

Evaluation of kidney injury biomarkers in rat amniotic fluid after gestational exposure to cadmium

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ABSTRACT: Cadmium is a well-characterized nephrotoxic agent that is also capable of accumulating and diffusing across the placenta; however, only a few studies have addressed its effects over fetal kidneys and none of them has used a panel of sensitive and specific biomarkers for the detection of kidney injury. The goal of this study was to determine cadmium renal effects in rat fetuses by the quantification of early kidney injury biomarkers. Pregnant Wistar rats were exposed by inhalation to an isotonic saline solution or to CdCl₂ solution (D_{Del}=1.48 mg Cd kg⁻¹ day⁻¹) during gestational days (GD) 8–20. On GD 21, dams were euthanized and samples obtained. Kidney injury biomarkers were quantified in amniotic fluid samples and fetal kidneys were microscopically evaluated to search for histological alterations. Our results showed that cadmium exposure significantly raised albumin, osteopontin, vascular endothelial growth factor and tissue inhibitor of metalloproteinases-1 levels in amniotic fluid, whereas it decreased creatinine. Clusterin, calbindin and IFN-inducible protein 10 did not show any change. Accordingly, histological findings showed tubular damage and precipitations in the renal pelvis. In conclusion, gestational exposure to cadmium induces structural alterations in fetal renal tissue that can be detected by some kidney injury biomarkers in amniotic fluid samples. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: fetal nephrotoxicity; metals; *in utero* exposure; osteopontin; VEGF; TIMP-1

Introduction

Cadmium is a heavy metal that is released into the environment by natural and anthropogenic activities such as mining, refining, and the manufacture and application of phosphate fertilizers. For humans, the major routes of exposure to cadmium are oral and inhalation. Cadmium absorption depends on the route of exposure, being relatively greater by inhalation (approximately 25%) than by the oral route (approximately 5%) (ATSDR, 2012). Oral exposure occurs via contaminated water and food (i.e. plants, mollusks and crustaceans). Cadmium inhalation occurs mainly through tobacco smoke (ATSDR, 2012). Smoking one cigarette increases cadmium blood levels by approximately 0.1–0.2 µg Cd l⁻¹ (Kosanovic *et al.*, 2002) because each cigarette contains from 1 to 2 µg of cadmium (Satarug *et al.*, 2004); nevertheless, cadmium content can vary depending on the origin of the tobacco leaves. In studies on Mexican cigarettes, it was found that each cigarette contained from 2.5 to 2.8 µg of cadmium (Elinder *et al.*, 1983; Saldívar *et al.*, 1991).

Air pollution is also an important source of cadmium exposure, especially in urban areas where the concentration of this metal ranges from 2 to 15 ng m⁻³ (ATSDR, 2012). However, some reports describe that the cadmium concentration in PM_{2.5} (particulate matter with a mass median diameter ≤ 2.5 µm) from certain zones in Mexico City and its metropolitan area is approximately 35 to 40 ng m⁻³ (Chow *et al.*, 2002; Guerra *et al.*, 2013).

Cadmium exposure affects several organs, including the bones and lungs; however, the kidneys are the most vulnerable organs because 99% of the filtered cadmium is reabsorbed in the

proximal tubule of the nephrons (Barbier *et al.*, 2005) where it accumulates and has a half-life that ranges from 6 to 38 years (ATSDR, 2012). In addition to its renal effects, cadmium also induces adverse developmental effects. In humans, it has been associated with a decrease in placental vascularization and a low birth weight (Peereboom-Stegeman *et al.*, 1983; ATSDR, 2012). In rats, the effects include the following: fetal and maternal death; necrosis, vascular congestion and hemorrhage in the placenta; and low birth weight, diminished ossification, cleft palate, unilateral anophthalmia, microphthalmia, renal cavitation, pulmonary hypoplasia, hydrocephaly and gastroschisis in the offspring (Pařizek, 1964, 1965; Prigge, 1978b; Samarawickrama

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and Webb, 1979; Levin and Miller, 1981; Barański, 1984; Salvatori *et al.*, 2004). Nevertheless, the routes of exposure of most of these studies are not environmentally relevant.

Despite the evidence that cadmium has nephrotoxic effects and that it is capable of diffusing across the placenta possibly through DMT-1 (Divalent Metal Transporter-1), ZIP-14 (Zrt-Irt-like protein 14) and ZnT2 (Zinc Transporter-2) (Nakamura *et al.*, 2012) metal transporters, only a few studies have addressed the effects of this metal on fetal kidney development or function. One of them demonstrated that exposure to low concentrations of cadmium ($500 \mu\text{g Cd}^{2+} \text{kg}^{-1}$) in drinking water throughout the entire gestation of Wistar rats causes the development of nephropathy and hypertension in the offspring on post-natal day (PND) 60 (Jacquillet *et al.*, 2007). Additionally, a different study shows that the repeated intraperitoneal injection of 1.2 and 1.5 mg $\text{Cd}^{2+} \text{kg}^{-1} \text{day}^{-1}$ in Sprague–Dawley rats on gestational days (GD) 8, 10, 12 and 14 caused a lower activity of γ -glutamyltransferase (γ -GT), alkaline phosphatase (ALP) and N-acetyl- β -D-glucosaminidase (NAG) and an increased excretion of β 2-microglobulin (β 2-MG) in urine samples of 3-day-old pups (Saillenfait *et al.*, 1991). Moreover, another study showed that a single intraperitoneal injection of 3 mg $\text{Cd}^{2+} \text{kg}^{-1}$ in Wistar rats on GD 10 caused alterations in proximal and distal tubules, as well as glomeruli in fetal kidneys (Roman *et al.*, 2004).

It is widely known that amniotic fluid is mainly composed of fetal urine in humans (Modena and Fieni, 2004; Tong *et al.*, 2009). In rats the origin is less clear, but because of the fact that the volume changes of amniotic fluid are similar to those found in humans and other mammals (Park and Shepard, 1994), that amniotic fluid composition can vary importantly in adjacent fetuses, which indicates that each fetus controls its composition (Adolph, 1967), and that at the time of birth neonate kidneys filter an approximate of $0.045 \text{ ml min}^{-1} \text{ g}^{-1}$ kidney (Larsson and Maunsbach, 1980), it is most likely that rat amniotic fluid is also conformed by fetal urine, at least, to some extent.

