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Maternal cadmium exposure reduces placental zinc transport and induces fetal growth restriction in mice

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ABSTRACT

Cadmium (Cd) is linked with increased risk of fetal growth restriction (FGR). Nevertheless, the mechanism remains unknown. This study established a mouse model of Cd-induced FGR through two exposure methods. Pregnant mice were either administered with $CdCl_2$ (5, 50 and 250 ppm) throughout pregnancy through drinking water or intraperitoneally injected with $CdCl_2$ (4.5 mg/kg) on GD9. As expected, fetal weight and crown-rump length were reduced in a gender-independent manner. Interestingly, *Mt1* and *Mt2*, two metallothionein genes, were up-regulated in maternal liver. Correspondingly, Cd accumulated mainly in maternal liver and kidney, and only trace amounts of Cd could pass from dam to placentas and fetuses. Further analysis showed that placental Zn concentration was elevated. Conversely, embryonic Zn concentration was reduced. Moreover, placental *Znt1* and *Znt2*, two zinc transporters, were down-regulated in Cd-exposed mice. These results suggest that maternal Cd exposure during pregnancy reduces placental Zn transport and induces fetal growth restriction.

1. Introduction

Cadmium (Cd) is an important occupational and environmental toxicant. Workers in electroplating, pigments, paints, welding and Ni–Cd batteries are frequently exposed to Cd at significantly higher level than the general population [1]. The general population is usually exposed to a low dose of Cd via drinking water, food and cigarette smoking [2]. Epidemiological investigations showed that Cd level in blood and seminal plasma was associated with male infertility and poor semen quality in humans [3–7]. At a high dose, Cd induces germ cell apoptosis in testes [8–13]. In addition, Cd (2.0 mg/kg, i.p.) inhibits the synthesis of testosterone in mouse testis [14,15].

An epidemiological report demonstrated that maternal urinary Cd level during pregnancy was negatively associated with birth weight and head circumference [16]. According to another birth cohort study, Cd level in maternal blood was positively associated with fetal growth restriction (FGR) [17]. In mice, maternal Cd exposure in mid- gestation resulted in a relatively specific forelimb ectrodactyly [18–23]. Moreover, maternal Cd exposure in middle and late gestational age induced FGR in mice [15,24,25]. Nevertheless, the molecular mechanism for Cd-induced FGR remains obscure.

The main objective of this study was to explore the effects of maternal Cd exposure during pregnancy on placental Zn transport and its mechanism. We established a mouse model of Cd-induced FGR through two different exposure methods. We showed that maternal Cd exposure through drinking water or ip injection significantly reduced fetal weight and crown-rump length in a gender-independent manner. We demonstrated that Cd accumulated mainly in maternal liver and kidney, whereas only trace amounts of Cd were found in placenta and fetus. We found that maternal Cd exposure during pregnancy reduced placental zinc (Zn) transport from maternal circulation to the fetuses through down-regulating the expression of Zn transporters.

2. Methods

2.1. Chemicals and reagents

Cadmium chloride ($CdCl_2$) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, OH, USA). AMV RT kits and RNase-free

Abbreviations: Cd, cadmium; FAAS, flame atomic absorption spectroscopy; GFAAS, graphite furnace atomic absorption spectrometry; FGR, fetal growth restriction; MT, metallothionein; ZIPs, zinc iron permeases; Zn, zinc; ZnTs, zinc transporters.

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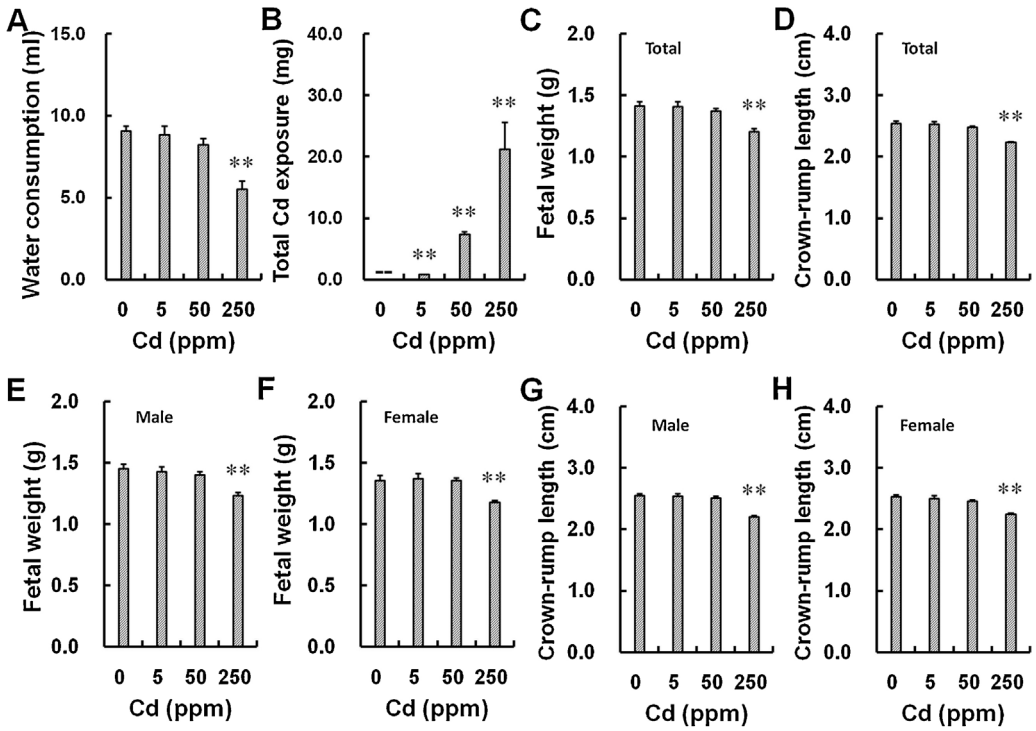

Fig. 1. Effects of maternal Cd exposure during pregnancy on maternal water consumption and fetal growth. Pregnant mice were administered with $CdCl_2$ (5, 50 and 250 ppm) throughout pregnancy through drinking water. All dams were sacrificed on GD18. Live fetuses were weighed and the crown-rump lengths were measured. (A) Maternal daily water consumption. (B) Total Cd exposure throughout pregnancy. (C) Fetal weight. (D) Crown-rump length. (E) The weight of male fetuses. (F) The weight of female fetuses. (G) The crown- rump length of male fetuses. (H) The crown-rump length of female fetuses. All data were expressed as mean ± SEM (n=10). $^{**}P < 0.01$ vs controls.

