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www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 2017, 1–10

doi: 10.1093/toxsci/kfx052
Advance Access Publication Date: March 23, 2017
Research article

Maternal Fenvalerate Exposure Induces Fetal Intrauterine Growth Restriction Through Disrupting Placental Thyroid Hormone Receptor Signaling

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ABSTRACT

Fenvalerate is an environmental endocrine disruptor that disrupts testosterone and estradiol synthesis. Nevertheless, whether fenvalerate disturbs placental TR signaling remains unclear. The aim of this study was to investigate whether maternal fenvalerate exposure causes fetal intrauterine growth restriction (IUGR) and to explore the role of placental thyroid hormone receptor (TR) signaling. Pregnant mice except controls were orally administered to fenvalerate (0.2, 2.0, or 20 mg/kg) daily throughout pregnancy. As expected, fetal weight was lowered in dams that were administered with 20.0 mg/kg of fenvalerate. Moreover, the rate of IUGR was elevated not only in male fetuses but also in female fetuses of dams exposed to 20.0 mg/kg of fenvalerate. Histopathology showed that the internal space of blood vessels in the labyrinth layer was smaller in placentas of mice exposed to fenvalerate. Mechanistic study found no significant difference on TT4 level in maternal serum, although TT3 level in maternal serum was slightly reduced in dams exposed to 2.0 mg/kg of fenvalerate. Interestingly, placental *TRα1* and *TRβ1* mRNAs were reduced in mice exposed to fenvalerate. Moreover, nuclear translocation of placental TRβ1 was suppressed in fenvalerate-exposed mice. Further analysis showed that placental *Vegfα* and *Igf2*, several target genes of TR signaling, were down-regulated in fenvalerate-exposed mice. In addition, mRNA level of placental *CD36*, *Snat1*, and *Snat2*, 3 nutrient transporters, were reduced in fenvalerate-exposed mice. These results suggest that maternal fenvalerate exposure induces fetal IUGR through disrupting placental TR signaling. These results provide a novel mechanistic explanation for fenvalerate-induced fetal IUGR.

Key words: fenvalerate; endocrine disruption; intrauterine growth restriction (IUGR); thyroid hormone receptors (TRs).

Fenvalerate, one of type II pyrethoid pesticides, is a class of neurotoxic chemical widely used for agricultural and residential pesticides. Due to its wide insecticidal range, superior insecticidal activity and a low toxicity, the consumption of fenvalerate has continuously increased in China (Li *et al.*, 2016). According to a recent report, fenvalerate and its metabolites were detected in bovine milk (Bedi *et al.*, 2015). In addition, fenvalerate and its metabolites were also detected in human samples, such as breast milk and urine (Corcellas *et al.*, 2012; Qi *et al.*, 2012). Fenvalerate is a reproductive toxicant. Several reports from rodent animals showed that fenvalerate induced germ cell apoptosis and permanently impaired spermatogenesis (Zhang *et al.*, 2009; Zhao *et al.*, 2011). According to an early epidemiological investigation, an increase in sperm DNA damage was observed among fenvalerate-exposed workers (Bian *et al.*, 2004). On the other hand, fenvalerate is also a developmental toxicant. An early report from our laboratory showed that prenatal fenvalerate exposure impaired testicular development in mice

(Zhang et al., 2010). Moreover, pubertal fenvalerate exposure impaired cognitive and behavioral development (Meng et al., 2011).

Increasing evidence demonstrates that fenvalerate is an environmental endocrine disruptor (EED). Two *in vitro* studies showed that fenvalerate inhibited release of steroid hormones in primary cultured rat ovarian follicles and mouse Leydig tumor cells (Fei et al., 2010; Qu et al., 2008). Several *in vivo* reports found that fenvalerate exposure reduced plasma steroid hormones and delayed sexual maturation in male and female animals (Moniz et al., 1999a, 2005b). In addition, pubertal fenvalerate exposure disrupted the synthesis of testosterone and estradiol in the developing brain (Liu et al., 2011). Thyroid hormone (TH), one of the most important hormones, is essential for fetal growth and development (Forhead and Fowden, 2014). Recently, several epidemiological reports found that maternal hyperthyroidism or hypothyroidism was associated with fetal intrauterine growth retardation (IUGR) (Aggarawal et al., 2014; Chen et al., 2014; Pearce et al., 2016). The actions of TH are mediated by thyroid hormone receptors (TRs) (Onigata and Szinnai, 2014; Ortiga-Carvalho et al., 2014). Indeed, TRs are highly expressed in human and rodent placentas (Kilby et al., 1998; Leonard et al., 2001). Nevertheless, whether maternal fenvalerate exposure disturbs placental TR signaling remains unclear.

The aim of this study was to investigate the effects of maternal fenvalerate exposure during pregnancy on fetal IUGR and to explore the role of placental TR signaling on fenvalerate-induced fetal IUGR. Our results showed that maternal fenvalerate exposure down-regulated the expression of placental *TRα1* and *TRβ1*. In addition, maternal fenvalerate exposure repressed nuclear translocation of placental TRβ1. The present study provides a novel mechanistic explanation for fenvalerate-induced fetal IUGR.

MATERIALS AND METHODS

Chemicals and reagents. Fenvalerate was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-thyroid hormone receptor (TR) α1 and β1 antibodies were from Abcam (Cambtidge, MA, USA). TRI reagent was from Molecular Research Center, Inc (Cincinnati, OH, USA). RNase-free DNase, and real time RT and polymerase chain reaction (PCR) kits were from Promega Corporation (Madison, WI, USA). All the other reagents were from Sigma or as indicated in the specified methods.