Because of its composition, amniotic fluid has been used to study congenital alterations in human fetal kidneys, as well as the maturation of renal function by quantifying certain kidney injury biomarkers, such as α 1-microglobulin (α 1-MG), β 2-MG, γ -GT, NAG and Cystatin C (Cys C) (Burghard *et al.*, 1987; Ring *et al.*, 1991; Gulbis *et al.*, 1996; Mussap *et al.*, 2002). Although these proteins have performed well in the detection of renal alterations in fetuses, it is important to study biomarkers that can help diagnose fetal kidney damage in a timelier manner and accurately provide treatment. Recently, a panel of new biomarkers that includes kidney injury molecule 1 (Kim-1), lipocalin-2, tissue inhibitor of metalloproteases-1 (TIMP-1), osteopontin (OPN), clusterin (CLU), cystatin C (Cys C) and microalbumin has been shown to be more sensitive and specific for the detection of acute kidney injury (AKI) in rat urine caused by diverse nephrotoxic agents, including cadmium (Ichimura *et al.*, 1998; Prozialek *et al.*, 2007; Rached *et al.*, 2008; Vaidya *et al.*, 2008a, b). Furthermore, they have shown good consistency with histopathological findings, but its usefulness to detect fetal kidney damage in amniotic fluid samples has not been widely studied, if at all.

The aim of this study was to determine the usefulness of early kidney injury biomarkers calbindin (Calb1), CLU, α -glutathione S-transferase (GST α), IFN-inducible protein 10 (IP-10), Kim-1, OPN, TIMP-1 and vascular endothelial growth factor (VEGF) to determine cadmium-induced renal effects in rat fetuses exposed during the gestational period.

Materials and methods

Animals

All experimental procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals (Comité Interno para el Cuidado y uso de los Animales de Laboratorio, CICAL; Protocol Number: 041-13) from CINVESTAV and they were performed in accordance with their guidelines.

Eight-week-old female Wistar rats (220–300 g) were housed in ventilated cages (One Cage; Lab Products, Inc., Seaford, DE, USA) with sawdust bedding. Cages were located in a room with filtered air (HEPA filters) (bioBUBBLE, Inc., Fort Collins, CO, USA), relative humidity of 50%, the light–dark cycle of 12 h and temperature between $22 \pm 2^\circ\text{C}$. The rats had access to standard chow (PMI 5008: Purina, San Antonio, TX, USA) and water *ad libitum* except during the inhalation exposure period in the whole body chamber (WBC).

Females were acclimatized for a week. Their sexual receptivity was assessed through the examination of their vaginal smears. When they were sexually receptive, they were mated with an untreated male of the same strain for one night. The next morning, copulation was evaluated by the presence of sperm on the vaginal smear. If there was sperm, that day was considered gestational day (GD) 1.

Once pregnant, rats were randomly divided into two groups of six rats each: negative control (CT) and cadmium-treated (Cd). Both groups of rats were acclimatized to the WBC daily for 2 h from GD 1 through GD 7.

From GD 8 to 20, rats from the CT group were exposed for 2 h to a mist of isotonic saline solution and those from the Cd group were exposed to a cadmium chloride (CdCl_2) mist (Sigma-Aldrich Co., St. Louis, MO, USA) solution ($1 \text{ mg CdCl}_2 \text{ ml}^{-1}$, dissolved in isotonic saline solution). Nebulization was achieved using an Aeroneb nebulizer (inExpose; SCIREQ, Inc., Quebec, Montreal, Canada) connected to a 10-l WBC. Nebulization was performed under a fume hood to avoid any contact with the cadmium mist throughout the exposure. Only three rats were placed in the chamber during the exposure period. Nebulization was controlled through the flexiWare software v.6.1. (SCIREQ, Inc.), according to the following parameters: bias flow of 3 l min^{-1} , 10% nebulization rate ($0.085 \text{ ml min}^{-1}$) and nebulization cycle time of 1 s. According to the manufacturer, the aerosolized particles had a volume median diameter (VMD) of $5 \mu\text{m}$, a mass median aerodynamic diameter (MMAD) between 2.5 and $3 \mu\text{m}$ and a geometric standard deviation (GSD) of $2 \mu\text{m}$. The dose concentration of the aerosol (C_{Dose}) and the delivered dose (D_{Del}) achieved with these conditions were $17.43 \text{ mg Cd}^{2+} \text{ m}^{-3}$ and $1.48 \text{ mg Cd}^{2+} \text{ kg}^{-1} \text{ day}^{-1}$, respectively. The weight of the rats was recorded daily through the entire gestation to assess their evolution.

Amniotic fluid, blood and tissue collection

On GD 21, pregnant rats were anesthetized with isoflurane (Sofloran Vet; PISA Farmacéutica, Hidalgo, Mexico) using the V-1 Table Top Lab Animal System (VetEquip, Inc., Pleasanton, CA, USA). Then, a caesarean section was performed, and a syringe was used to collect amniotic fluid from the sac of each fetus. The amniotic fluid from each sac of the litter was pooled into one vial. Samples were centrifuged at $9300g$ for 10 min at 4°C to remove blood traces. Then, they were stored at -70°C until their further use.

Later, fetuses and placentae were obtained and kept on an ice-cold isotonic saline solution until they were weighed. Fetal kidneys were weighed, and one kidney from one fetus from four different litters was submerged in 10% buffered formalin. Kidneys were kept at 4 °C until histological processing and examination.

Additionally, dam blood samples were obtained by cardiac puncture (terminal exsanguination) with a heparinized syringe; fetus blood samples were obtained by decapitation, and the blood was collected in heparinized capillary tubes. Blood samples were centrifuged at 2500 *g* for 15 min at 4 °C to obtain plasma samples, which were stored at –70 °C until use.

After exsanguination, dam kidneys and liver were perfused with an isotonic saline solution via the renal artery before they were extracted. Lungs were also obtained. All tissues were kept on an ice-cold saline solution until they were weighed and snap frozen in liquid nitrogen for the subsequent measurement of their cadmium content.