DNase were from Promega Co. (Madison, WI, USA). LightCycler® 480 SYBR Green I Master kit was from Roche Diagnostics Co. (Indianapolis, IN, USA). Ultrapure HNO_3 was from Aladdin Reagents Co., LTD (Shanghai, China). Matrix modifiers colloid palladium was from Xinda Measuring & Control Technology Co., Ltd (Colpd™, Chengdu, China).

2.2. Animals and experimental procedures

Adult CD-1 mice (8–10 week-old; male mice: 32–36 g; female mice: 26–28 g) were purchased from Beijing Vital River (Beijing, China) whose foundation colonies were all introduced from Charles River Laboratories, Inc. All mice were allowed free access to food (Beijing Keao Xieli Feed Co., LTD., Beijing 100107) and ultrapure 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 a period of one week before use. For mating purposes, four females were housed overnight with two males starting at 21:00. Females were checked at 7:00 am the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. Four pregnant mice were housed per cage. The present study consisted of two independent experiments. *Experiment 1.* Forty pregnant mice were randomly divided into four groups. All pregnant mice were administered with different concentrations of $CdCl_2$ (0, 5, 50 and 250 ppm, dissolved in ultrapure water) through drinking water throughout pregnancy. The dose of $CdCl_2$ used in the current study referred to a previous study with minor revision [26]. Maternal toxicity was assessed according to maternal weight and general signs. Maternal water consumption was measured. All dams were sacrificed on GD18. The uterine horns were incised and weighed. Live fetuses were counted. The gender of fetal mice was determined by anogenital distance (AGD). Male and female fetuses per litter were weighed. Crown-rump length was measured. *Experiment 2.* Sixty pregnant mice were randomly divided into two groups. In the Cd-treated group, pregnant mice were intraperitoneally (i.p.) injected with $CdCl_2$ (4.5 mg/kg) on GD 9. Normal saline-treated pregnant mice served as controls. The dose of $CdCl_2$ used in the current study referred to our previous study [23]. Forty dams were sacrificed at different time points (0, 2, 12 and 24 h) after Cd injection. Maternal serum, maternal liver, maternal kidney, placenta and embryo were collected and stored at −80 °C for measurement of Cd and Zn. Twenty dams were sacrificed on GD18. The uterine horns were exposed and weighted. Live and dead fetuses were counted. Live fetuses were weighed. Maternal serum, maternal liver, maternal kidney, placenta, fetal serum and fetal liver were collected and stored at −80 °C for measurement of Zn and Cd. Maternal liver and placenta were collected for real-time RT-PCR. Placental cross sections were stained with Hematoxylin & Eosin (H&E). The present study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University. All animal experimental procedures were performed in accordance with the guidelines for humane treatments established by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

2.3. Cd measurement

The levels of Cd in maternal serum, maternal liver, maternal kidney, placenta, embryo, fetal serum and fetal liver were measured by graphite furnace atomic absorption spectrometry (GFAAS; model: TAS-990; Purkinje General Instrument Co., Ltd, Beijing, China) coupled with a deuterium-lamp background correction system. All samples were prepared and analyzed according to a slightly modified method as previously described [15]. For serum samples, maternal serum and fetal serum were diluted with 1% HNO_3 according to 1:4 (v/v). Matrix modifiers colloid palladium were added to each standard, blank and sample dilution. For tissue sam-

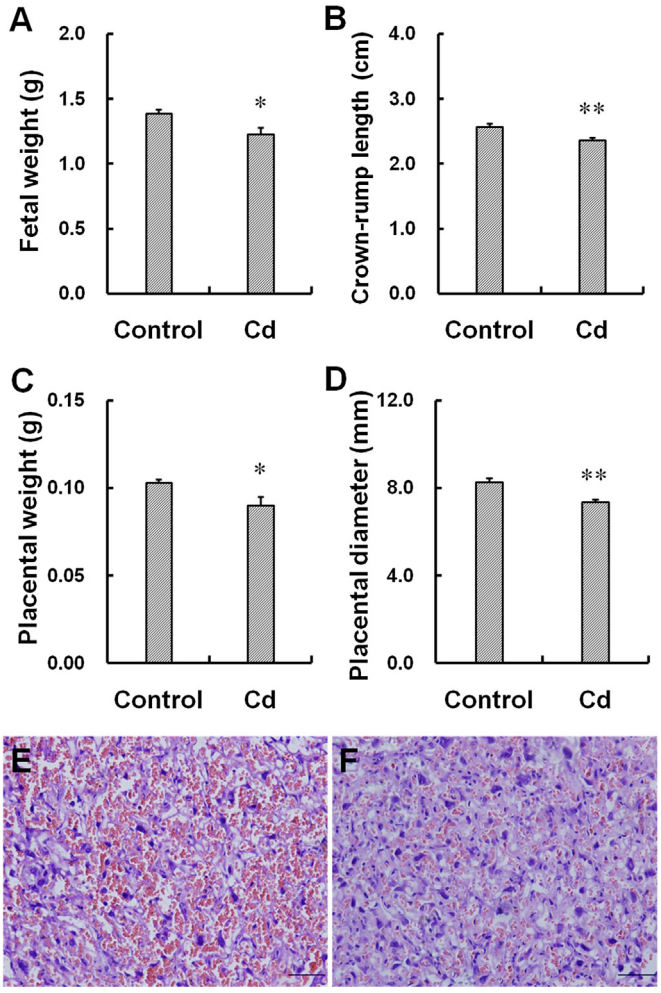

Fig. 2. Effects of maternal Cd exposure at middle gestational stage on placental and fetal development. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. All dams were sacrificed on GD18. (A-D) Live fetuses and placentas were weighed. The crown-rump length and the placenta diameter were measured. (A) Fetal weight. (B) Crown-rump length. (C) Placental weight. (D) Placental diameter. All data were expressed as mean ± SEM (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the control. (E and F) Four placentas from four pregnant mice in each group were fixed using 4%PFA. Placental cross sections were stained with H&E. (E) Representative section from controls. (F) Representative section from the Cd-treated mice. Original magnification: 400 ×. Scare bars: 50 μm.