Animals and treatments. The ICR mice (8–10 weeks old; male mice: 32–34 g; female mice: 28–30 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food (Beijing Keao Xieli Feed Co, LTD, Beijing 100107) and water at all times and were housed in a room with controlled lighting (12 h light/12 h dark cycle) and temperature (20–25 °C) for 1 week before use. For mating purposes, 4 females were housed overnight with 2 males starting at 9:00 PM. Females were checked by 7:00 AM the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. To investigate the effects of maternal fenvalerate exposure during pregnancy on fetal development, 56 pregnant mice were divided into 4 groups randomly. In fenvalerate pregnant mice, pregnant mice were daily administered with fenvalerate (0.2, 2.0, and 20 mg/kg, dissolved in corn oil) by gavage from GD0 to GD17. The control pregnant mice were daily administered with corn oil by gavage from GD0 to GD17. According to our previous study (Zhang et al., 2010), the dose of 20 mg/kg, about 1/10 LD50 of fenvalerate, was chosen as the highest dose. Our preliminary data showed that no signs of maternal toxicity were observed in dams that were administered with fenvalerate (20 mg/kg) during pregnancy. All dams were sacrificed on GD18. Gravid uterine weights and the number of implantation, live fetuses, dead fetuses, and resorption sites were counted. Male and female live fetuses were weighed respectively. Anal reproductive distance is used to distinguish the sex of fetal mice. If this way could not clearly identify the sex of the fetus, we identify fetal sex through the uterus and testicles. In this study, the threshold of IUGR was further determined through evaluating the distribution of male and female fetal weights in control group. Fetuses with weights <10th percentile were designated as IUGR (Cotechini et al., 2014; Chen et al., 2016). In our present study, fetuses with weight below a value (male < 1.229 g; female < 1.186 g) were designed as IUGR. Placentas from male and female live fetuses were weighted respectively. For each group, 6 placentas from 6 different pregnant mice were aseptically removed for RT-PCR. Fetal serum was collected for measurement of TT4 and TT3. To investigate the effects of fenvalerate on placental thyroid hormone receptor signaling, 24 pregnant mice were divided into 4 groups randomly. In fenvalerate group, pregnant mice were daily administered with fenvalerate (0.2, 2.0, and 20 mg/kg, dissolved in corn oil) by gavage from GD0 to GD14. The control pregnant mice were daily administered with corn oil by gavage from GD0 to GD14. All dams were sacrificed on GD15. Maternal serum was collected for measurement of TT4 and TT3. For each group, 6 placentas from 6 different pregnant mice were aseptically removed for RT-PCR. For each group, 30 placentas from 6 different pregnant mice were aseptically removed for immunoblots. For each group, 6 placentas from 6 different pregnant mice were collected for placental histopathology.

This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 13-0012). All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Hefei, China).

Electrochemiluminescence immunoassay (ECLIA). Maternal blood and fetal blood were collected respectively. The blood was then centrifuged at 3000 r for 10 min and the serum was collected for −80 °C. Serum TT4 and TT3 were measured by ECLIA (Iwaku et al., 2013) on Cobas Elecsys 601 (Roche Diagnostics, Germany). TT4 and TT3 ECLI kits were purchased from Roche Applied Science. Results were determined via a calibration curve that is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

Histology in labyrinth. Freshly collected placentas were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded placentas were serially sectioned. Hematoxylin and eosin (H&E) stained placental sections were analyzed for blood sinusoidal area quantification according to previous study (Neres et al., 2008). In each section, at least 8 fields were randomly selected in the labyrinthine region in each placenta. We performed an image analysis using the public domain NIH Image J Program. Briefly, the images were given a color threshold to cover the internal space of maternal and fetal blood vessels in the labyrinthine layer after noise removal. The percentage of blood sinusoidal area was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image. The results in the present study represent the average results for 6 placentas from 6 pregnant mice in each group.

Isolation of total RNA and real-time RT-PCR. Total RNA in placenta tissue was extracted using TRI reagent. RNase-free DNase-

treated total RNA (1.0 μg) was reverse-transcribed with AMV reverse transcriptase (Promega). Real-time RT-PCR was performed with a Light Cycler 480 SYBR Green I kit (Roche Diagnostics GmbH) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on Light Cycler 480 Instrument (Roche Diagnostics GmbH) with an initial hold step (95 °C for 5 min) and 50 cycles of a 3-step PCR (95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s).

Immunoblots. For nuclear protein extraction from placenta, 400 mg placenta tissue was homogenized in 5 mL ice-cold buffer A [0.6% NP-40, 150 mM NaCl, 10 mM HEPES (pH7.9), 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] on ice. The homogenate was centrifuged at 270× g for 30 s and the precipitate was discarded. The supernatant was kept on ice for 20 min and centrifuged again at 750× g for 10 min at 4 °C. The supernatant was then mixed with 1 mL ice-cold buffer A and centrifuged again at 750× g for 10 min. The nuclear pellet obtained was reserved and homogenized in 100 μL Buffer B [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 25% glycerol, and 1% Protease Inhbitor Cocktail (P8340, Sigma)] for 60 min on ice. Nuclear lysate was centrifuged at 11 000× g for 10 min at 4 °C. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL, USA) according to manufacturer's instructions. For immunoblots, same amount of protein (16 μg) was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were blocked in 5% skimmed milk for 1.5 h and incubated with rabbit polyclonal antibodies (Abcam, MA, USA) for 2 h. After washes in DPBS containing 0.05% Tween-20 4 times for 10 min each, the membranes were incubated with goat anti-rabbit IgG (1:80 000) for 2 h. The membranes were then washed for 4 times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL detection kit. Lamin A/C was used as a loading control.

Statistical analysis. The litter was considered the unit for statistical analysis among different groups. For fetal weight, the means were calculated per litter and then averaged per group. Quantified data were expressed as means ± S.E.M. at each point. $P<0.05$ was considered statistically significant. Normally distributed data was performed using ANOVA and the Student–Newmann–Keuls post hoc test. Non-normally distributed data was performed using Kruskal–Wallis test.

RESULTS

Effects of Fenvalerate Exposure During Pregnancy on Maternal Food Consumption and Body Weight Growth

No significant difference on food consumption and body weight growth was observed among different groups (Figs. 1A–B). Despite the upward trend in a dose-dependent manner, no significant difference on maternal weight gain was observed among different groups (Figure 1C).