Cadmium content quantification

The cadmium content in dam lungs, liver, kidneys and placentae, and fetal kidneys was measured by atomic absorption as previously described (Santoyo-Sanchez *et al.*, 2013). Briefly, up to 3 g of each tissue were weighted (for fetal kidneys, a pool of kidneys from fetuses of each litter was used) and then digested in a 2:1:6 solution of nitric acid (Reactive grade, 69.9% of purity; J.T. Baker®, Avantor Performance Materials, Center Valley, PA, USA), hydrochloric acid (Reactive grade, 37.1% of purity; J.T. Baker®, Avantor Performance Materials) and hydrogen peroxide (Reactive grade, 30% of purity; J.T. Baker®, Avantor Performance Materials) overnight. The next morning, the samples were heated until they reached their boiling point and released nitrous fumes (yellow-orange). Then, two more milliliters of hydrogen peroxide was added until full degradation of organic matter and concentration of the samples. At the end of this period, the solution was filtered and diluted to 5 or 10 ml with deionized water depending on the organ digested. Cadmium content in the final solution was measured using an atomic absorption direct aspiration flame (AAAnalyst 100; Perkin Elmer®, Waltham, MA, USA).

Amniotic fluid cadmium content was also quantified by atomic absorption. To every 1.5 ml of sample, 100 µl of nitric acid (J.T. Baker®, Avantor Performance Materials) was added prior to their aspiration and measurement.

Kidney injury biomarkers assessment

Creatinine was measured in the plasma and amniotic fluid samples using a commercial kit (CR510; Randox Laboratories, Lakewood, CA, USA) based on the Jaffe method.

Albumin levels in amniotic fluid samples were measured using the HemoCue Albumin 201 system (HemoCue AB, Ängelholm, Sweden), whereas in dam plasma samples they were quantified using a commercial kit based on a colorimetric reaction (AB362; Randox Laboratories).

Amniotic fluid samples were employed to quantify kidney injury biomarkers, Calb1, CLU, GSTα, IP-10, Kim-1, OPN, TIMP-1 and VEGF, using the Milliplex® Map Magnetic Rat Kidney Toxicity Panel 1 (Millipore Corp., St. Charles, MO, USA). Manufacturer's instructions were followed; 12.5 µl of each undiluted sample were used for the detection. All samples were analyzed in duplicate. The plate was read on a Magpix® System (Millipore Corp.).

To improve the detection of VEGF and Calb 1, amniotic fluid samples were concentrated with a 30k Amicon® Ultra Centrifugal Filter Device (Merck Millipore Corp., Darmstadt, Germany) according to the manufacturer's instructions.

The levels of albumin and early kidney injury biomarkers were corrected with total protein levels in amniotic fluid samples. The protein content was quantified using the Bradford method with the Quick Start™ Bradford Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Histological analysis

Fetal kidneys were removed from the 10% buffered formalin solution and transferred into a sterile 30% buffered sucrose solution for 48 h at 4 °C. Then, they were dehydrated in a series of ethanol dilutions before being embedded in paraffin. The samples were then longitudinally and serially sectioned in 5 µm slices. Finally, the kidneys were stained with hematoxylin and eosin (H&E) for histological evaluation. Two sections from four different kidneys of each group were examined. Histological changes were graded on a scale from 0 to 5 based on microscopic observations of a pathologist, where 0 represents normal histology, 1, 2, 3, 4 and 5 represents mild, moderate, high and severe changes, respectively. All observations were made in a blinded fashion.

For tubular necrosis the presence and severity of cytoplasm eosinophilia, desquamation of cells into tubular lumen, and shrinkage, hyperchromic, fragmentation or absence of nuclei was assessed. With regard to tubular degeneration, the intensity of vacuolization, paleness of the cytoplasm and cell membrane protrusions were evaluated. Finally, in terms of hyaline cylinders in the tubular lumen and protein deposits in the renal pelvis, the extent of material deposited was examined.

Statistical analyzes

All the statistical analyzes were performed using the GraphPad Prism software v.5.0c (GraphPad Software, La Jolla, California, USA). Data that met the criteria to be analyzed through parametric tests were analyzed with Student's *t*-test (unpaired, two-tailed). Data that did not meet the criteria for parametric tests were analyzed with a Mann–Whitney *U*-test (unpaired, two-tailed). $P \leq 0.05$ was considered statistically significant.

Results

Cadmium content

To ensure that cadmium was being absorbed and distributed among the organs of the pregnant rats, the levels of this metal in kidneys, livers and placentae, which serve as cadmium major reservoirs, were quantified. Cadmium levels in the lungs were also quantified because the exposure was by inhalation.

Similarly, to make sure that cadmium was transferred from the dam to the fetuses, the levels of this metal in fetal kidneys and amniotic fluid were also quantified. As expected, the exposure to a mist of cadmium increased the content of the metal in all the organs evaluated, both maternal and fetal, compared with the levels found in the CT group (Table 1), whereas no change in the content of amniotic fluid samples was observed (Table 1).

Table 1. Cadmium content in dam and fetus organs, and amniotic fluid samples

Organ/Group	CT	Cd
Dam lungs ($\mu\text{g Cd}^{2+} \text{ g}^{-1}$ wet weight)	0.008 \pm 0.00	11.73 \pm 1.12 ^a
Dam liver ($\mu\text{g Cd}^{2+} \text{ g}^{-1}$ wet weight)	0.019 \pm 0.01	1.4 \pm 0.24 ^b
Dam kidneys ($\mu\text{g Cd}^{2+} \text{ g}^{-1}$ wet weight)	0.101 \pm 0.05	3.17 \pm 0.34 ^c
Placentae ($\mu\text{g Cd}^{2+} \text{ g}^{-1}$ wet weight)	0.008 \pm 0.00	0.17 \pm 0.01 ^d
Fetal kidneys ($\mu\text{g Cd}^{2+} \text{ g}^{-1}$ wet weight)	0.397 \pm 0.07	0.803 \pm 0.14 ^e
Amniotic fluid ($\mu\text{g Cd}^{2+} \text{ ml}^{-1}$)	0.036 \pm 0.001	0.038 \pm 0.001

Data represent the mean \pm SEM, $n = 5$ (at least).
^a $P = 0.0042$ vs. CT,
^b $P = 0.0042$ vs. CT,
^c $P = 0.0024$ vs. CT,
^d $P < 0.0001$ vs. CT and
^e $P = 0.0335$ vs. CT. Data were analyzed with a Mann–Whitney U -test (Dam lungs, liver and kidneys, and placentae cadmium content) and a Student's t -test (fetal kidneys and amniotic fluid cadmium content).

