hongyun Liu: Placental tissue section analysis, Writing-Review & Editing. Qi An, Linping Kang, Qing Dong and Zhugen Yang: Data analysis, Writing-Review & Editing. Chengwei Lei: Methodology, Data analysis. Chuanhai Li: Detection of GenX concentration, Resources. Yongfeng Lin: Detection of GenX concentration. Wendong Liu: Term, Conceptualization, Writing - Review & Editing. Wenchao Han: Term, Conceptualization, Writing-Review & Editing, Funding acquisition.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81903271, 82103875).

Declaration of competing interest

The authors declare they have no actual or potential competing financial interest.

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2024-01-05

Elsevier

<https://doi.org/10.1016/j.jhazmat.2023.132536>

Lv D, Liu H, An Q, et al., (2024) Association of adverse fetal outcomes with placental inflammation after oral gestational exposure to hexafluoropropylene oxide dimer acid (GenX) in Sprague-Dawley rats. *Journal of Hazardous Materials*, Volume 461, January 2024, Article Number 132536