ples (maternal liver, maternal kidney, placenta, embryo and fetal liver), 100–200 mg of samples were accurately weighed in a digestion tube, a 3 ml freshly prepared mixture of HNO_3 and H_2O_2 was added to each tube, and the solutions were kept at room temperature for 12 h, the clear transparent digests were obtained. After this period, the mixture was boiled nearly to dryness, and the residue was re-dissolved with 1.0% HNO_3. The following solution was then analyzed using GFAAS by taking 10 μl of the solution. Each sample was analyzed in triplicate. The limit of detection was 0.01 μg/L. To avoid exogenous Cd contamination, all polypropylene tubes and pipette tips (Free DNase and RNase, and Sterile; Sangon Biotech Co., Ltd., Shanghai, China) were soaked overnight in 10% ultrapure HNO_3 at room temperature and then rinsed in deionized water.

2.4. Zn measurement

The levels of Zn in maternal serum, maternal liver, placenta, embryo, fetal serum and fetal liver were measured by flame atomic absorption spectroscopy (FAAS). For serum samples, maternal serum and fetal serum were diluted with 1% HNO_3 according to 1:35 (v/v). For tissue samples (maternal livers, placentas, embryos and fetal livers), 100–200 mg of samples were accurately weighed in a digestion tube, a 3 ml freshly prepared mixture of HNO_3 and H_2O_2 was added to each tube, and the solutions were kept at room temperature for 12 h, the clear transparent digests were obtained. After this period, the mixture was boiled nearly to dryness, and the residue was re-dissolved with 1.0% HNO_3. The diluted solution was then analyzed using FAAS. Each sample was analyzed in triplicate. The detection limit of this method was 0.2 μg/dL.

2.5. Quantitative RT-PCR

Total RNA from maternal liver and placenta was extracted using TRI reagent and treated with RNase-free DNase for 30 min at 37 °C according to protocols supplied by the manufacturers. After heat inactivation of DNase, 2 μg of purified RNA was reverse-transcribed with AMV reverse transcriptase according to the manufacturer's supplied protocols. Real-time PCR was performed with a LightCycler® 480 SYBR Green I Master kit using gene-specific primers as listed in Table S1. The amplification reactions were carried out on a LightCycler® 480 instrument (Roche Diagnostics GmbH, Indianapolis, IN, USA) with an initial hold step (95 °C for 5 min) and 50 cycles of a three-step PCR (95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s). The comparative C_T method was used to determine the amount of target, normalized to an endogenous reference (*Gapdh*) and relative to a calibrator ($2^{-\Delta\Delta Ct}$) using the Lightcycler® 480 software (Roche, version 1.5.0).

2.6. Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M.), and sample sizes are presented in figure legends. All statistical analyses were performed using SPSS 16.0 software. The normalcy of data distributions was evaluated using one-sample Kolmogorov-Smirnov test. Differences between two groups were analyzed by means of a Student's *t*-test. Differences among multiple groups were analyzed using *ANOVA* and the following *Bonferroni's* or *Tamhane's T2 post hoc* test. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Pregnant outcomes and fetal development

Effects of maternal Cd exposure through drinking water on maternal water consumption were analyzed. Although maternal low Cd exposure (≤50 ppm) did not influence maternal daily water consumption, maternal high Cd exposure (250 ppm) significantly reduced maternal daily water consumption (Fig. 1A). Total Cd exposure level increased gradually with an increase of Cd concentration in drinking water (Fig. 1B). No signs of maternal toxicity were observed in dams exposed to Cd through drinking water. There was no significant effect of Cd on maternal weight gain (Table S2). Moreover, no dead fetus was observed among all groups. Effects of maternal Cd exposure through drinking water on fetal growth were then analyzed. Fetal weight was decreased in high-Cd (250 ppm) group (Fig. 1C). In addition, crown-rump length was reduced in high-Cd (250 ppm) group (Fig. 1D). Effects of maternal Cd exposure through drinking water on fetal weight and crown-rump length were analyzed in different genders. As shown in Figs. 1E–H, maternal Cd exposure through drinking water significantly reduces fetal weight and crown-rump length in a gender-independent manner.

Effects of maternal Cd exposure through i.p. injection on fetal growth were then analyzed. A single dose of $CdCl_2$ did not affect body weight gain of the pregnant mice (Table S2). No signs of maternal toxicity were observed in dams ip exposed to Cd. Moreover,

