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Cadmium-induced neural tube defects and fetal growth restriction: Association with disturbance of placental folate transport

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ABSTRACT

Previous studies found that maternal Cd exposure on gestational day (GD)9 caused forelimb ectrodactyly and tail deformity, the characteristic malformations. The aim of the present study was to investigate whether maternal Cd exposure on GD8 induces fetal neural tube defects (NTDs). Pregnant mice were intraperitoneally injected with $CdCl_2$ (2.5 or 5.0 mg/kg) on GD8. Neither forelimb ectrodactyly nor tail deformity was observed in mice injected with $CdCl_2$ on GD8. Instead, maternal Cd exposure on GD8 resulted in the incidence of NTDs. Moreover, maternal Cd exposure on GD8 resulted in fetal growth restriction. In addition, maternal Cd exposure on GD8 reduced placental weight and diameter. The internal space of maternal and fetal blood vessels in the labyrinth layer was decreased in the placentas of mice treated with $CdCl_2$. Additional experiment showed that placental PCFT protein and mRNA, a critical folate transporter, was persistently decreased when dams were injected with $CdCl_2$ on GD8. Correspondingly, embryonic folate content was markedly decreased in mice injected with $CdCl_2$ on GD8, whereas Cd had little effect on folate content in maternal serum. Taken together, these results suggest that maternal Cd exposure during organogenesis disturbs transport of folate from maternal circulation to the fetuses through down-regulating placental folate transporters.

1. Introduction

Cadmium (Cd) is one of major occupational and environmental toxicants. Cd is frequently used in electroplating, pigments, paints, welding, and Ni-Cd batteries, where workers are exposed to Cd at a higher level (Beveridge et al., 2010). On the other hand, the general population is exposed to a low level of Cd via drinking water, food and cigarette smoking (Honda et al., 2010). Several epidemiological data demonstrate that maternal Cd exposure during pregnancy is associated with fetal growth restriction (Kippler et al., 2012; Menai et al., 2012; Wang et al., 2016). Animal experiments indicate that Cd is a potent teratogen in rodent animals (Barr, 1973; Thompson and Bannigan, 2008). Several studies showed that maternal Cd exposure at early limb development caused forelimb ectrodactyly and tail deformity, the characteristic malformations in fetuses (Hovland et al., 1999; Scott et al., 2005; Paniagua-Castro et al., 2007; Robinson et al., 2009). In addition, maternal Cd exposure during pregnancy resulted in fetal growth restriction in rodent animals (Ahokas et al., 1980; Ji et al., 2011). Nevertheless, the mechanism by which maternal Cd exposure induces fetal malformation and growth retardation remains obscure.

Increasing evidence demonstrated that placenta could deter most of Cd from passing from dams to fetuses. According to several earlier reports, only <0.1% of Cd was passed from dams to fetuses when pregnant mice were exposed to tracer levels of ^{109}Cd in drinking water (Whelton et al., 1993; Brako et al., 2003). Recently, we observed no significant elevation of blood Cd level in fetuses whose mothers were exposed to Cd during late pregnant period (Ji et al., 2011). These results indicate that Cd-induced fetal malformation and growth restriction cannot be completely attributed to its direct toxic effect on the fetuses. Indeed, the placenta is essential for the growth and development of the fetuses. Several studies demonstrates that the defects in placental function result in fetal growth restriction or even malformation and fetal demise (Watson and Cross, 2005). Therefore, we hypothesize that Cd induces fetal malformation and growth restriction through impairing placental development and function.

Previous studies focused on the effect of maternal Cd exposure at early limb development on forelimb structure. However, the aim of the present study was to explore the effect of maternal Cd exposure at neural tube development on neural tube formation and its mechanism. We found that maternal Cd exposure on gestational day (GD) 8 resulted in the incidence of neural tube defects (NTDs) and fetal growth restriction in mice. We demonstrate for the first time that maternal Cd exposure during organogenesis disturbs transport of folate from maternal circulation to the fetuses through down-regulating placental folate transporters.

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2. Materials and methods

2.1. Chemicals and reagents

$CdCl_2$ was from Sigma Chemical Co. (St. Louis, MO). TRI reagent was from Molecular Research Center, Inc. (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise stated.

