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Mother-fetus transference of lead and cadmium in rats: involvement of metallothionein

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Summary. This study was designed to assess the effect of Cadmium (Cd) and lead (Pb) exposure during pregnancy in rats and their correlation with metallothionein (MT). Rats were exposed to either 10 ppm Cd or 300 ppm Pb through drinking water during pregnancy. Both metals were measured in placenta, fetus brain and fetal and maternal blood. MT was quantified in placenta and fetus brain and it was also observed in placenta by immunohistochemical technique. Offspring weight was found to be significantly lower for the Cd exposure group than for the control group. A Cd increase in the placenta of the exposed group was accompanied by MT induction; these effects were related to a limited accumulation of Cd in fetus brain. In contrast, dam Pb exposure caused an accumulation of Pb in the fetus brain and induced damage to placenta. The results account for differences in the transference of these metals during pregnancy that could be related to their toxicity.

Key words: Cadmium, Lead, Placenta, Fetus, Transference, Metallothionein

Introduction

The placenta supplies the fetus with oxygen, water, carbohydrates, amino acids, lipids, vitamins and essential trace elements such as zinc (Zn), iron (Fe) and copper (Cu) (Shennan and Boyd, 1988; Gude et al., 2004). The placenta also protects the fetus from certain toxicants that could be present in maternal blood. Nevertheless, many small xenobiotics are able to cross the placenta by simple diffusion via transcellular or paracellular routes (Iyengar and Rapp, 2001; Gude et al., 2004). Female smokers increase their Cd body burden that, in the case of pregnancy, has been associated with increased Cd content in the placenta and with low birth weight (Galicia-García et al., 1997; Ronco et al., 2005; Jauniaux and Burton, 2007). In pregnant rats, Cd administration can be teratogenic or fetotoxic depending on the dose, chemical species and administration during gestational period (Salvatori et al., 2004). It has been observed that the placenta is a natural defense against Cd toxicity during pregnancy, because it acts as a barrier for Cd transfer from mother to fetus by sequestering its excess from the blood and minimizing its transfer to the fetus (Osman et al., 2000; Trottier et al., 2002; Ronco et al., 2005; Sorkun et al., 2007).

The primary targets for Pb toxicity include: cardiovascular, central nervous, gastrointestinal, renal, immunological and reproductive systems, as well as red blood cells (Carrington et al., 1993; Goyer, 1993). Epidemiological studies have shown that Pb exposure during pregnancy is associated with reduced growth during infancy and early childhood (Little et al., 1990; Osman et al., 2000). Further evidence from experimental animal studies shows that Pb exposure affects the brain of the developing fetus (Antonio et al., 2003). Experiments with Pb exposure during the late gestation in rats showed the entrance of Pb into trophoblasts through Ca^{2+} transport mechanisms and that this effect enhances the potential of interactions between Pb and calcium-binding-proteins expressed by undifferentiated trophoblasts (Evans et al., 2003). However, the placenta cannot prevent the fetus from exposure to Pb, as this metal is able to cross the placental barrier easily (Osman et al., 2000; Lafond et al., 2004). Metallothioneins

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(MTs) show high affinity for both essential and nonessential metals (Nordberg and Nordberg, 2000). The presence of MT during pregnancy has been demonstrated in human placenta and fetal membranes (Goyer et al., 1992; Sorkun et al., 2007). The metal binding characteristics, localization and inducibility of placental MT suggest that it may play a role in modulating the transfer of essential and non-essential metals, mainly Cd, from mother to fetus (Wade et al., 1986; Goyer and Cherian, 1992; Ronco et al., 2006; Sorkun et al., 2007). There are several studies that report the role of placental MT in restricting the transfer of Cd from mother to fetus (Goyer et al., 1992, Itoh et al., 1996; Peterson-Grawe and Oskarsson, 2000). However this hypothesis is not fully sustained, since it was demonstrated in studies using MT-null mice that MT is not the main barrier to transplacental Cd transport (Lau et al., 1998; Brako et al., 2003). The present study explores the implications of oral exposure to relatively low levels of Cd or Pb during pregnancy. The objectives were to determine the association of MT levels with Pb and Cd in placenta and to quantify and evaluate the immunohistochemical localization of MT in term placenta during pregnancy in rats. Results may help to better understand the differences between the toxicity mechanism of these metals during pregnancy.