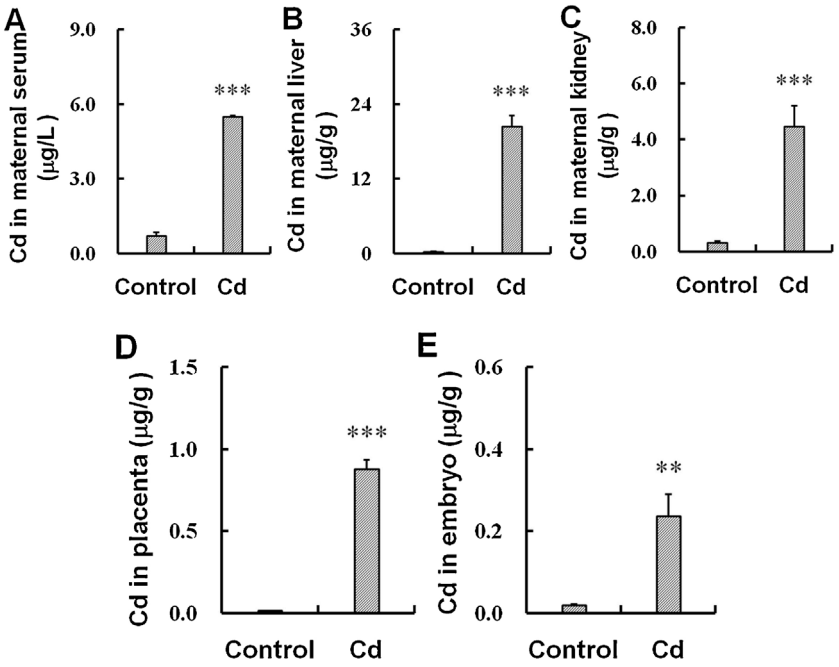

Fig. 3. Short-term Cd distribution in mother, placenta and fetus. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal serum, maternal liver, maternal kidney, placenta and embryo were collected 24 h after maternal Cd exposure. Cd content was measured by GFAAS. (A) Cd content in maternal serum. (B) Cd content in maternal liver. (C) Cd content in maternal kidney. (D) Cd content in placenta. Four placentas per litter were pooled for each sample. (E) Cd content in embryo. All embryos per litter were pooled for each sample. Data were expressed as mean ± SEM (n = 10). $^{**}P < 0.01$, $^{***}P < 0.001$ vs controls.

no significant difference on number of dead fetuses per litter and live fetuses per litter was observed between Cd-treated mice and controls (Table S2). Fetal weight was decreased in Cd-treated mice (Fig. 2A). Moreover, crown-rump length was reduced in Cd-treated mice (Fig. 2B). Effects of maternal Cd exposure on placental development were also analyzed. As shown in Figs. 2C and D, placental weight and diameter were significantly reduced in Cd-treated mice. Histopathology showed that the internal space of fetal and maternal blood vessels was reduced in placentas of Cd-treated mice (Figs. 2E and F).

3.2. Cd distribution in mother, placenta and fetus

To investigate the short-term effects of maternal Cd exposure on Cd distribution in mother, placenta and fetus, the levels of Cd in maternal serum, maternal liver, maternal kidney, placenta and embryo were measured at 24 h after maternal Cd injection. The level of Cd in maternal serum was increased at 24 h after Cd injection (Fig. 3A). Cd content in maternal liver and maternal kidney was increased at 24 h after Cd injection (Figs. 3B and C). Although placental Cd content was elevated by more than 60-fold, placental Cd content was only 4.3% of maternal liver and 19.7% of maternal kidney at 24 h after maternal Cd injection (Figs. 3B–D). Moreover, embryonic Cd content was increased by more than 11-fold, embryonic Cd content was only 1.2% of maternal liver, 5.3% of maternal kidney, and 26.9% of the placenta at 24 h after maternal Cd injection (Figs. 3B–E).

To investigate the long-term effects of maternal Cd exposure on Cd distribution in mother, placenta and fetus, the levels of Cd in maternal serum, maternal liver, maternal kidney and placenta were first measured on GD18 in mice ip exposed to Cd on GD9. As shown in Figs. 4A–C, the levels of Cd in maternal serum, maternal liver and maternal kidney remained elevated on GD18. Although placental Cd content remained elevated on GD18, placental Cd content was only 2.4% of maternal liver and 6.1% of maternal kidney in Cd-exposed mice (Fig. 4D). The effect of maternal Cd exposure on the level of Cd in fetal serum and fetal liver was further analyzed. As shown in Fig. 4E, no significant difference in fetal serum Cd level was observed between the two groups. Although Cd content in fetal liver was increased by 4.9-fold in Cd- exposed mice, Cd content in fetal liver was only 0.26% of maternal liver (Fig. 4F).

3.3. Expression of metallothionein 1 (Mt1) and Mt2 in maternal liver and placenta

The short-term effects of maternal Cd exposure on *Mt1* and *Mt2* mRNA in maternal liver and placenta were analyzed. As shown in Figs. 5A and B, *Mt1* and *Mt2* mRNA in maternal liver were up-regulated at 2 h after maternal Cd exposure and remained elevated at 24 h after maternal Cd exposure. However, there was no significant difference on placental *Mt1* mRNA among different time points after Cd treatment (Fig. 5C). As shown in Fig. 5D, placental *Mt2* mRNA was slightly up-regulated at 24 h after maternal Cd injection. To investigate the long-term effects of maternal Cd exposure on *Mt1* and *Mt2* expression, *Mt1* and *Mt2* mRNA in maternal liver and placenta were then measured on GD18. Although no significant difference on *Mt1* mRNA in maternal liver was observed between two groups (Fig. 6A), *Mt2* mRNA in maternal liver remained elevated in Cd-treated mice (Fig. 6B). There was no significant difference on placental *Mt1* and *Mt2* mRNA between two groups (Figs. 6C and D).

3.4. Zn levels in maternal serum, maternal liver, placenta, embryo, fetal serum and fetal liver

The levels of Zn in maternal liver and maternal serum were measured at 24 h after maternal Cd exposure (GD10). As shown in Fig. 7A, Zn level in maternal serum was reduced at 24 h after Cd injection. There was no difference in hepatic Zn concentration between Cd-treated dams and controls (Fig. 7B). The levels of Zn in placenta and embryo were then measured at 24 h after maternal Cd exposure (GD10). As shown in Fig. 7C, placental Zn level was