Effect of cadmium on fetal development

As an indirect way of detecting problems during the evolution of pregnancy, the dam weight was recorded daily throughout the entire gestation. No difference in weight gain relative to the CT group was observed (Table 2). Additionally, the exposure to cadmium did not change either the number of fetuses per litter or the dam liver and kidney relative weight. Nevertheless, the relative weight of the lungs increased with the exposure to cadmium compared with the CT group (Table 2).

Moreover, the fetuses from the Cd group had no evident external malformations, but they were smaller than those from the CT group (Table 3); however, the relative weight of their kidneys did not change (Table 3). Similarly, the placentae from the Cd group were significantly smaller compared with the CT group (Table 3).

Effect of cadmium on the levels of plasma and amniotic fluid creatinine

Dam and fetus plasma creatinine levels were quantified to assess their glomerular function. The exposure to cadmium significantly reduced fetal creatinine plasma levels ($0.22 \pm 0.01 \text{ mg dl}^{-1}$) compared with those found in the CT group ($0.33 \pm 0.02 \text{ mg dl}^{-1}$) (Fig. 1). No statistically significant changes were observed in maternal plasma levels ($0.55 \pm 0.10 \text{ mg dl}^{-1}$ found in the CT group vs. $0.37 \pm 0.07 \text{ mg dl}^{-1}$ in the Cd group) (Fig. 1).

Because amniotic fluid is mainly composed of fetal urine, the creatinine levels in this fluid were quantified to assess whether

Table 2. Effect of the exposure to cadmium during gestation over dam weight gain and organ relative weight

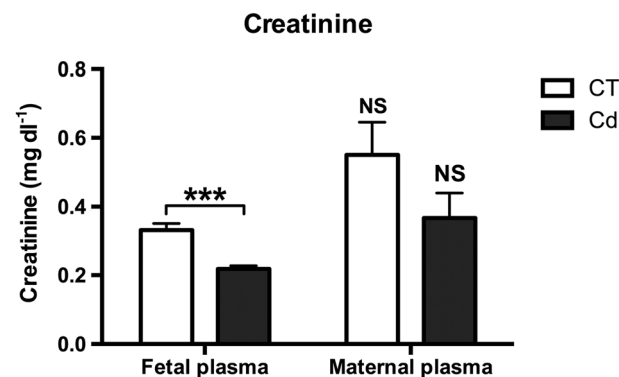
Group	CT	Cd
Weight gain (g)	149.9 \pm 5.34	115.9 \pm 14.57
Lungs relative weight (%)	0.363 \pm 0.01	0.557 \pm 0.03 ^a
Liver relative weight (%)	4.329 \pm 0.32	4.268 \pm 0.42
Kidneys relative weight (%)	0.629 \pm 0.02	0.626 \pm 0.02
Number of fetuses per litter	14 \pm 1	13 \pm 1

Data represent the mean \pm SEM, $n = 5$ (at least).
^a $P = 0.0043$ vs. CT. Data were analyzed with a Mann–Whitney U -test.

Table 3. Effect of the exposure to cadmium over fetal body weight, fetal kidney relative weight and placental weight

Group	CT	Cd
Body weight (g)	4.14 \pm 0.02	3.8 \pm 0.05 ^a
Kidneys relative weight (%)	0.75 \pm 0.01	0.75 \pm 0.01
Placental weight (g)	0.62 \pm 0.01	0.55 \pm 0.01 ^b

Data represent the mean \pm SEM, $n = 70$ (at least).
^a $P < 0.0001$ vs. CT,
^b $P < 0.0001$ vs. CT. Data were analyzed with a Mann–Whitney U -test (body weight and kidneys relative weight) and a Student's t -test (placental weight).

**Figure 1.** Effect of cadmium gestational exposure on creatinine levels in fetus plasma ($P < 0.0001$, Mann–Whitney U -test) and dam plasma ($P = 0.1581$, Student's t -test). The bars represent the mean \pm SEM, $n = 6$ (at least). *** $P < 0.001$. NS, not statistically significant.

the changes observed in fetal plasma correlated with the amount they excreted in urine. As observed in plasma samples, cadmium significantly reduced the amount of creatinine in amniotic fluid compared with the CT group (0.28 ± 0.03 and $0.57 \pm 0.07 \text{ mg dl}^{-1}$, respectively) (Fig. 2a).

Effect of cadmium on the levels of early kidney injury biomarkers

Similarly to creatinine, kidney injury biomarkers were quantified in amniotic fluid samples because this matrix mainly represents