Materials and methods

Chemicals

Cadmium chloride ($CdCl_2$) and lead acetate were obtained from J.T. Baker (México), all other reagents were from E. Merck (Mexico). Deionized water (Milli R/Q, Millipore, Milli-Q water system) was used for the preparation of all reagents and solutions.

Animals

Experiments were performed using female Wistar rats; NIH bred in-house strain, weighing 250-300 g. Animals were maintained in a room with standard conditions (light period 7:00 AM-7:00 PM, at 22-24°C, humidity was 40%) during all the experimental period. All animals were fed on a standard chow diet (Purine Chow) and given water *ad libitum*. Rats were housed individually in polycarbonate cages.

Treatment

Adult female rats were mated during the night with male rats from the same strain. The following morning, rats were examined, and those in which a sperm plug was found, were considered to be in day 0 of gestation. Pregnant rats were then sorted into experimental groups and exposed to the metals through drinking water with a solution containing either 10 ppm of Cd ($CdCl_2$), or 300 ppm of Pb acetate. The control group received deionized water. The treatment lasted 21 days (from day 1 to day 21 of pregnancy) for all the groups. On 21st day of gestation, the pregnant rats (7-9 animals per treatment per assay) were anaesthetized and blood was collected by cardiac puncture. The uterine horns were examined for the number of total implants and resorptions. The fetuses were sacrificed by decapitation, their brains were quickly removed and the fetus blood was collected and stored at 4°C, placentas and fetus brains were weighed. The tissue was stored at -80°C until analyzed for Pb, Cd or MT contents.

Microscopical and immunohistochemical study

The placenta was removed and fixed in 10% buffered formalin for at least 24 h. Tissue samples were processed separately in a Histokinette 200 apparatus (Reichert Jung). The sections were paraffin-embedded, cut into 5 µm slices and stained with hematoxylin-eosin; slices were examined under light microscope. Deparaffinized tissue sections were processed for immunohistochemical staining. In brief, sections were incubated with monoclonal antibody against MT I and II 1:100 (Metallothionein, DAKO, Carpinteria, CA). The sections were then incubated with a Biotin-conjugated secondary antibody and Streptavidin-Enzyme Conjugate (LSAB System HRP, DAKO, Carpinteria; CA).

The immune reaction resulted in the oxidation of the 3,3'-diaminobenzidine by peroxidase (Liquid DAB, DAKO Carpinteria CA) into an insoluble brown precipitate. The reaction sites were visualized as a brown staining of the tissue. Counterstaining with hematoxylin was performed after immunostaining.

Cadmium and lead determinations

Cd and Pb in blood, placenta and brain were analyzed by graphite furnace atomic absorption spectrophotometry (GFAAS), using a Perkin-Elmer 3110 spectrophotometer equipped with an HGA-600 furnace and AS-60 auto sampler according to the technique described by Stoeppler and Brandt (1980). Blood samples (200 µl) were added to 800 µl concentrated nitric acid (Merck), centrifuged at 15,000 rpm for 15 min, and a 100 µl aliquot was taken from clear solution for analyses. Tissue samples were digested in 1 ml of concentrated HNO_3. Calibration curves were constructed using an aqueous Cd reference standard (NBS-3108, National Bureau of Standards, Gaithersburg, MD).

Bovine blood standards with high, medium and low concentrations of Cd served as quality control for the Cd measurement. In the case of Pb, calibration curves were constructed using an aqueous Pb standard (3128, National Bureau of Standards, Gaithersburg, MD). The laboratory conducting blood Pb analysis participates in the blood proficiency program of the Center for Disease Control (CDC). All glassware was cleaned by soaking in nitric acid and rinsing several times in deionized water.

Metallothionein quantification

The content of MT was determined as described by Rojas and Rios (1997). Briefly, tissue samples of about 0.02 g were homogenized in 300 µl phosphate buffer (0.05 M), 0.375 M NaCl mixture (1.5:1 v/v). Then, 250 µl of silver nitrate solution (20 ppm) and 400 µl of glycine buffer (0.5 M, pH 8.5) were added. After 5 min, 100 µl of rat hemolyzed erythrocytes were added and the mixture was allowed to boil for 2 min, then it was centrifuged at 4,000 g for 5 min. The centrifugation was repeated twice. MT was estimated by measuring the silver content of the supernatant fractions (diluted 1:10 with 3% HNO_3 v/v) with an atomic absorption spectrophotometer (Perkin-Elmer 3110) equipped with a HGA-600 furnace and AS60 auto sampler. MT results were expressed as micrograms of MT/g wet tissue.