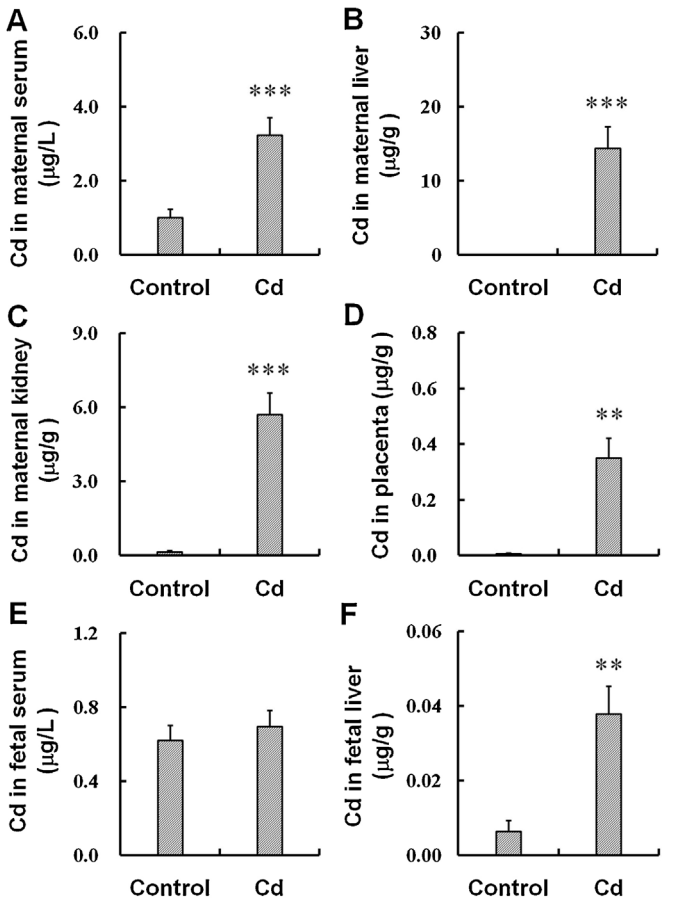

Fig. 4. Long-term Cd distribution in mother, placenta and fetus. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal serum, maternal liver, maternal kidney, placenta, fetal serum and fetal liver were collected on GD 18. Cd content was measured by GFAAS. (A) Cd content in maternal serum. (B) Cd content in maternal liver. (C) Cd content in maternal kidney. (D) Cd content in placenta. Two placentas per litter were pooled for each sample. (E) Cd content in fetal serum. All fetal sera per litter were pooled for each sample. (F) Cd content in fetal liver. Two fetal livers per litter were pooled for each sample. Data were expressed as mean ± SEM (n = 10). $^{**}P < 0.01$, $^{***}P < 0.001$ vs controls.

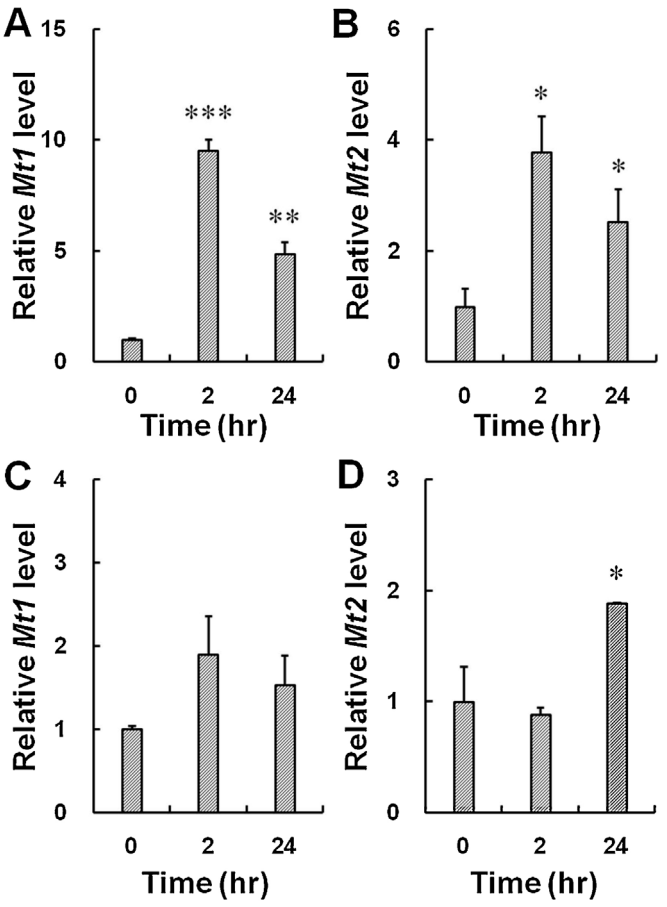

Fig. 5. Short-term effects of maternal Cd exposure on the expression of *Mt1* and *Mt2* in maternal liver and placenta. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal liver and placenta were collected at 0, 2 and 24 h after maternal Cd exposure. *Mt1* and *Mt2* mRNAs were detected using real-time RT-PCR. (A) *Mt1* in maternal liver. (B) *Mt2* in maternal liver. (C) Mt1 in placenta. (D) *Mt2* in placenta. Six samples were randomly chosen from six different litters. Data were expressed as mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs controls.

increased at 24 h after maternal Cd injection. Embryonic Zn level was decreased at 24 h after maternal Cd injection (Fig. 7D).

To investigate the long-term effects of maternal Cd exposure on Zn level in mother, Zn levels in maternal liver and maternal serum were measured on GD18. Although no significant difference in Zn level of maternal serum was observed between two groups (Fig. 8A), Zn level in maternal liver remained elevated on GD18 (Fig. 8B). The levels of Zn in placenta, fetal serum and fetal liver were then measured on GD18. As shown in Figs. 8C and D, there was no significant difference in Zn levels of placenta, fetal serum and fetal liver between two groups.

3.5. *Expression of zinc transporters (*ZnTs*) and zinc iron permeases (*ZIPs*) in placenta*

The expression of placental ZnTs and ZIPs was analyzed at 24 h after maternal Cd injection (GD10). As shown in Fig. 9A, placental *Znt1* mRNA was down-regulated at 12 h after maternal Cd injection. Moreover, placental *Znt2* mRNA was down-regulated at 12 h after maternal Cd injection and remained decreased at 24 h after maternal Cd injection (Fig. 9B). There was no significant difference on placental *Znt5*, *Zip1 and Zip4* mRNA among different time points after Cd treatment (Figs. 9C–E). The expression of placental ZnTs and ZIPs was further analyzed on GD18. In the Cd group, placental *Znt2* mRNA level remained reduced on GD18 (Fig. 10B). There was no significant difference on placental *Znt1*, *Znt5*, *Zip1 and Zip4* between the two groups (Figs. 10A,C–E).