Effects of Maternal Fenvalerate Exposure During Pregnancy on Pregnant Outcomes

No significant difference on the number of implantation sites, resorptions per litter, live fetuses per litter and dead fetuses per litter was observed among different groups (Table 2). The effects of maternal fenvalerate exposure during pregnancy on fetal

TABLE 1. Primers for Real-Time RT-PCR

Genes	Sequence	Sizes (bp)
18S	Forward:5′-GTAACCCGTTGAAGGGATT-3′ Reverse:5′-CCATCCAATCGGTAGTAGAG-3′	151
TRα	Forward:5′-GACAAGGCCACCGGTTATCA-3′ Reverse:5′-CTTGTCGATGACACAGCAGC-3′	132
TRβ	Forward:5′-CTGATCCGTGTTTTCCCTCTC-3′ Reverse:5′-TCTGTACTGGCATTCCCTCTG-3′	101
Vegfα	Forward:5′-TATTCAGCGGACTCACCAGC-3′ Reverse:5′-AACCAACCTCCTCAAACCGT-3′	156
Vegfr-1	Forward:5′-TCAAGCTAGAGGTGTCCCCG-3′ Reverse:5′-CTCGGCACCTATAGACACCC-3′	152
Igf-1	Forward:5′-AAGGCAGTTTACCCAGGCTC-3′ Reverse:5′-GGCCGAGGTGAACACAAAAC-3′	125
Igf-2	Forward:5′-CTTCAGCAGCGTCCACTTCA-3′ Reverse:5′-TTGGTACCACAAGGCCGAAG-3′	105
Fatp-1	Forward:5′-CGCCGATGTGCTCTATGACT-3′ Reverse:5′-ACACAGTCATCCCAGAAGCG-3′	138
CD36	Forward:5′-CACAGCTGCCTTCTGAAATGTGTGG-3′ Reverse:5′-TTTCTACGTGGCCCGGTTCTAATTC-3′	171
Snat-1	Forward:5′-AACCCGGCCTTTTACCTTCC-3′ Reverse:5′-CCCGGCAGTTAGATGTCCTT-3′	122
Snat-2	Forward:5′-ACCTCACCTGCTCGTCAAAG-3′ Reverse:5′-TGGTTGTCATGGCACCTCTC-3′	117

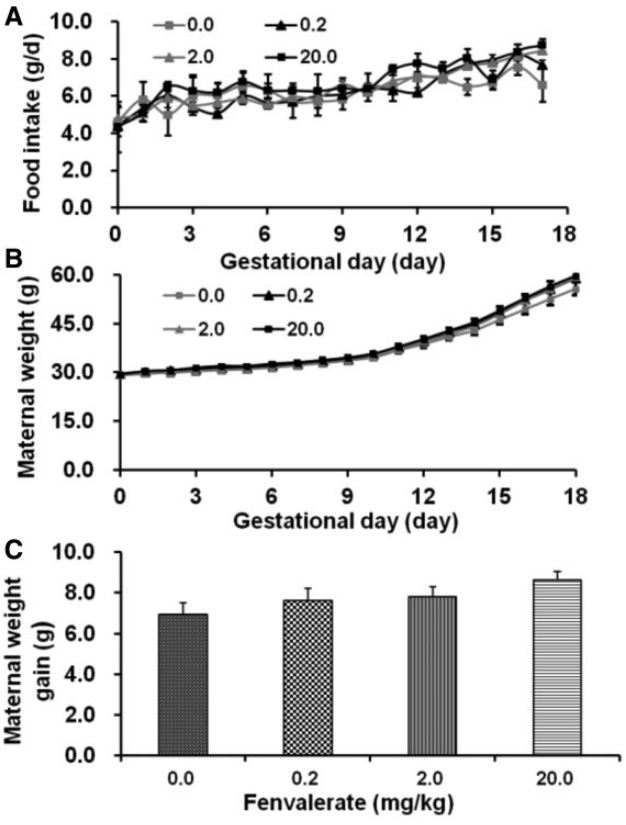

FIG. 1. The effects of fenvalerate exposure during pregnancy on food consumption and body weight growth. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0 or 20.0 mg/kg) daily throughout pregnancy. A, Food consumption and B, maternal weight. C, Maternal weight gain. All data were expressed as mean ± S.E.M ($n = 11$–16).

TABLE 2. The Effects of Fenvalerate Exposure During the Whole Pregnancy on Fetal Outcomes

	Fenvalerate (mg/kg/day)				*F/H*	*P*
	0.0	0.2	2.0	20.0		
Number of pregnancy mice	11	16	15	14		
Implantation sites per litter	12.36 ± 0.88	13.25 ± 0.40	13.47 ± 0.36	13.93 ± 0.56	1.382	.259
Live fetuses per litter	11.73 ± 0.90	12.75 ± 0.46	12.80 ± 0.44	13.29 ± 0.61	1.141	.341
Resorptions per litter	0.27 ± 0.20	0.31 ± 0.12	0.53 ± 0.24	0.14 ± 0.10	2.084	.555
Dead fetuses per litter	0.36 ± 0.20	0.19 ± 0.10	0.13 ± 0.09	0.50 ± 0.23	4.081	.253

The data are expressed as the means ± SEM. The results of implantation sites per litter and live fetuses per litter were analyzed by using one-way ANOVA followed by Student–Newman–Keuls post hoc test. Resorptions per litter, dead fetuses per litter were analyzed using Kruskal–Wallis test.