2.2. Animals and treatments

The ICR mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 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 and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 21:00 h. Females were checked by 7:00 h the next morning, and the presence of a vaginal plug was designated as gestational day (GD) 0. The present study consisted of three independent experiments. Experiment 1. To investigate Cd-induced neural tube defects in mice, pregnant mice were divided randomly into three groups. In Cd group, pregnant mice were intraperitoneally (i.p.) injected a single dose of $CdCl_2$ (2.5 or 5.0 mg/kg) between 08:00 and 09:00 h on GD8. The saline-treated pregnant mice served as controls. The doses of $CdCl_2$ used in the present study were determined by preliminary experiments. The critical period of neural tube development is on GD8. In order to establish the mouse model of Cd-induced NTDs, the time of Cd exposure was chosen on GD8. For mechanical studies, a single dose of Cd by intraperitoneal injection was chosen in the current study. All animals were inspected daily for clinical signs and determined whether a pregnancy loss had occurred according to clinical signs and maternal weight. The dams were sacrificed on GD18. The uterine horns were exposed and weighed. Live, dead and resorbed fetuses were counted. Placentas were collected for histological examination. Live fetuses were sexed, weighed, and examined for external morphological malformations. Experiment 2. To investigate the effects of maternal Cd exposure during pregnancy on placental folate transport, twenty-four pregnant mice were divided randomly into two groups. In Cd group, pregnant mice were ip. injected with a single dose of $CdCl_2$ (5.0 mg/kg) between 08:00 and 09:00 h on GD8. The saline-treated pregnant mice served as controls. Pregnant mice were sacrificed 24 h after Cd injection. Maternal serum and embryo were collected for measurement of folate contents. Experiment 3. To investigate the effects of maternal Cd exposure during pregnancy on the expression of placental folate transporters, forty-eight pregnant mice were divided randomly into eight groups. In Cd group, all pregnant mice were ip. injected with a single dose of $CdCl_2$ (5.0 mg/kg) between 08:00 and 09:00 h on GD8. The saline-treated pregnant mice served as controls. Cd-treated pregnant mice were sacrificed at different time points (2, 12, 24, 48 and 72 h) after Cd injection. Normal saline-treated pregnant mice were killed on GD8, GD9, GD10 and GD11. Placentas were collected for real-time RT-PCR and Western blotting. 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.

2.3. Histology examination

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 vascular space quantification according to the previous study (Neres et al., 2008). In each section, 5 fields were randomly selected in the labyrinthine region at magnification ×400. 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 labyrinth layer after noise removal. The blood sinusoids area in the labyrinthine region was estimated from the analysis of two nonconsecutive sections in each placenta. The coverage percentage 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 reported results in the present study represent the average results for six placentas from six pregnant mice in each group.

2.4. Isolation of total RNA and real-time RT-PCR

Total RNA was extracted using TRI reagent (MRC, Inc.). RNase-free DNase-treated total RNA (1.0 μg) was reverse-transcribed with AMV (Pregmega). Real-time RT-PCR was performed with a LightCycler® 480 SYBR Green I kit (Roche Diagnostics GmbH) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler® 480 Instrument (Roche Diagnostics GmbH) 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, 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).

2.5. Western blotting

Mouse placentas were homogenized in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 1 mM phenylmethylsulfonyl fluoride. The homogenates were then centrifuged at 15,000*g* for 15 min. Supernatants from each sample were added to a gel loading buffer (100 mM Tris, pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 0.03% bromophenol blue) and boiled for 10 min. Proteins (20 μg per sample) in loading buffer were subjected to electrophoresis in 12.5% SDS-polyacrylamide gel (PAGE) for 3 h. The gel was transferred electrophoretically onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore powdered milk in Dulbecco's PBS (DPBS)). The membranes were blocked by non-fat milk for 2 h at room temperature, and then incubated with primary antibodies PCFT or β-actin for 2 h at room temperature. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each and PBS for 10 min once, the membranes were incubated with goat anti-rabbit or goat anti-mouse IgG antibody for 1.5 h at room temperature. The membranes were then washed four times in DPBS containing 0.05% Tween-20 for 10 min each and PBS for 10 min once, followed by signal development using an enhanced chemiluminescence (ECL) detection kit from Pierce (Pierce Biotechnology, Rockford, IL, USA).

2.6. Measurement of folate

Maternal sera and embryos on GD9 were collected. Maternal serum was centrifuged at 4000 rpm for 10 min and stored at −80 °C until the

Table 1
Primers for real-time RT-PCR.

Gene	Sequences	Product length
Gapdh	Forward:5′-ACCCCAGCAAGGACACTGAGCAAG-3′ Reverse:5′-GGCCCCTCCTGTTATTATGGGGGT-3′	109
Pcft	Forward: 5′-CTACCCTACCTCACCAGCCT-3′ Reverse: 5′-GCAAACGCAAAGACCACCAT-3′	119
Rfc-1	Forward: 5′-TGGGTGTTGTAGTCTGCGTG-3′ Reverse: 5′-CACTCCACCTTGCACTACCC-3′	114
Frα	Forward: 5′-GTGGAGACAAAGAAGCCCGA-3′ Reverse: 5′-CTCCACTCCCTGCTTAGGGT-3′	104