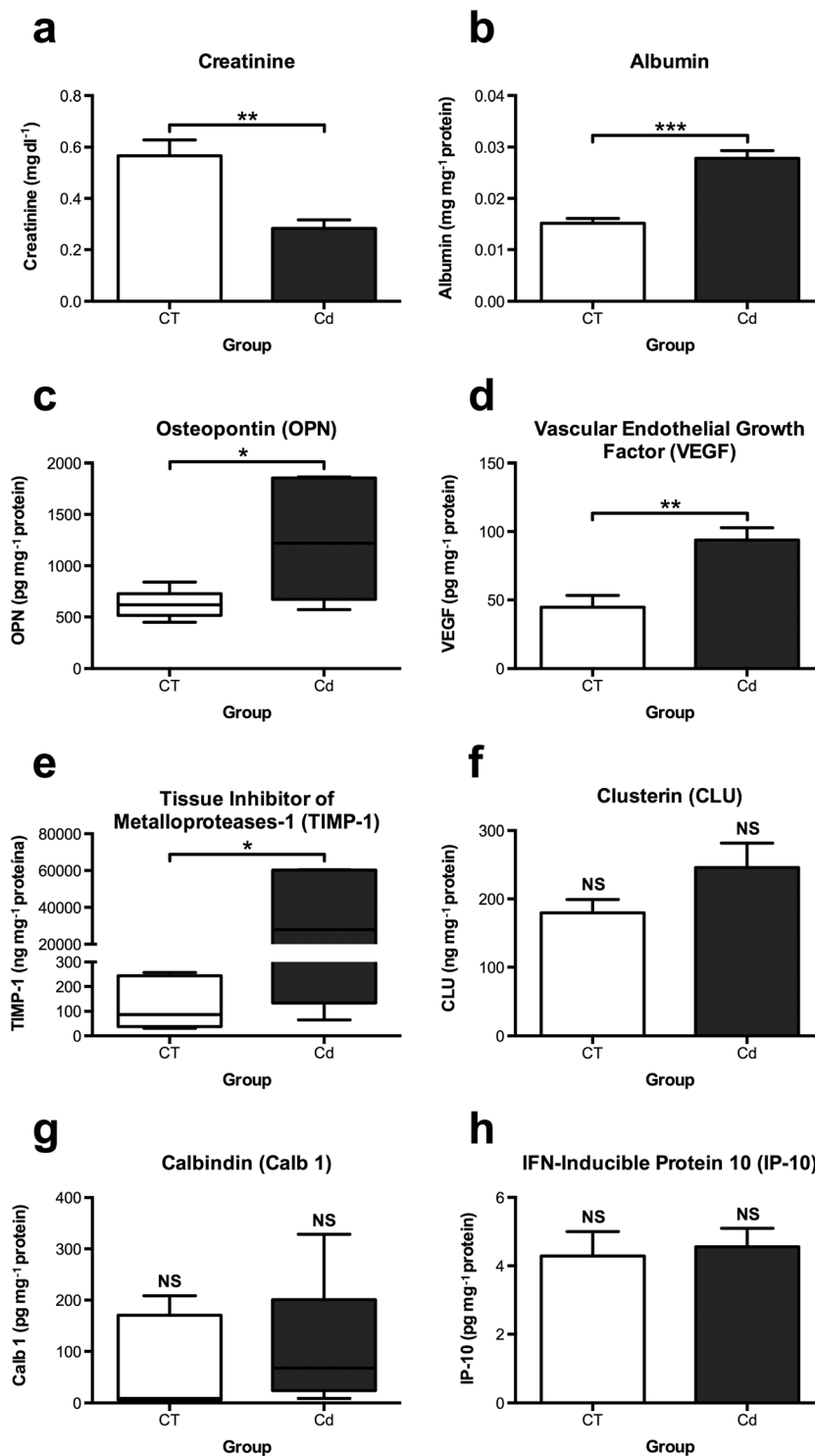


Figure 2. Effect of cadmium gestational exposure on the levels of kidney injury biomarkers in amniotic fluid samples. (a) Creatinine levels ($P=0.0023$, Student's t -test), (b) albumin levels ($P < 0.0001$, Student's t -test), (c) osteopontin (OPN) levels ($P=0.0260$, Mann-Whitney U -test), (d) vascular endothelial growth factor levels (VEGF) ($P=0.0036$, Student's t -test), (e) tissue inhibitor of metalloproteases-1 levels (TIMP-1) ($P=0.0411$, Mann-Whitney U -test), (f) clusterin (CLU) levels ($P=0.1357$, Student's t -test), (g) calbindin (Calb 1) levels ($P=0.3290$, Mann-Whitney U -test) and (h) IFN-inducible Protein 10 (IP-10) levels ($P=0.7676$, Student's t -test). All biomarkers were corrected by protein levels in the samples. Parametric data are presented in the bar graphs. The bars represent the mean \pm SEM. Non-parametric data are presented in the box and whiskers plots. The boxes represent the 25th, 50th and 75th centiles. The whiskers represent the 5th and 95th centiles. $n=5$ (at least). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not statistically significant.

fetal urine composition. Biomarkers presented different patterns after cadmium exposure (Fig. 2). The levels of albumin, OPN and VEGF in amniotic fluid showed a statistically significant

increase of 1.83-, 1.98- and 2.10-fold, respectively, compared with the CT group (Fig. 2b-d), whereas dam plasma albumin was not significantly ($P=0.1592$, Student's t -test) altered by cadmium

exposure ($2.10 \pm 0.18 \text{ g dl}^{-1}$ in the CT group vs. $2.48 \pm 0.16 \text{ g dl}^{-1}$ in the Cd group).

The TIMP-1 levels had a steeper increase (238.24-fold) after exposure to cadmium (Fig. 2e). In contrast, the CLU, Calb 1 and IP-10 levels did not show any change compared with the CT group (Fig. 2f–h).

Finally, the levels of GST α and Kim-1 were below the detection limit of the equipment in all samples, regardless the group (data not shown).

Effect of cadmium on fetal kidney histology

To seek correspondence between the biomarker levels and any structural alteration, fetal renal tissue was evaluated.

On a macroscopic level, there was no visible damage to any of the kidneys dissected. On a microscopic level, kidneys from the CT group presented normal histological structure corresponding to their gestational age (Fig. 3a–d). Only mild tubular degeneration and necrosis were observed (Table 4).

Cadmium-exposed fetuses presented higher levels of tubular degeneration and necrosis than the CT group (Table 4, Fig. 3g and h). Additionally, they presented deposits of proteinaceous material in the renal pelvis (Fig. 3e and f) and hyaline cylinders in the lumen of some tubules (Fig. 3h).

As shown in Table 4, the lesion score of the Cd group is significantly higher than the one from the CT group.

Discussion

The aim of this study was to quantify the levels of early kidney injury biomarkers in amniotic fluid samples to determine cadmium-induced fetal nephrotoxicity.

Cadmium exposure was by inhalation to mimic an environmentally relevant route of exposure because growing industrialization and high smoking prevalence around the world are making this route increasingly important. In this study, the concentration of cadmium achieved within the WBC was $17 \text{ mg Cd}^{2+} \text{ m}^{-3}$. This was higher than previous studies performed with pregnant (Prigge, 1978b; Barański, 1984; Trottier *et al.*, 2002) and non-pregnant (Prigge, 1978a) rodents; nevertheless, it is important to

Table 4. Histopathologic lesions scores found in fetal kidneys after gestational exposure to cadmium

Lesion/Group	CT	Cd
Tubular necrosis	0.62 ± 0.12	3.0 ± 0.20
Tubular degeneration	1.4 ± 0.12	3.25 ± 0.14
Hyaline cylinders in tubules	0.62 ± 0.12	2.34 ± 0.12
Proteinaceous material in renal pelvis	0.0 ± 0.0	1.38 ± 0.24
Total score	2.62 ± 0.12	10.0 ± 0.35^a

Data represent the mean \pm SEM, $n = 4$.