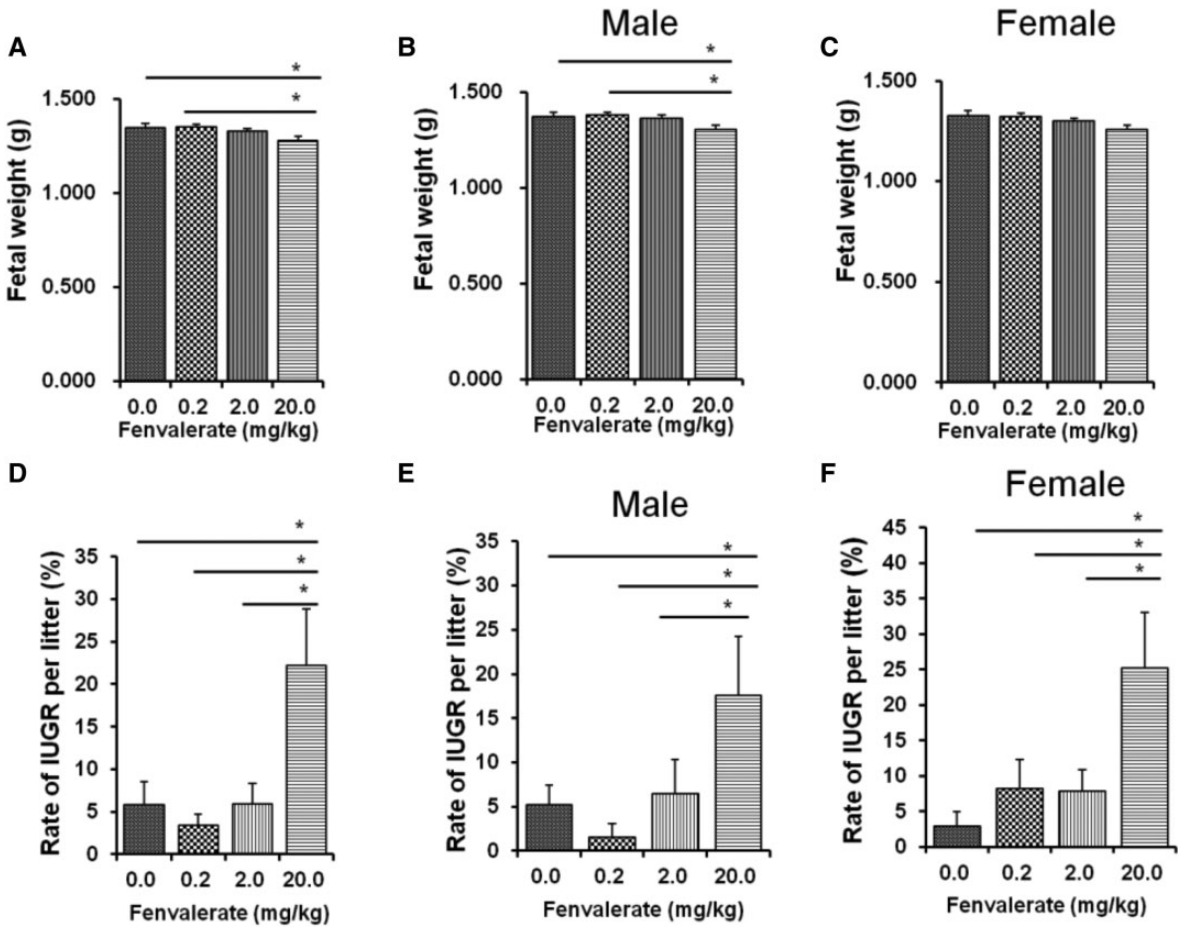

FIG. 2. The effects of maternal fenvalerate exposure during pregnancy on fetal weight. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0, or 20.0 mg/kg) daily throughout pregnancy. All dams were sacrificed on GD18. Male and female fetuses were identified and weighted. A, Body weight of all fetuses. B, Body weight of male fetuses. C, Body weight of female fetuses. D, Rate of IUGR in all fetuses. E, Rate of IUGR in male fetuses. F, Rate of IUGR in female fetuses. All data were expressed as mean ± S.E.M ($n = 11$–16). *$P < 0.05$.

weight were analyzed. As shown in Figure 2A, body weight was significantly reduced in fetuses whose mothers were daily exposed to 20.0 mg/kg fenvalerate from GD0 to GD17. Further analysis showed that body weight of male fetuses was significantly reduced in dams that were daily exposed to 20.0 mg/kg fenvalerate from GD0 to GD17 (Figure 2B). Despite no statistically significant difference on body weight among different groups, there was a downward trend on body weight of female fetuses in fenvalerate-exposed mice as compared with controls (Figure 2C). The effects of maternal fenvalerate exposure during pregnancy on the rate of IUGR per litter were analyzed. The results showed that the rate of IUGR was significantly increased in dams treated with 20.0 mg/kg of fenvalerate (Figure 2D). Further analysis showed that the rate of IUGR was significantly increased not only in male fetus but also in female fetus of mice exposed to 20.0 mg/kg of fenvalerate (Figs. 2E–F).

Effects of Fenvalerate Exposure During Pregnancy on Thyroid Hormone Levels in Maternal and Fetal Sera

No significant difference on the level of TT4 in maternal serum was observed (Figure 3A), despite the level of TT3 in maternal

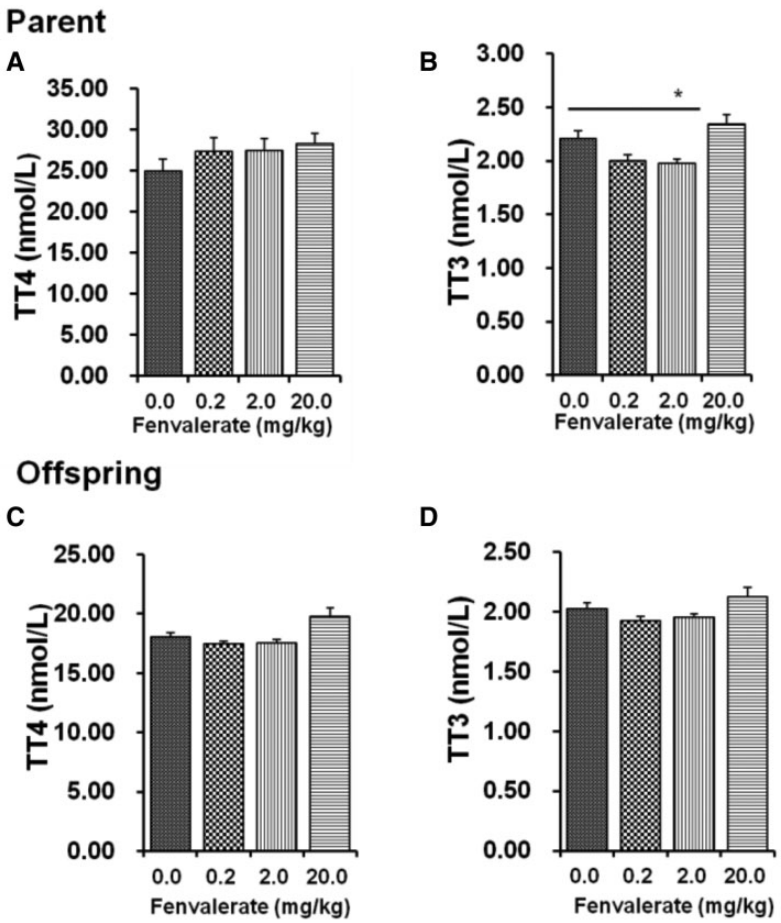

FIG. 3. The effects of maternal fenvalerate exposure during pregnancy on thyroid hormone levels in maternal and fetal serum. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0, or 20.0 mg/kg) daily throughout pregnancy. A and B, Pregnant mice were sacrificed on GD15. Maternal serum was collected for measurement of TT4 and TT3. (A) TT4 in maternal serum. (B) TT3 in maternal serum. All data were expressed as mean ± S.E.M ($n = 6$). C and D, Pregnant mice were sacrificed on GD18. Fetal serum was collected for measurement of TT4 and TT3. (C) TT4 in fetal serum. (D) TT4 in fetal serum. All data were expressed as mean ± S.E.M ($n = 11$–16). *$P < 0.05$.