4. Discussion

Epidemiological investigations demonstrated that maternal Cd exposure during pregnancy elevated the risks of small for gestational age (SGA) and low birth weight (LBW) infants [17,27]. The present study investigated Cd-induced FGR through two different exposure methods. We showed that pregnant mice injected with a single dose of $CdCl_2$ in mid- gestation led to FGR in mice. Moreover, maternal exposure to Cd (250 ppm) in drinking water throughout pregnancy reduced fetal weight and crown-rump length. These results are in agreement with an earlier report, in which maternal Cd exposure through drinking water significantly reduced fetal weight in rats [26]. According to a prospective cohort study, there was a gender difference in the association between maternal Cd exposure and birth size, which was apparent only in girls [16]. The present study analyzed whether maternal Cd exposure throughout pregnancy impairs fetal growth in a gender-dependent manner. Our results showed that no significant difference on the adverse effects of maternal Cd exposure on fetal weight and crown- rump length was observed between male and female fetuses. This result

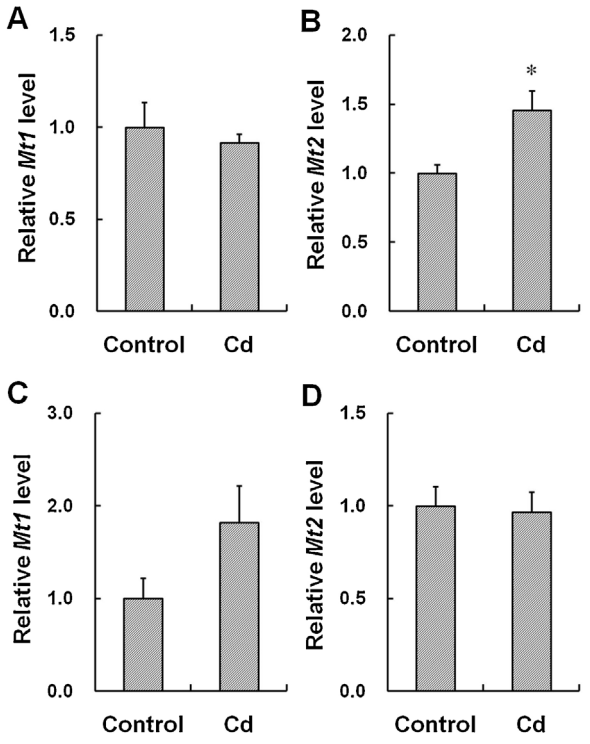

Fig. 6. Long-term effects of maternal Cd exposure on the expression of *Mt1* and *Mt2* in maternal liver and placenta. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal liver and placenta were collected on GD18. *Mt1* and *Mt2* mRNAs were detected using real-time RT-PCR. (A) *Mt1* in maternal liver. (B) *Mt2* in maternal liver. (C) *Mt1* in placenta. (C) *Mt2* in placenta. Six samples were randomly chosen from six different litters. Data were expressed as mean ± SEM (n = 6). $^*P < 0.05$ vs controls.

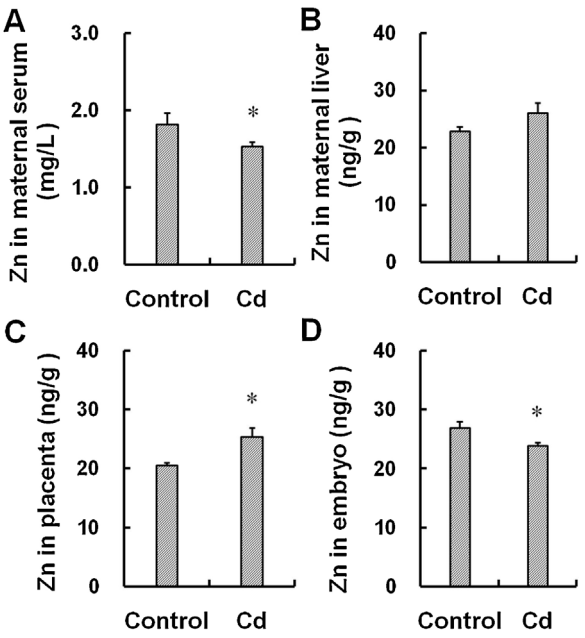

Fig. 7. Short-term effects of maternal Cd exposure on Zn content in maternal serum, maternal liver, placenta and embryo. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal serum, maternal liver, placenta and embryo were collected 24 h after maternal Cd exposure. Zn content was measured using FAAS. (A) Zn content in maternal serum. (B) Zn content in maternal liver. (C) Zn content in placenta. Four placentas per litter were pooled for each sample. (D) Zn content in embryo. All embryos per litter were pooled for each sample. Data were expressed as mean ± SEM (n = 10). $^*P < 0.05$ vs controls.

suggests that maternal Cd exposure throughout pregnancy through drinking water induces FGR in a gender-independent manner.

It remains controversial whether Cd can pass the placental barrier. Several reports showed that the placenta prevented most Cd from passing from maternal circulation to the fetuses [15,28]. To explore Cd distribution in mother, placenta and fetus, the present study analyzed Cd content in maternal serum, maternal liver, maternal kidney, placenta and embryo at 24 h after maternal Cd exposure. We showed that most Cd rapidly accumulated in maternal liver and kidney. Placental Cd content was only 4.3% of maternal liver and 19.7% of maternal kidney. Moreover, embryonic Cd content was only 1.2% of maternal liver, 5.3% of maternal kidney, and 26.9% of placenta. To investigate the long-term distribution of Cd in mother, placenta and fetus, we analyzed Cd content in maternal serum, maternal liver, maternal kidney, placenta, fetal serum and fetal liver on GD18. Cd content in maternal liver and kidney remained elevated on GD18. By contrast, placental Cd content was only 2.4% of maternal liver and 6.1% of maternal kidney. Moreover, Cd content in fetal liver was about 0.26% of maternal liver. These results suggest that most Cd is sequestered mainly in maternal liver and kidney, and only trace amounts of Cd can pass from maternal circulation to placenta and fetus. Therefore, it is unlikely that Cd-induced FGR can be completely attributed to its direct toxic effect on the fetus.