^a $P = 0.0256$ vs. CT. Total score was analyzed with a Mann–Whitney *U*-test.

note that cadmium levels found in the lungs, liver and kidneys were lower than those found in the studies cited above (Prigge, 1978a, b). This can be explained by a shorter length of exposure and a larger aerodynamic diameter of the aerosolized particles generated in our study. This last parameter is the primary physical–chemical characteristic that influences deposition efficiency, as well as the portion of the bronchial tree in which the cadmium is deposited. The smaller the MMAD is, the further into the bronchial tree the particles will go, increasing the probability of cadmium being absorbed. Although the MMAD of the particles in this study falls in the respirable range of rats (Asgharian *et al.*, 2003), it was notably larger ($2.5\text{--}3 \mu\text{m}$) than in previous studies ($0.19\text{--}0.6 \mu\text{m}$) (Prigge, 1978a, b; Barański, 1984; Trottier *et al.*, 2002), which might have increased nasal deposition and reduced pulmonary deposition and thus cadmium burden.

In addition, we evaluated cadmium content in fetal kidneys and amniotic fluid samples to assess maternal–fetal transfer. Our results showed a significant increase in fetal kidneys content but no change in amniotic fluid. These findings confirm the capability of cadmium to diffuse across the placenta and reach the fetuses, specifically their kidneys.

Cadmium reaches the kidney by being filtered and reabsorbed at the proximal tubule portion of the nephron (ATSDR, 2012). Our findings are consistent with the functionality of some

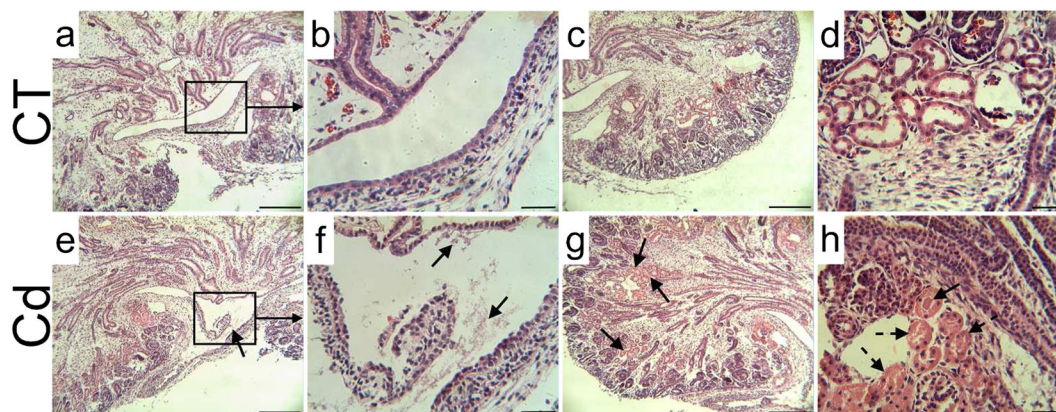


Figure 3. Representative microphotographs of fetal kidney sections ($5 \mu\text{m}$) stained with hematoxylin and eosin (H&E) from the CT group (a, b, c and d) and the Cd group (e, f, g and h). (a) and (e) Renal pelvis and corticomedullary area. Protein material is deposited in the renal pelvis (e, black arrow) ($10\times$ magnification), (b) and (f) Renal pelvis. Black arrows in (f) indicate deposition of protein material ($40\times$ magnification), (c) and (g) Nephrogenic zone and cortex. Tubular degeneration in (g) is pointed by black arrows ($10\times$ magnification), (d) and (h) fully developed nephron from the outer medulla. In (h), the black arrow points at a hyaline cylinder deposited in the lumen of a tubule, whereas the dashed arrows show tubular necrosis and degeneration ($40\times$ magnification). Scale bars in (a), (c), (e) and (g) represent $150 \mu\text{m}$. Scale bars in (b), (d), (f) and (h) represent $30 \mu\text{m}$.

nephrons in fetal kidneys, which explains the lack of cadmium in amniotic fluid samples. Cadmium was filtered and reabsorbed at proximal tubules; hence, it started to accumulate there instead of being eliminated through urine.

In the study, we evaluated the evolution of pregnancy by a follow-up of dam weight from GD 1 to 21, as well as the determination of the relative weight of the cadmium target organs in the dams (liver and kidney). Because cadmium exposure did not affect either of these parameters, and there were no clinical signs of overt toxicity in the dams, we concluded that the findings of this study were not maternally induced, at least not substantially. Only the relative weight of the lungs increased at the end of the exposure period. This finding is consistent with previous studies where the weight increase was correlated with pulmonary hyperplasia (Prigge, 1978a, b). Additionally, in a different study, the exposure of CD-1 mice to cadmium increased the proliferation of non-ciliated bronchiolar cells (Fortoul *et al.*, 2005). Also, cadmium exposure significantly reduced placenta weight. This supported the findings in several studies that showed the placenta as one of the cadmium target organs because it causes edema, necrosis and decreased utero-placental blood flow (Samarawickrama and Webb, 1979; Levin and Miller, 1980, 1981; Pařizek, 1964, 1965). Additionally, a recent study found that cadmium chloride reduced the placental weight, reduced proliferation and increased apoptosis in the labyrinthine region in placenta from mice, which can account for the reduced weight (Wang *et al.*, 2012). Similarly, cadmium reduced the fetal weight, as previously reported (Prigge, 1978a, b; Barański, 1984; Trottier *et al.*, 2002; Jacquillet *et al.*, 2007; Ronco *et al.*, 2009). Although the mechanism has not yet been elucidated, it is well known that cadmium impairs copper, calcium, sodium, potassium, zinc and iron transport across the placenta, altering the concentration of those trace metals in fetal liver and kidneys, and possibly affecting fetal development and metabolism (Kuriwaki *et al.*, 2005). Moreover, it has been shown that cadmium exposure reduces the internal space of maternal and fetal blood vessels in the labyrinth layer of the placenta, the place where oxygen and nutrient exchange occurs between the mother and fetus, which might explain the reduced fetal weight (Wang *et al.*, 2012). Furthermore, cadmium exposure elevates cortisone levels in dam and fetus plasma, which has been related to the presence of intrauterine growth restriction (IUGR) (Ronco *et al.*, 2009).