Statistical analysis

Data analysis included descriptive statistics, mean comparisons and linear regression models. Two-way ANOVA fixed effects was used in order to asses the differences of fetus, fetus brain and placenta weight and MT in placenta and in fetus brain, between control and treated groups. This procedure allowed us to discern the litter from treatment effects. Tukey's test was subsequently used for specific mean comparisons. Metal contents in exposed and non-exposed groups were compared using independent samples t test. Log transformation was performed to normalize data distribution when necessary. A level of $p<0.05$ was considered as statistically significant. Analysis was performed by using SPSS V. 13 software.

Results

Dam, fetus and placenta weights

The dams in the groups exposed to Cd and Pb did not differ from control; in gross appearance they were responsive to tactile stimuli and showed normal behavior at parturition day. None gave birth prior to day 20. Average weights of dams, fetuses, brain and placenta are shown in Table 1. There were no differences between dam, fetus brain and placenta weights. Fetus weight, however, was significantly lower for the Cd group (3.35±0.10 g) than for the control (4.21±0.15 g) and Pb treated groups ($p<0.05$, two-way ANOVA fixed effects, followed by Tukey). Covariance analysis showed that the decreased fetus weight on the Cd exposed group was independent of maternal weight.

Maternal and fetal cadmium and lead concentrations

Table 2 shows Cd and Pb concentration in dam and fetus blood of the exposed and control animals. As expected, Cd levels in dam and fetus blood, metal

Table 1. Weights of dam, fetuses, placenta, and fetus brain.

Treatment	Dam Weight (g)	Number of fetus per rat	Fetus Weight (g)	Fetus Brain Weight (g)	Placenta Weight (g)
Control	417.74±19.03 (n=9)	13±2.65	4.21±0.15 (n=111)	0.171±0.010 (n=22)	0.9087±0.018 (n=111)
Cd	360.70±21.52 (n=7)	10±4.04	3.35±0.10* (n=58)	0.1587±0.016 (n=13)	0.8611±0.029 (n=58)
Pb	417.48±18.50 (n=8)	12±3.10	4.12±0.13 (n=87)	0.1495±0.005 (n=16)	0.9541±0.018 (n=87)

Number of pups are also shown. Pregnant rats were exposed either to Cd (10 ppm) or Pb (300 ppm) in drinking water from day 1 of pregnancy until birth. Animals form control group received only deionized water. Data are expressed as mean ±SEM. *p<0.05= statistically different from control group (two-way ANOVA fixed effects, followed by Tukey).

Table 2. Pb and Cd levels in dam blood, placenta, fetus blood and brain.

Treatments	Dam Blood (µg/L)	Fetus Blood (µg/L)	Placenta (µg/g tissue)	Fetus Brain (µg/g tissue)
Cd				
Control	1.23±0.18 (n=9)	1.37±0.21 (n=6)	(25.11±11.12) (n=27)	(4.83±0.92) (n=11)
Cd exposure	15.95±4.95* (n=6)	3.38±0.69* (n=6)	(436.06±68.90)* (n=16)	(14.85±4.39*) (n=10)
Pb				
Control	11.06±3.0 (n=9)	25.55±6.60 (n=9)	0.022±0.008 (n=24)	0.008±0.004 (n=9)
Pb exposure	219.28±17.87* (n=7)	284.28±20.41* (n=7)	1.24±0.26* (n=19)	1.459±0.39* (n=13)

Samples were obtained from pregnant rats after exposure either to Cd (10 ppm) or Pb (300 ppm) in drinking water from day 1 of pregnancy until birth, control group received deionized water. Data are expressed as litter mean ±SEM. *p< 0.05= statistically different from control, independent samples t test.