The mechanism through which most Cd is mainly sequestered in maternal liver remains obscure. MT-I and MT-II, the major forms of mouse MTs, are key players in the detoxification of Cd [29]. According to an early study, MT-I/II over-expression results in an increased resistance to Cd-induced toxic effects [30]. By contrast, MT-I/II-deficient mice display an increased susceptibility to Cd-induced FGR [25]. Thus, we hypothesize that MTs sequester Cd in maternal liver and reduce its adverse effects on the fetus. To test this hypothesis, the present study analyzed the expression of *Mt1* and *Mt2 in maternal liver and placenta. As expected, Mt1* and *Mt2* mRNAs in maternal liver were rapidly up-regulated at 2 h after maternal Cd exposure and remained elevated at 24 h after maternal Cd exposure. Unexpectedly, there was no significant difference on placental *Mt1* mRNA between Cd-exposed mice and controls. Moreover, the level of placental *Mt2* mRNA was elevated at 24 h after maternal Cd exposure. These results explain why most Cd is mainly sequestered in maternal liver with only a low level of Cd reaching the placenta.

According to an early epidemiological report, there was a negative association between maternal zinc level during pregnancy and birth weight [31]. A large population-based birth cohort study from our laboratory demonstrated that maternal zinc deficiency elevated the risks of FGR [32]. Indeed, pretreatment with Zn protected against Cd-induced embryonic death and teratogenic effects in mouse and chick embryos [33,34]. Moreover, pretreatment with Zn significantly ameliorated Cd-activated apoptosis in embryonic cells and prevented embryonic growth restriction in mice [35]. The present study investigated whether maternal Cd exposure disturbed Zn metabolism in mother, placenta and fetus. Although Zn level in maternal serum was reduced in Cd-exposed mice, maternal Cd exposure significantly elevated placental Zn content. On the contrary, maternal Cd exposure reduced embryonic Zn content. These results suggest that maternal Cd exposure during pregnancy reduces placental Zn transport.

Zn transporters (ZnTs) and Zinc Iron Permeases (ZIPs), two Zn transporter families, play key roles in Zn mobilization of influx, efflux, compartmentalization or sequestration across biological membranes [36]. ZnTs and ZIPs are highly expressed in mouse placentas and rat trophoblast cells [37]. An early study found that mRNA and protein levels of ZIP8 were significantly down-regulated in Cd-resistant MT-null mouse fibroblast cells [38]. The present study showed that mRNA levels of placental *Znt1* and *Znt2* were down-regulated at 12 h after maternal Cd exposure, which was

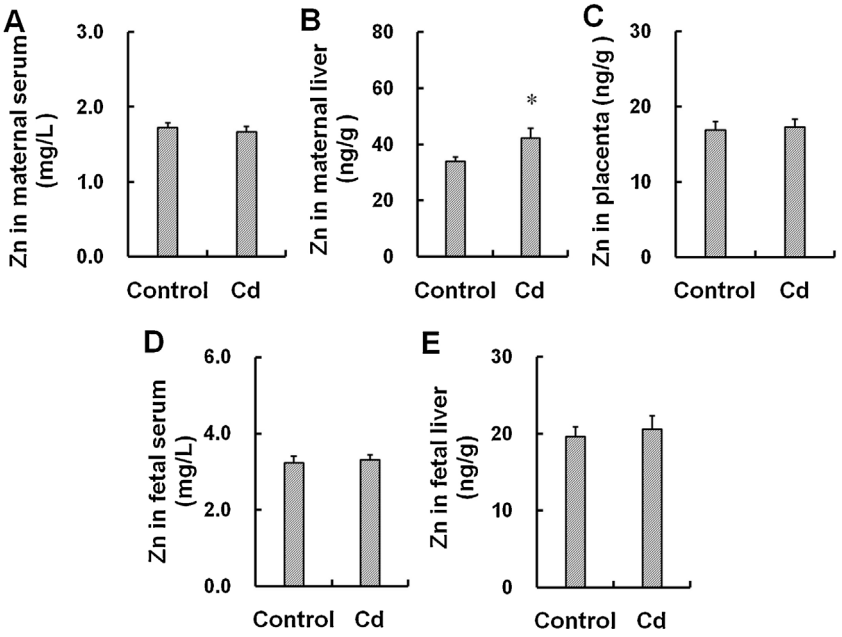

Fig. 8. Long-term effects of maternal Cd exposure on Zn content in maternal serum, maternal liver, placenta and embryo. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Maternal serum, maternal liver, placenta, fetal serum and fetal liver were collected on GD 18. Zn content was measured using FAAS. (A) Zn content in maternal serum. (B) Zn content in maternal liver. (C) Zn content in placenta. Two placentas per litter were pooled for each sample. (D) Zn content in fetal serum. All fetal sera per litter were pooled for each sample. (E) Zn content in fetal liver. Two fetal livers per litter were pooled for each sample. Data were expressed as mean ± SEM (n = 10). $^{*}P < 0.05$ vs controls.