Histological findings in embryonic kidneys after cadmium exposure included tubular necrosis and degeneration, and the presence of hyaline cylinders in tubular lumen. These findings are similar to those reported in male Wistar rats after a 4-week oral exposure to cadmium (Tripathi and Srivastav, 2011) with the exception that we did not find glomerular alterations. In addition to tubular lesions, we observed protein precipitations in the renal pelvis. To our knowledge, this is the first report of an alteration in this portion of the kidney after cadmium exposure. This could be the result of an increase in the excretion of proteins due to tubular damage, which precipitated on their way of elimination.

For many years, the glomerular function has been evaluated through the quantification of creatinine levels in urine and plasma because it is a molecule that is freely filtered by the glomerulus and is not reabsorbed in the proximal tubule. Whenever there is a decrease in the rate of glomerular filtration, creatinine accumulates in the plasma and its urinary levels decrease. In this study, we quantified creatinine levels in amniotic fluid, as well as in fetus and dam plasma.

Cadmium exposure significantly reduced creatinine levels in amniotic fluid; nevertheless, histologic examination did not show

any glomerular alteration in fetal kidneys, so this reduction may be the result of the decrease in creatinine content in fetal plasma. In addition, this alteration can be explained by the fact that creatinine correlates with muscle mass (Narayanan and Appleton, 1980). Thus, because cadmium reduced fetal weight, there was a decrease in muscle mass that could have affected creatinine levels. Additionally, this decrease might be explained by a change in maternal–fetal transference rate because this molecule is capable of passively diffusing from dam plasma, through the placenta into fetal blood (Koszalka *et al.*, 1975).

Albumin is a functional biomarker that can indicate glomerular or tubular damage. In normal physiological conditions, a small amount of albumin is filtered by the glomerulus, and it is almost entirely reabsorbed at the proximal tubule by endocytosis; thus, only minimal amounts of this protein are found in urine (Toto, 2004). Glomerular damage can result in an excessive protein filtration or tubular damage can result in a reduced protein reabsorption, thus, the levels of urinary albumin will be increased. Plasma albumin in fetuses has two possible origins: the embryonic liver (where it is synthesized from GD 12 on) (Muglia and Locker, 1984) and the maternal plasma from where it is transferred (Morgan, 1964). In this study, the albumin levels in amniotic fluid were significantly elevated after cadmium exposure. This finding is consistent with the presence of tubular degeneration and necrosis found in fetal kidneys, which might have altered the tubular capacity to reabsorb proteins, including albumin. Because we did not find a significant modification in albumin plasma levels of the dams after cadmium exposure, an increased contribution from this source can be discarded.

OPN is a glycoprophosphoprotein that is normally expressed in the loop of Henle and distal tubule, and is eliminated in the urine. After ischemia/reperfusion (I/R) events, OPN gene and protein expressions are up-regulated in the entire tubular portion of the nephron promoting tissue repair and regeneration: consequently, its urinary levels increase (Vlasakova *et al.*, 2014). In the developing kidney, OPN can be detected from GD 13 in maturing ureteral epithelium (Denda *et al.*, 1998), metanephric blastema, S-shaped bodies (Rogers *et al.*, 1997) and developing tubules (Nomura *et al.*, 1988). It is thought to participate in the tubulogenesis process by inhibiting apoptosis mainly through the integrin receptor $\alpha_8\beta_1$ (Rogers *et al.*, 1997; Denda *et al.*, 1998). Our results showed a two-fold increase of OPN in amniotic fluid after cadmium inhalation, which is consistent with our histological findings of tubular degeneration and necrosis. This finding suggests the presence of a repair and regeneration process in the fetal kidney due to injury. A contribution from other sources where OPN is also expressed [i.e. calvaria, backbone and rib bones (Yoon *et al.*, 1987; Denda *et al.*, 1998; Nomura *et al.*, 1988)] seems unlikely because it is considerably modified post-translationally (Kazanecki *et al.*, 2007), which increases its molecular weight, making it more difficult to be filtered by the glomerulus. Although, to our knowledge, there are no studies that address the maternal transfer of OPN, a maternal contribution cannot be totally dismissed because of the very fine equilibrium between dam and fetus.

VEGF is a molecule that is involved in angiogenesis and vasculogenesis. In the fetal kidney, VEGF mRNA is detected from GD 14 but its protein is expressed until GD 19 in visceral and parietal epithelial cells from the glomerulus and in developing tubular cells where it promotes cellular proliferation, vasculogenesis, angiogenesis and tubulogenesis (Tufro *et al.*, 1999; Tufro, 2000). Its neutralization during kidney development causes fewer nephrons and abnormal glomeruli (Kitamoto *et al.*, 1997). Its use as a

biomarker of kidney injury has shown some variations depending on the cause of the nephropathy. Its urinary levels normally increase after kidney injury, but they may or may not relate to an up-regulation of the protein. For instance, treatment with cyclosporine induces VEGF expression mainly in proximal and distal tubules and sometimes in podocytes (Schrijvers *et al.*, 2004), which results in an increase of its urinary levels, whereas an I/R do not increase VEGF expression but rather promotes its redistribution from the cytoplasm to the basolateral surface (Kanellis *et al.*, 2000). Additionally, an increase in its levels in urine has been associated with podocyte loss rather than an up-regulation of its expression (Schrijvers *et al.*, 2004). In amniotic fluid, we detected a two-fold increase in VEGF levels after cadmium exposure, but because we did not observe glomerular damage, it is most likely that this increase reflects an overexpression or a redistribution of the protein in the tubular portion from the nephron due to tubular degeneration and necrosis.