serum was slightly reduced in dams exposed to 2.0 mg/kg of fenvalerate (Figure 3B). No significant difference on the level of TT4 and TT3 in fetal serum was observed among different groups (Figs. 3C–D).

Effects of Maternal Fenvalerate Exposure During Pregnancy on Placental Development

The effects of maternal fenvalerate exposure during pregnancy on placenta weight were analyzed. Unexpectedly, no significant difference on placenta weight was observed among different groups (Figure 4A). The effects of maternal fenvalerate exposure during pregnancy on placenta weight of male and female fetuses were then analyzed. Despite a downward trend in a dose-dependent manner, there was no statistically significant difference on placenta weight of male fetuses among different groups (Figure 4B). In addition, no significant difference on placenta weight of female fetuses was observed among different groups (Figure 4C). To investigate the effects of maternal fenvalerate exposure during pregnancy on placental vascular space, cross-sectional areas of blood sinusoids were analyzed in placental labyrinthine region using computerized morphometry method. As shown in Figures 4D and E, blood sinusoid area in the labyrinth layer was reduced in a dose-dependent manner.

Effects of Maternal Fenvalerate Exposure During Pregnancy on Thyroid Hormone Receptors in Placenta

The effects of maternal fenvalerate exposure during pregnancy on the expression of placental thyroid hormone receptors were analyzed. As shown in Figures 5A and B, the expressions of placental *TRα1* and *TRβ1* mRNAs were down-regulated in fenvalerate-exposed mice. The effects of maternal fenvalerate exposure during pregnancy on nuclear translocation of placental TRα1 and TRβ1 were then analyzed. As shown in Figures 5C and 5D, there was no significant difference on the level of placental nuclear TRα1 level among different groups. Interestingly, the level of placental nuclear TRβ1 level was significantly reduced in dams that were daily exposed to 20.0 mg/kg of fenvalerate from GD0 to GD17 (Figs. 5C and E).

Effects of Maternal Fenvalerate Exposure During Pregnancy on the Expression of Placental Nutrient Transporters and Growth Factors

The effects of maternal fenvalerate exposure during pregnancy on placental nutrient transporters were analyzed. Although no significant difference on placental *Fatp1*, a fatty acid transporter gene, was observed among different groups (Figure 6A), placental *CD36*, another fatty acid transporter gene, was significantly down-regulated in fenvalerate-exposed mice (Figure 6B). Interestingly,