concentration in placenta and in fetus brain were, on average, significantly higher for the Cd treated group than values from control group (independent-samples t test, $p<0.05$). Note that Cd exposure elicited a three fold increase of this metal in brain fetuses. Similarly, Pb exposure during pregnancy induced an increase of this metal in dam and fetus blood and placenta and fetus brain as compared to control (independent-samples t test $p<0.05$). It can also be observed that Pb exposure during pregnancy produced a build up of this metal in fetus brain, as Pb increased by about 170 fold as compared to control groups. Regression analysis, considering only exposed subjects, showed that Pb content in fetal blood was positively correlated to Pb dam blood (data not shown) with a relatively high correlation coefficient ($r^2=0.80$) and a 1.12 slope which indicates higher fetal than maternal exposure. The same relationship for Cd showed $r^2=0.42$ and a 0.69 slope, indicating a disruption in the transfer of Cd from mother to fetus as well as a higher Cd exposure for mothers than for fetuses.

Metallothionein levels in placenta and brain exposed to Cd and Pb

The MT levels in placenta and fetus brain are shown in figure 1. There were no differences in its levels in fetus brains from the different groups: control (3.27±0.07 µg/g), Cd (4.63±1.1 µg/g), and Pb (3.08±0.86 µg/g). Levels of MT in placenta were significantly increased in the group exposed to Cd (4.39±0.6 µg/g) in comparison to those from Pb (2.03±0.23 µg/g) or control groups (2.96±0.28 µg/g), showing the induction of MT by Cd in placenta (Two-Way ANOVA Fixed effects followed by Tukey).

Figure 2 shows the association between MT levels and both Cd (A) and Pb (B) content in placenta. As expected, a significant ($p=0.017$) direct linear

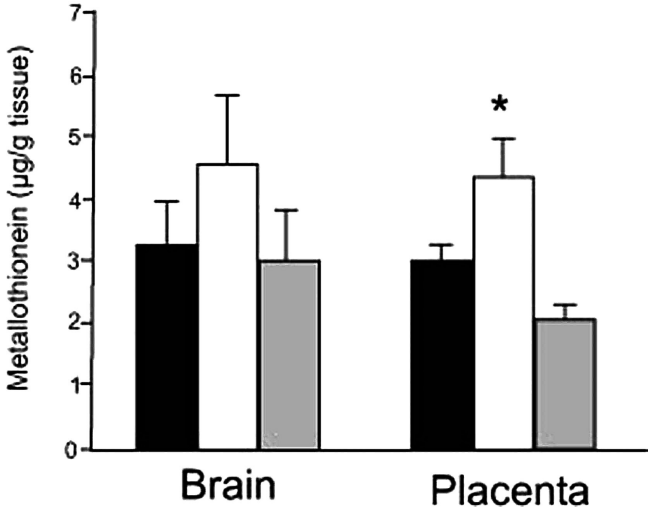

Fig. 1. Metallothionein levels in placenta and fetus brain in rats after exposure to either to Cd (10 ppm) or Pb (300 ppm) in drinking water from day 1 of pregnancy until birth in the different treatments. Animals form control group received only deionized water. Bars represent mean ±SEM of 6-12 independent experiments. Solid bars: control; empty bars: cadmium exposure; grey bars: lead exposure. *Significantly different from control and lead group ($p<0.05$), two way ANOVA fixed effects, followed by Tukey.