TIMP-1 is an inhibitor of all matrix metalloproteinases (MMPs), particularly of MMP-2 and MMP-9, but it has also been shown to promote cell proliferation and differentiation and to inhibit apoptosis and angiogenesis in a manner independent of its activity over MMPs (Ries, 2014). TIMP-1 is expressed during kidney development, but its concrete function during this period is not completely understood; nevertheless, it is known that its expression pattern changes as the nephrogenic stage progress. On GD 11.5 and 12.5, TIMP-1 can be detected in non-induced and induced mesenchyme; by GD 16.5, it is mainly expressed in the ascendant loop of Henle and distal tubules, and only slightly expressed in proximal tubules; from birth until adulthood, it shows a similar expression pattern (ascendant loop of Henle and distal tubules, and a mild expression in collecting tubules) (Legallicier *et al.*, 2001). It is used as a kidney injury biomarker because elevated urinary and serum levels of TIMP-1 have been found in nephropathies of diverse etiologies (Hörstrup *et al.*, 2002). This increase in its urinary levels has been associated with damage in the proximal tubule (Minowa *et al.*, 2012) as well as collecting ducts (renal papilla) (Uehara *et al.*, 2013). TIMP-1 has also been detected in amniotic and extra-embryonic fluids from human and rabbit origin. Amniotic fluid TIMP-1 comes from different sources that include the epithelium of the amnion, the chorion, the placenta, the decidua and the fetus (Riley *et al.*, 1999a, b; Goldman *et al.*, 2003). Therefore, an alteration in any of these elements can change the levels of TIMP-1. An increase in its amniotic fluid levels has been related to chorioamnionitis, intra-amniotic infection, twin-to-twin transfusion syndrome and the fetal membrane repair process (Devlieger *et al.*, 2000; Locksmith *et al.*, 2001; Fox *et al.*, 2014), whereas a decrease is associated with the onset of labour and premature rupture of membranes (Vadillo-Ortega *et al.*, 1996; Goldman *et al.*, 2003). In this model, we found an important increase in the levels of TIMP-1 in amniotic fluid after cadmium exposure. This increase, like with OPN and VEGF, is consistent with the presence of tubular degeneration and necrosis. As mentioned before, the sources of amniotic fluid TIMP-1 are diverse, and they include the placenta, which was altered in our model by cadmium exposure; thus, a contribution from this source cannot be entirely discarded.

In contrast to the biomarkers mentioned so far, Calb 1, CLU and IP-10 did not show any statistically significant modifications in their protein levels in amniotic fluid. Calb 1 is a protein whose expression is confined to the distal and collecting tubules in adult as well as developing kidneys (Davies, 1994; Georgas *et al.*, 2008). Its use as a kidney injury biomarker has shown specificity for these portions of the nephron because its expression changes when these

structures are injured but not when the proximal tubules are injured (Iida *et al.*, 2014). Cadmium affects mainly the proximal tubules and glomeruli but not the distal tubules (ATSDR, 2012); therefore, the fact that calb1 was not changed indicates that cadmium-induced tubular damage in embryonic kidneys may be confined to the proximal tubule portion of the nephron and that cadmium affects similar structures in metanephric and fully developed kidneys.

Additionally, CLU is a glycoprotein that is expressed in comma- and s-shaped bodies as well as the tubular epithelia of embryonic kidneys (French *et al.*, 1993). In adult kidneys, CLU is expressed in the apical portion of the distal tubules and the cytoplasm of the proximal tubules (Betton *et al.*, 2005). It is up-regulated after tubular injury induced by different types of stimuli and it does not indicate injury to a particular tubular portion (Hidaka *et al.*, 2002; Vlasakova *et al.*, 2014). Unlike Kim-1 and OPN, CLU levels increase after the insult until they peak after which they start to decrease (Vinken *et al.*, 2012; Nguan *et al.*, 2014). It is possible that the moment at which we evaluated the CLU levels, its expression had already started to decrease almost reaching its basal levels because our results showed a mild increase in CLU levels that was not statistically significant.

IP-10 is a chemokine that attracts Th1 lymphocytes through its binding to the CXCR3 receptor. Studies have shown an increase in its renal expression after I/R events (Furuichi *et al.*, 2008; Stroo *et al.*, 2010), and its urinary levels in patients with acute kidney injury of diverse etiology, diabetic nephropathy and kidney allograft rejection (Vaidya *et al.*, 2008b); however, its role in kidney injury and reparative processes is not fully understood.

In this exposure model, IP-10 amniotic fluid levels were not altered by cadmium. This could be because after the I/R events, IP-10 is mainly expressed by infiltrated macrophages in the interstitium (Furuichi *et al.*, 2008). Our histological analysis did not show signs of interstitial infiltration with inflammatory cells, so the lack of an alteration of IP-10 levels is reasonable. Additionally, it is important to note that IP-10 renal expression after I/R is biphasic, showing a peak at the beginning of the inflammatory and recovery phases after which it decreases (Furuichi *et al.*, 2008). Therefore, it is possible that when we obtained amniotic fluid samples, IP-10 expression had already declined.

Unlike the rest of the biomarkers evaluated, Kim-1 and GST- α could not be quantified in this study. This could seem rather unexpected as traditional biomarker creatinine was readily quantified; nevertheless, it is important to remember that creatinine can be transferred from the dam to the fetus, which can contribute, in a certain degree, to the amount found in amniotic fluid.

The information about Kim-1 and GST- α in embryonic kidneys is extremely limited; however, possible explanations of the lack of these biomarkers in this matrix are described as follows. For instance, Kim-1 mRNA levels in rat embryonic kidney are low (Ichimura *et al.*, 1998), which can lead to an equally low level of the protein, making it difficult to be quantified with the technique we employed regardless of the amount of damage caused by cadmium exposure. Additionally, it is possible that the MMP-1- and MMP-3-dependant shedding of Kim-1 ectodomain was reduced because the amniotic fluid levels of TIMP-1 (inhibitor of MMPs) increased after cadmium exposure. Considering that Kim-1 ectodomain is the fraction quantified by the kit we used, the lack of results is reasonable.

GST- α expression in rat fetal kidney has not been reported to date, at least to our knowledge; nevertheless, in human embryonic kidney, its expression is low until the end of gestation and

increases after birth (Harrison *et al.*, 1990). As a result of the fact that nephrogenesis in mammals is similar, it is possible that this protein presents a similar pattern in rat embryonic kidney which might explain the lack of detection.

These facts should not be interpreted as the complete absence of these biomarkers in amniotic fluid and let alone as a lack of change on its expression in fetal kidney but as an encouragement to seek for alternative methods for their quantification in future studies so that an appropriate conclusion can be drawn regarding its usefulness for the detection of fetal kidney damage.

Also, it is important to remember that it is always advisable to quantify a panel of biomarkers when searching for kidney damage, due to interindividual variability.

In conclusion, cadmium inhalation during pregnancy induced tubular damage and protein deposits in the renal pelvis of fetal kidneys. These structural alterations correlated with an increase in the levels of several early kidney injury biomarkers in amniotic fluid samples. The increase in these biomarkers in amniotic fluid suggests their potential use in the diagnosis of kidney injury before birth, providing the possibility of timely treatment.

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Conflict of interests

The authors declare that they have no conflict of interest.

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