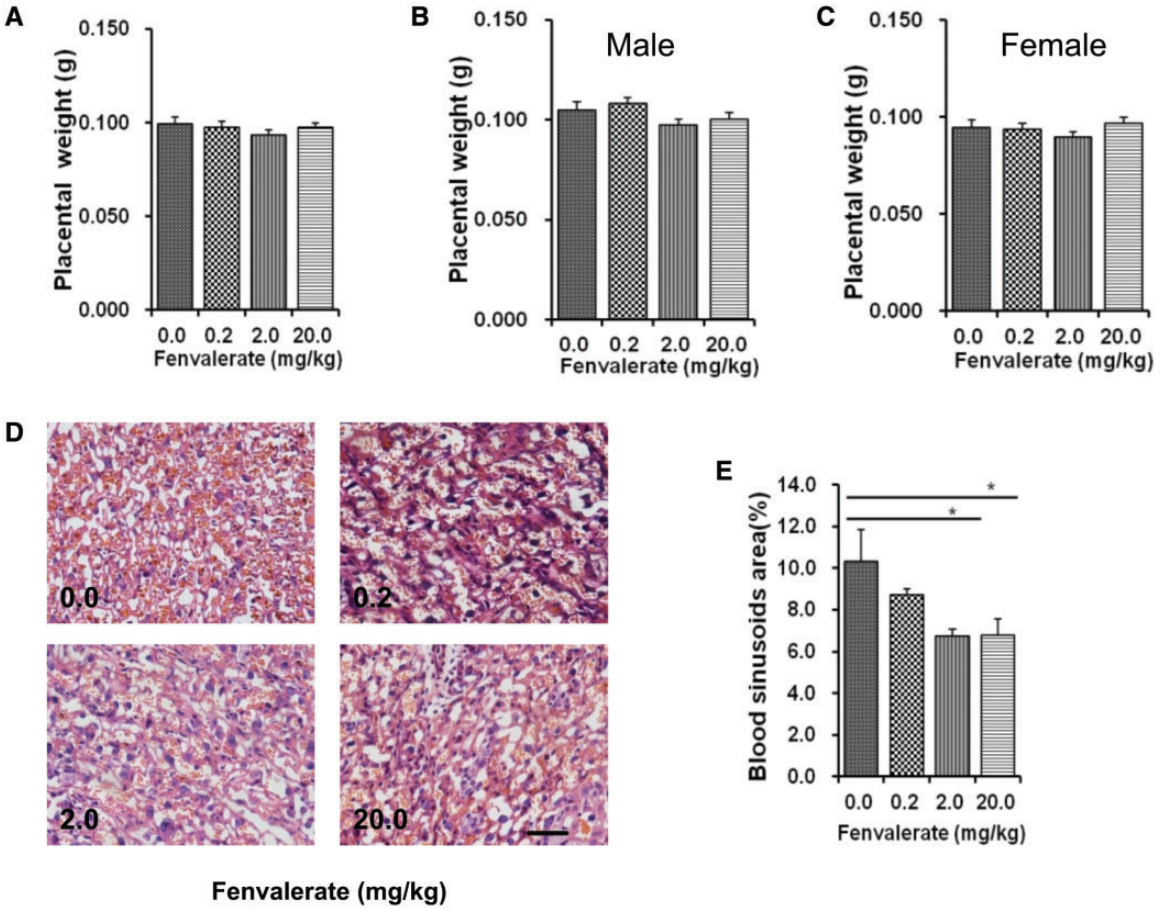

FIG. 4. The effects of maternal fenvalerate exposure during pregnancy on placental development. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0, or 20.0 mg/kg) daily throughout pregnancy. A–C, Pregnant mice were sacrificed on GD18. Male and female fetuses were identified. Placenta weights of male and female fetuses were measured. (A) Placenta weight of all fetuses. (B) Placenta weight of male fetuses. (C) Placenta weight of female fetuses. All data were expressed as mean ± S.E.M ($n = 11$–16). D and E Some pregnant mice were sacrificed on GD15. (D) Placentas were collected and placental cross sections were stained with H&E. Original magnification: 400×. Scale bar: 50 μm. (E) Vascular area in the labyrinthine region was estimated from at least 8 non-consecutive sections in each placenta using the public domain NIH Image J Program. The rate of blood sinusoid area was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image. Bar size: 50 μm. All data were expressed as mean ± S.E.M ($n = 6$). * $P<0.05$.

placental *Snat1 and Snat2*, 2 amino acid transporter genes, were significantly down-regulated in fenvalerate-exposed mice (Figs. 6C and D). The effects of maternal fenvalerate exposure during pregnancy on placental growth factors were then analyzed. As shown in Figures 6E and F, placental *Vegfα* and its receptor *Vegfr1* were significantly down-regulated in fenvalerate-exposed mice. Although no significant difference on placental *Igf1* was observed among different groups (Figure 6G), the level of placental *Igf2* mRNA was significantly reduced in dams that were daily exposed to 20.0 mg/kg fenvalerate from GD0 to GD17 (Figure 6H).

DISCUSSION

In the present study, we investigated the effects of maternal fenvalerate exposure during pregnancy on fetal IUGR. Our results showed that fetal weight was reduced when dams were exposed to fenvalerate throughout pregnancy. We further analyzed whether maternal fenvalerate exposure induced fetal IUGR in a gender-dependent manner. We found that body weight of male fetuses was reduced in dams that were exposed to fenvalerate throughout pregnancy. Despite no statistically significant difference on body weight among different groups, there was a downward trend on body weight of female fetuses in fenvalerate-exposed mice as compared with controls. Further analysis found that the rate of IUGR was significantly increased not only in male fetuses but also in female fetuses of dams exposed to 20.0 mg/kg of fenvalerate. This results suggested fenvalerate-induced IUGR is not gender specific.

The placenta is essential for sustaining the growth and development of fetuses. Placental labyrinth is the site of oxygen and nutrient exchange between the mother and the fetus and is a highly developed tissue of blood vessels. It is well known that placenta insufficiency is a major cause of fetal IUGR (Cetin and Alvino, 2009; Scifres and Nelson, 2009). In addition, a reduction in placenta size can directly reduce the size of the fetus, due to placenta's inability to transfer nutrients from the mother to the fetus (Barker and Thornburg, 2013). In the present study, we measured the effects of maternal fenvalerate exposure on placental development. Although maternal fenvalerate exposure had little effect on placenta size, the internal space of blood vessels in the labyrinth layer was smaller in placentas of dams with fenvalerate throughout pregnancy. These results suggest that fenvalerate-induced IUGR may be partially attributed to reduction of placental transport capacity.