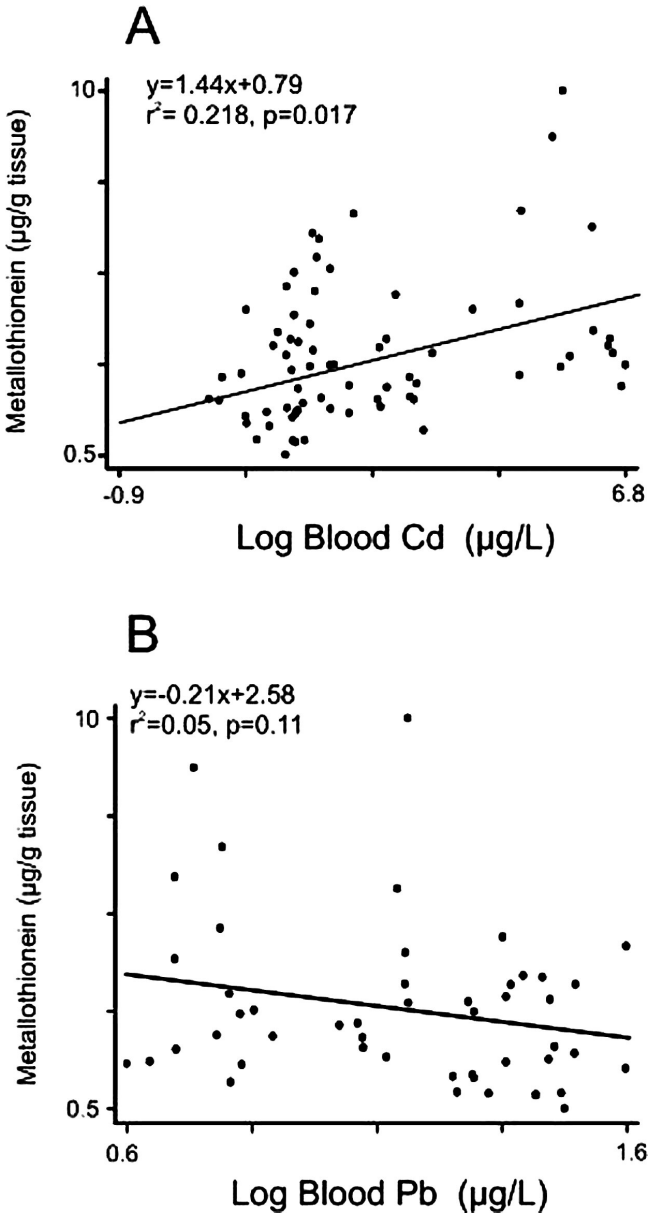

Fig. 2. Scatter plot displaying the relationship between metallothionein levels and either Cd **(A)** or Pb **(B)** concentration in placenta. Samples were obtained from rats after exposure either to Cd (10 ppm) or Pb (300 ppm) in drinking water from day 1 of pregnancy until birth; control group received deionized water. Data were analyzed by linear regression adjustment after log transformation for metal levels, relationship between metallothionein and Cd showed statistical significance ($p<0.05$).