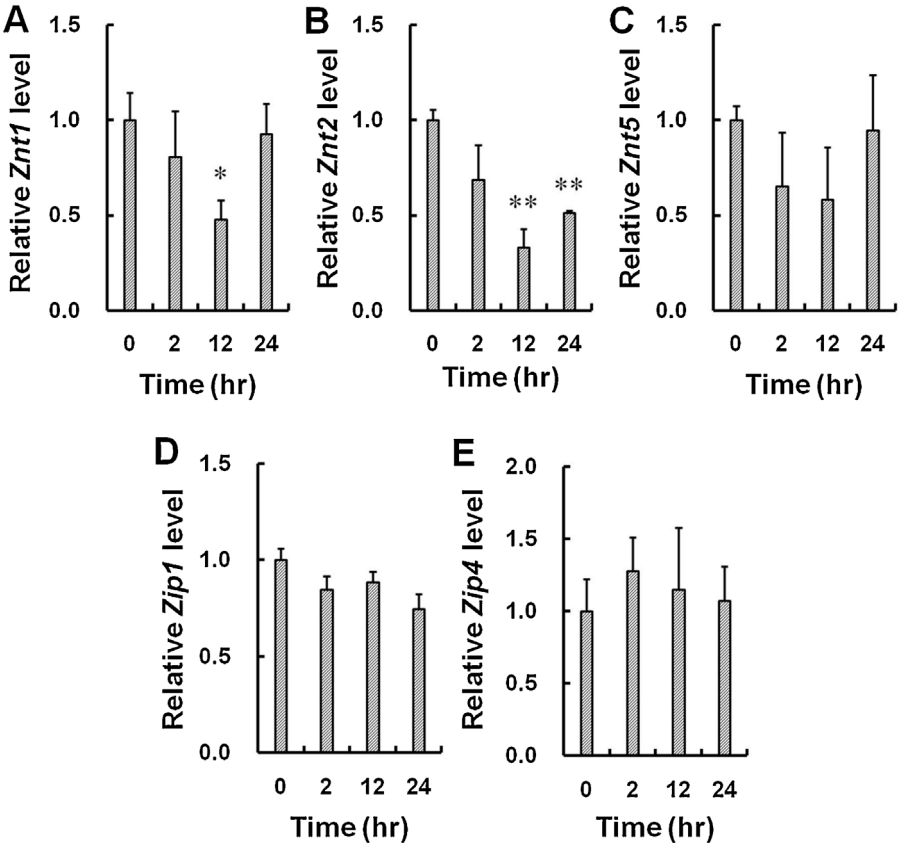

Fig. 9. Short-term effects of maternal Cd exposure on the expression of placental ZnTs and ZIPs. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Mouse placentas were collected at 0, 2, 12 and 24 h after maternal Cd exposure. *Znts* and *Zips* mRNAs were detected using real-time RT-PCR. (A) *Znt1*. (B) *Znt2*. (C) *Znt5*. (D) *Zip1*. (E) *Zip4*. Six placentas were randomly chosen from six different litters. Data were expressed as mean ± SEM (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ vs controls.

in agreement with the reduction of embryonic Zn content in Cd-exposed mice. These results suggest that maternal Cd exposure reduces Zn transport from maternal circulation to the fetus through

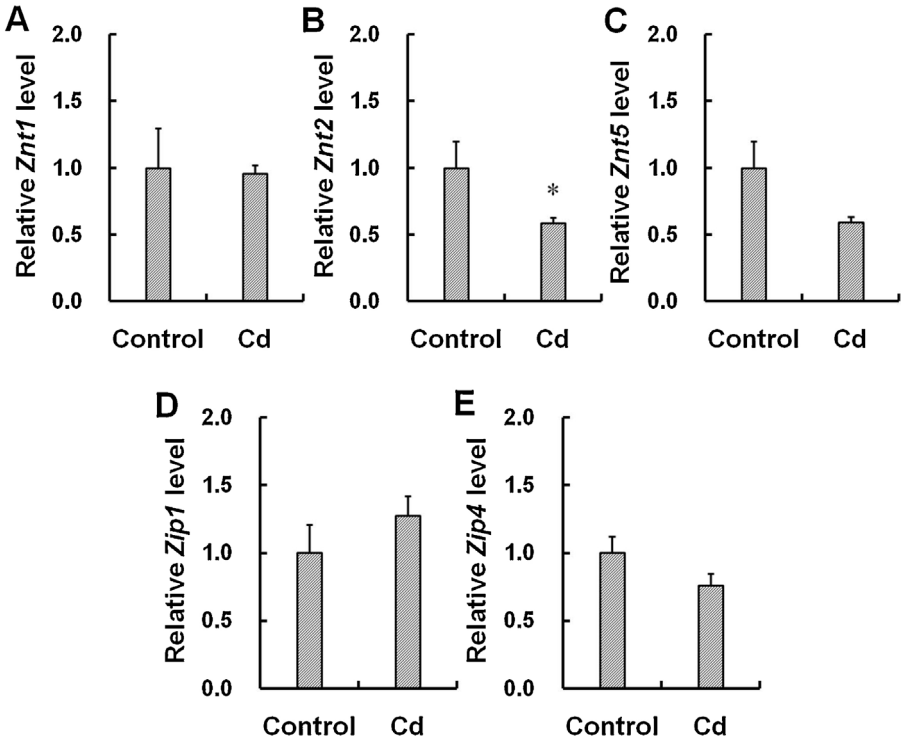

Fig. 10. Long-term effects of maternal Cd exposure on the expression of placental ZnTs and ZIPs. The pregnant mice were i.p. injected with $CdCl_2$ (4.5 mg/kg) on GD9. Mouse placentas were collected on GD 18. *Znts* and *Zips* mRNAs were detected using real-time RT-PCR. (A) *Znt1*. (B) *Znt2*. (C) *Znt5*. (D) *Zip1*. (E) *Zip4*. Six placentas were randomly chosen from six different litters. Data were expressed as mean ± SEM (n = 6). $^*P < 0.05$ vs controls.

down- regulating the expression of Zn transporters in mouse placenta.

In the current study, we mainly explored the effects of maternal Cd exposure during pregnancy on fetal growth and placental Zn transport. This study has several limitations. First, the present study did not investigate the effects maternal Cd exposure in drinking water on placental Zn transport. Second, the present study did not investigate the effects maternal Cd exposure in drinking water on Cd distribution in mothers, placenta and fetus. Third, the present study did not explore the mechanism through which Cd down-regulated placental Zn transporters. Additional work is required to explore the effect of maternal Cd exposure in drinking water on Cd distribution, placental Zn transport and its mechanism.

In summary, the present study established a mouse model of Cd-induced FGR through two different exposure methods. We showed that maternal Cd exposure through drinking water and ip injection significantly reduced fetal weight and crown-rump length in a gender-independent manner. We demonstrated that Cd accumulated mainly in maternal liver and kidney, whereas only trace amounts of Cd were found in placenta and fetus. We found that maternal Cd exposure during pregnancy reduced placental Zn transport from maternal circulation to the fetuses through down-regulating Zn transporters such as Znt1 and Znt2.

Competing interest

All authors declare no competing financial interest.

Author contributions

Hua Wang and Ying Wang contributed equally to this work. De-Xiang Xu and Hua Wang designed the research; Hua Wang, Ying Wang, Qing-Li Bo, Yan-Li Ji, Lu Liu, Yong-Fang Hu, Jun Zhang and Ling-Li Zhao conducted the research, Hua Wang and Ying Wang analyzed data; De-Xiang Xu and Hua Wang wrote this manuscript. All authors have read and approved the final version of the manuscript.

Transparency document

The transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2016.06.010.

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