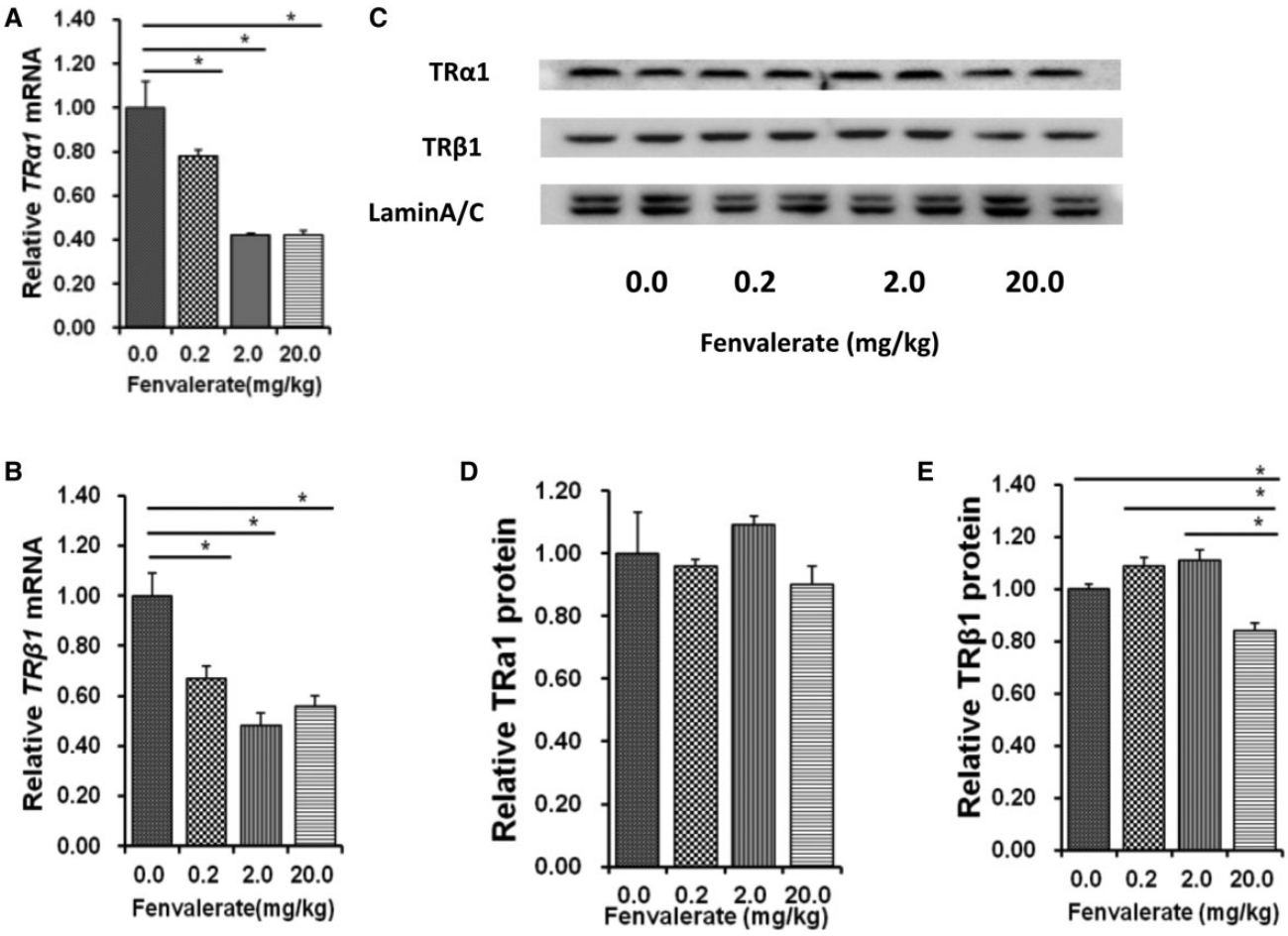

FIG. 5. The effects of maternal fenvalerate exposure during pregnancy on placental thyroid hormone receptor signaling. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0, or 20.0 mg/kg) daily throughout pregnancy. All dams were sacrificed on GD15. Placentas were collected. A and B, Placental *TRα1* and *TRβ1* mRNAs were measured using real-time RT-PCR. C, Nuclear TRα1 and TRβ1 were measured using immunoblots. All experiments were repeated for 3 times. D and E, Quantitative analyses of scanning densitometry on 6 samples from 6 different litters were performed. (D) TRα1. (E) TRβ1. All data were expressed as mean ± S.E.M ($n = 6$). *$P<0.05$.

Increasing evidence demonstrates that TR signaling plays an important role in the maintenance of placental function and fetal development (Chan *et al.*, 2009; Chen *et al.*, 2015). According to an early study, maternal exposure to anti-thyroid agents in pregnancy and lactation resulted in growth retardation lasting into the adult stage, which was particularly prominent in male offspring (Shibutani *et al.*, 2009). On the other hand, several studies demonstrated that environmental endocrine disruptors, such as polychlorinated biphenyl, bisphenol A, and arsenic, disturbed TR signaling (Davey *et al.*, 2008; Tabuchi *et al.*, 2006; Yang and Chan, 2015). Recently, an in vitro report showed that bifenthrin or λ-cyhalothrin, 2 synthetic pyrethroids, disrupted hypothalamus-pituitary-thyroid axis in zebrafish embryos (Tu *et al.*, 2016). The present study investigated the effects of maternal fenvalerate exposure on TT3 and TT4 in maternal serum. Although the level of TT3 in maternal serum was slightly reduced in dams exposed to 2.0 mg/kg of fenvalerate, no significant difference on the level of TT4 in maternal serum was observed among different groups. Fenvalerate reduced TT3 in maternal serum may likely reflects a variation and not a truly biological significant change. A recent epidemiological report also found that the concentration of fenvalerate main metabolite 3-PBA in urine was not associated with TT3 and TT4 in general US population (Jain, 2016). The present study then investigated the effects of maternal fenvalerate exposure on placental TR signaling. Interestingly, maternal fenvalerate exposure reduced the expression of placental *TRα1* and *TRβ1* mRNAs. In addition, maternal fenvalerate exposure during pregnancy inhibited nuclear translocation of TRβ1 in placenta in 20.0 mg/kg fenvalerate group. These results suggest that maternal fenvalerate exposure disturbs placental TR signaling through down-regulating placental *TRα1* and *TRβ1* and suppressing nuclear translocation of placental TRβ1 but not through altering TT3 and TT4 in maternal serum. Future research should focus on exploring whether fenvalerate disturbs interaction between T3 with placental TRβ1.

The mechanism by which TR signaling regulates placental function and fetal development remains obscure. Increasing data demonstrate that insulin-like growth factors (IGF) are downstream target genes of TR signaling (Dong *et al.*, 2009; Kim and Mohan, 2013; Xing *et al.*, 2012). Recently, 2 reports showed that vascular endothelial growth factor (VEGF) was up-regulated in T3-treated mouse trophoblast cells and T4-treated rat placenta (Silva *et al.*, 2015a,b). Indeed, IGF-2 plays important roles in both decidual angiogenesis and placental development (Constância *et al.*, 2002; Herr *et al.*, 2003; Pringle and Roberts, 2007). Moreover, VEGF is a key regulator for placental angiogenesis (Reynolds and Redmer, 2001). A recent study showed that