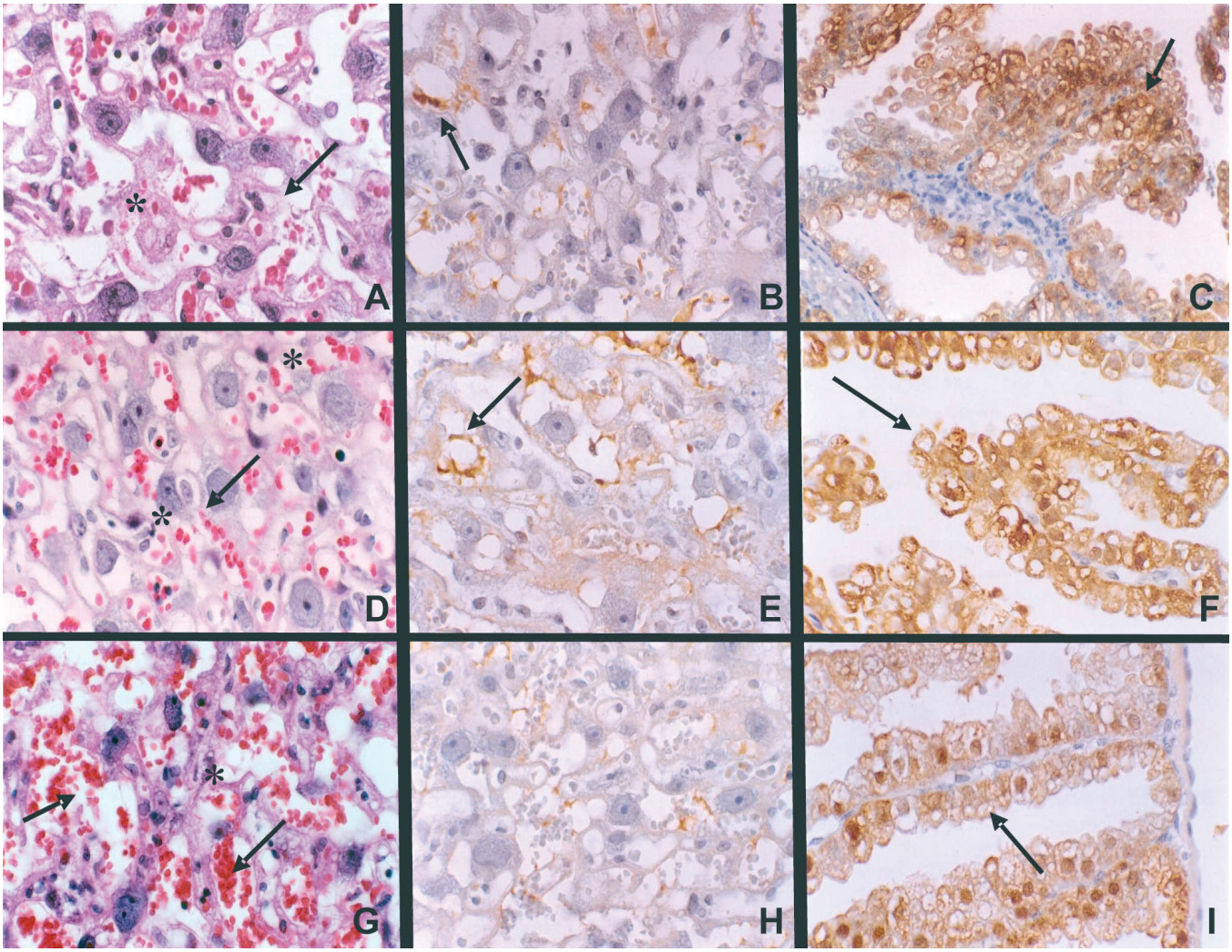

Fig. 3. Representative microphotographs of placenta from control group **(A-C)**, Cd exposure group **(D-F)** and Pb exposure group **(G-I)**. It can be observed that in vasculosyncytial membranes (*), the villi are mainly occupied by sinusoidal capillaries (arrows) **(A, D, G)** and congestion and initial necrosis **(G)**. Immunolocalization of MT in placenta can also be noticed. The MT was mainly localized in the cytoplasm of spongiotrophoblasts **(B, E, H)** and cells of yolk sac (arrows) **(C, F, I)**. Immunostaining MT was scarcely detectable in trophoblastic cells. x 400

relationship between MT concentrations in placenta and Cd content was observed (Fig. 2A). On the contrary, Pb was not related at all with MT in placenta.

Immunohistochemical evaluation

Microscope examination of placenta from the control group showed that the terminal villi, fetus blood vessels and trophoblastic layer had a normal aspect (Fig. 3A). The group treated with Cd showed trophoblastic cells and blood vessels with normal appearance (Fig. 3D); however in the group treated with Pb the villi are mainly occupied by sinusoidal capillaries with congestion and initial necrosis (Fig. 3G). In all three groups the immunohistochemical localization of MT in different cell types was identical: it was mainly located in the cytoplasm of spongiotrophoblasts and yolk sac (Fig. 3B,C,F,H,I). The MT staining was more intense in the group treated with Cd, which confirmed an increased concentration of MT in this group (Fig. 3E,F).

Discussion

Results from the present study indicate that experimental exposure to a relatively low concentration of Cd (Ishitobi and Watanabe, 2005) through drinking water during pregnancy produces a decrease in the fetus weight. This result is in agreement with other studies. Sorell and Graziano (1989) utilized 0, 5, 50 and 100 ppm of $CdCl_2$ and they found an effect on fetus birth weight only in the 50 and 100 ppm groups. Ali et al. (1986) exposed pregnant rats to 4.2 and 8.4 ppm of $CdCl_2$ and

they found an effect on birth weight only in the 8.4 ppm group. From those studies, it can be drawn that the effect of Cd oral exposure on fetus birth weight is only observed in concentrations higher than 5 ppm. The mechanism by which Cd exposure of the mother influences product birth weight remains controversial and several hypothesis have been raised to explain it: interference with transport of essential minerals (Osman et al., 2000; Ronco et al., 2006), placental damage (Peereboom-Stegeman et al., 1983; Osman et al., 2000), endocrine disruption (Kawai et al., 2002; Henson and Chedrese, 2004) or a combination of those. Fetus weight from the Pb pregnancy-exposed group was not affected. Such a result is in concordance with a previous experimental study, showing that different doses of Pb acetate injected intraperitoneally did not affect fetal body weight (Fuentes et al., 1996; Nampoothiri and Gupta, 2008). In regard to metal content, it was observed that Pb concentrations in blood from fetuses were in agreement, in quantity, with those in blood from exposed mothers (Table 2), which suggests a direct transference. Those findings are consistent with previous studies (Trottier et al., 2002; Butler-Walker et al., 2006; Villeda-Hernández et al., 2006). In conjunction, results reinforce the idea that Pb could freely cross through the placenta in a dose response manner. Results from the present research indicate that dam Pb exposure not only produced an increase of this metal in fetus blood, but it also elicited an rise of Pb in the central nervous system of the developing fetus.