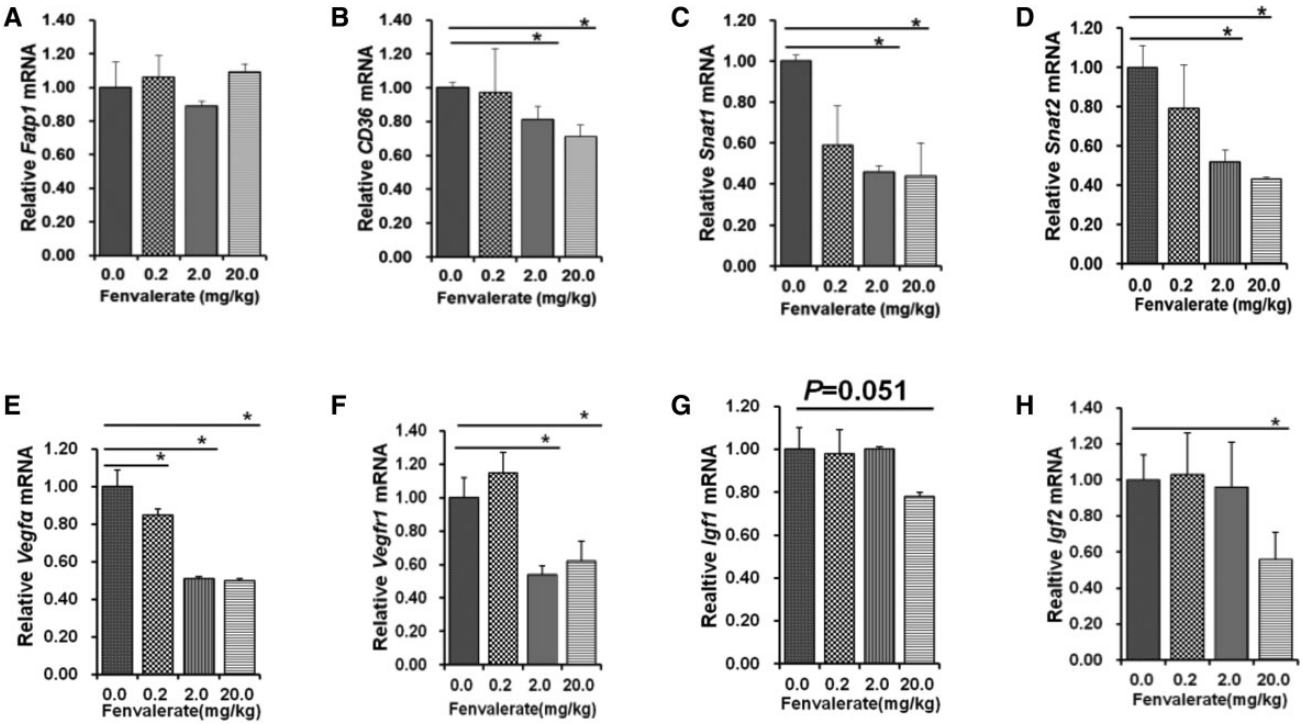

FIG. 6. The effects of maternal fenvalerate exposure during pregnancy on the expression of placental nutrient transporters and growth factors. The pregnant mice were administered with fenvalerate (0, 0.2, 2.0, or 20.0 mg/kg) daily throughout pregnancy. A–D and G–H, Pregnant mice were sacrificed on GD18. Placentas were collected. The expression of placental nutrient transporters and growth factors were measured using real-time RT-PCR. (A) *Fatp1*. (B) *CD36*. (C) *Snat1*. (D) *Snat2*. (G) *Igf1*. (H) *Igf2*. All data were expressed as mean ± S.E.M ($n = 6$). *$P<0.05$. (E–F) Pregnant mice were sacrificed on GD15. Placentas were collected. The expression of placental *vegfa* and *vegfr1* were measured using real-time RT-PCR. (E) *Vegfa*. (F) *Vegfr1*. All data were expressed as mean ± S.E.M ($n = 6$). *$P<0.05$.

fetal IUGR in hypothyroidism was associated with down-regulation of placental *VEGF* and alteration of vascular development of placental labyrinth in rats (Silva *et al.*, 2012). The present study investigated the effects of maternal fenvalerate exposure during pregnancy on placental IGFs and VEGF. We showed that placental *Igf2* and *Vegfα* mRNAs were down-regulated in fenvalerate-exposed mice. These results suggest that maternal fenvalerate exposure impairs placental function and fetal development, at least partially, through inhibiting TR-mediated placental IGF-2 and VEGF expression. A recent study found that T4-treated rats showed reduced *Vegfr1* expression (Silva *et al.*, 2015b). The present study showed that placental *Vegfr1* were down-regulated in fenvalerate-treated mice. These results suggest that other mechanism is involved in fenvalerate-induced fetal IUGR. Additional experiment is required to explore the mechanism through which maternal fenvalerate exposure down-regulates placental *Vegfr1* gene.

The present study showed that blood sinusoid area in the labyrinth layer was reduced not only in high-dose group but also in middle-dose group, whereas IUGR fetuses were observed only in high-dose group. These results suggest that fenvalerate-induced IUGR cannot be completely attributed to reduction of the internal space of blood vessels in the labyrinth layer. Indeed, placenta exerts its nutrient transport function by nutrient transporters. The density of nutrient transporters often determines the efficiency of nutrient transport across the placenta. Fatty acid transport proteins (FATP) and fatty acid translocase (FAT/CD36) are the key transporters for fatty acid (Brass *et al.*, 2013; Dube *et al.*, 2012; Duttaroy 2009). Sodium-dependent neutral amino acid transporter (SNAT) transfers neutral amino acid from maternal circulation to the fetus (Kavitha *et al.*, 2014). The present study analyzed the effects of maternal fenvalerate exposure on placental nutrient transporters. Although it had little effect on placental *Fatp1*, maternal fenvalerate exposure during pregnancy reduced expression of placental *CD36*, *Snat1*, and *Snat2* in a dose-dependent manner. These results suggest that fenvalerate-induced fetal IUGR is partially attributed to reduced expression of placental nutrient transporters.

The aim of the present study was to explore the role of placental TR signaling on fenvalerate-induced IUGR. The present study also investigated the effects of maternal fenvalerate exposure during pregnancy on TH level in fetal serum. Our results showed that no significant difference on the level of TT4 and TT3 in fetal serum was observed among different groups. However, our present study has several deficiencies. First, the present study did not investigate the effects of maternal fenvalerate exposure during pregnancy on neurobehavioral development in fetuses. Second, the present study did not investigate the effects of maternal fenvalerate exposure during pregnancy on TR target genes in fetal brain. Additional experiment is required to explore the effects of maternal fenvalerate exposure on abnormal brain development and TH target gene expression.

In summary, the present study investigated the effects of maternal fenvalerate exposure on placental function and fetal development. Our results showed that maternal fenvalerate exposure throughout pregnancy caused fetal IUGR. Moreover, maternal fenvalerate exposure disturbed placental TR signaling. We demonstrate that maternal fenvalerate exposure impairs placental function and fetal development, at least partially, through inhibiting TR-mediated placental IGF-2 and VEGF expression. These results provide a novel mechanistic explanation for fenvalerate-induced IUGR.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the help of Cheng-Wei Yang, Inspection Physician from Second People 's Hospital of Anhui Province, for measurement of TT4 and TT3.

FUNDING

This work was supported by National Natural Science Foundation of China (81102155) and Major projects of Natural Scientific Research in Universities and Colleges, Anhui, China (KJ2016SD27).

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