The relationship between dams and fetus Cd blood levels was not as consistent as observed for Pb. In fact, as shown in table 2, the Cd content in blood from fetuses reached only 30% of that in the blood from the exposed mothers. It is noteworthy that Cd exposed dams tended to accumulate this toxicant metal in placenta, as Cd concentration in placenta was 30-fold from that observed in fetus brain; Pb contents in those tissues reached similar values. The difference in the transference of the two metals to tissues may be due to their ability to induce MT in placenta. Herein, MT levels in placenta from Cd treated group were higher than those from Pb and control groups, which confirms the findings obtained by several authors (Goyer and Cherian, 1992; Itoh et al., 1996; Lau et al., 1998; Peterson-Grawe and Oskarsson, 2000). This indicates that Cd is a strong inducer of MT biosynthesis in placenta. The functional significance of the presence of MT in human placental tissues and fetal membranes cannot be discerned from the present results, although several authors (Goyer et al., 1992; Kuriwaki et al., 2005; Sorkun et al., 2007) have suggested that it could be preventing the transference of Cd from mother to fetus. In contrast, studies using MT-I and II deficient transgenic mice exposed to Cd during pregnancy concluded that MT did not play a major role in restricting the transfer of Cd from dam to fetus via placenta (Conrad et al., 1997; Brako et al., 2003). In the case of the group treated with Pb, levels of MT did not increase although Pb is a divalent metal with similar chemical characteristics to those of Cd. Although high levels of Pb in the brain of fetuses of exposed dams were observed, this accumulation did not lead to the induction of metallothionein, in contrast to the limited accumulation of Cd of fetal brain. There was no increased MT biosynthesis of statistical significance, probably due to the fact that the homeostasis and transport of Pb is associated to Ca^{2+} transport mechanisms (Evans et al., 2003).

Results obtained from microscope observation of placenta include congestion, hemorrhage and initial necrosis for the group exposed to Pb. Goyer (1990) and Fuentes et al. (1996) have already reported histopathological damage in the placenta (trophoblast hyperplasia, vascular congestion); however, they used higher Pb doses than those used in the present work and they administered it intraperitoneally. A possible explanation for the damage observed in placenta is the ability of Pb to induce oxidative stress by generating free radicals which lead to lipid peroxidation with consequent damage to the cytoplasmic membranes (Villeda-Hernandez et al., 2006). Meanwhile, microscopic observation of placenta from the group exposed to Cd did not show apparent abnormalities. The absence of major morphologic alterations may be due to the differences in Cd exposure route and the relatively low Cd level of exposure compared to those observed in other experimental studies (Peereboom-Stegeman et al., 1983; Wier et al., 1990) in which toxicity was observed in the maternal vasculature by morphological techniques. Some of the morphologic alterations found were: congestion, hemorrhage, necrosis of trophoblasts, infiltration by polimorphonuclear leucocytes, thickening of the trophoblastic and reduced density of blood vessels (Levin et al., 1987) and thickening of vasculo-syncytial membrane (Sorkun et al., 2007). In conclusion, the results presented in this paper show that oral exposure of pregnant rats to relatively low doses of Cd is associated with a decrease of fetus birth weight, an important induction of MT levels in placenta and a consequent Cd accumulation in this tissue. Pb exposure during pregnancy induced higher exposure for the fetus than for mothers, which suggests a poor Pb mother-fetus barrier and an easy metal transference through the placenta which results in important Pb levels in the developing brain of the fetus. Moreover, levels of MT were not increased by Pb, which suggests that Pb transport is not associated with MT. Additional studies are required to clarify the exact role of MT in placenta and other metal transporters which may help to understand the accumulation and transfer of Cd and Pb in the placenta.

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