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Effect of a Single Dose of Cadmium on Pregnant Wistar Rats and their Offspring

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Contents
Cadmium (Cd) is a well-known toxicant targeting many organs, among them placenta. This heavy metal also has embryonary and foetal toxicity. This study was undertaken to analyse the effect of a single Cd dose administered at 4, 7, 10 or 15 days of gestation on the offspring of pregnant rats sacrificed at 20 days of gestation. Cadmium chloride was administered subcutaneously at 10 mg/kg body weight to Wistar pregnant dams; control animals received a proportionate volume of sterile normal saline by the same route. Maternal uteri, livers, kidneys and lungs, and foetuses were examined at necropsy. Samples of maternal organs and whole foetuses were collected for histopathologic examination, determination of Cd levels and staining by the Alizarin red S technique. Results revealed a clear embryotoxic and a teratogenic effect of this heavy metal, the former as a significant increase in the number of resorptions, and the latter as significant decrease of the gestational sac weight, and the size and weight of foetuses of Cd-treated dams as well as induced malformations in skull bones, vertebrae and thoracic, and pelvian limbs. The deleterious effects found were similar to those previously reported for other animal models suggesting a high conservation of the pathogenic mechanisms of Cd. Additionally, many of the addressed aspects showed a slight dependence on the time of administration of the toxic that might be due to the accumulation of the metal in different organs, as we were able to demonstrate by the analysis of its concentration.

Introduction

Cadmium (Cd) is a non-essential metal for the animal life (IARC 1993). It is widely distributed in the environment as a pollutant from industrial and agricultural sources (EFSA 2009). This heavy metal is employed to manufacture insecticides, fungicides, phosphate fertilizers, paints and batteries (ATSDR 1999). Tobacco smoking is another important source of Cd exposure (Dechanet et al. 2011).

In regard to toxicity, Cd is a well-known nephrotoxic (Edwards and Prozialeck 2009; Klaassen et al. 2009) and hepatotoxic agent (Koyu et al. 2006; Fahim et al. 2012). It has also been recognized as a toxicant targeting lungs (Klaassen et al. 2009; Stosic et al. 2010), thymus and spleen (Gonçalves et al. 2012) and bones (Bhattacharyya 2009; Klaassen et al. 2009). The toxicity of Cd on reproductive tissues was first reported in the second decade of the past century (Nordberg 2009). Since then a vast body of toxicological evidence both *in vivo* and *in vitro* has confirmed its deleterious effects on male and female gonads by a variety of mechanisms (Thompson and Bannigan 2008). Placental maternal and foetal components are also disrupted by Cd (Padmanabhan and Hameed 1990; Díaz et al. 2006). Recently, exposure to Cd has been associated to endocrine effects leading to its consideration as a metallohormone acting as an oestrogen and an androgen (Byrne et al. 2009; Kippler et al. 2010).

This metal also has embryonary and foetal toxicity affecting the development of pre-and post-implantation rodent embryos (Thompson and Bannigan 2008). Effects of Cd on rat foetuses reported comprise deformities in brain (hydrocephalus), eyes (anophthalmia, microphthalmia), gastroschisis, umbilical hernia (Samarawickrama and Webb 1979), urogenital abnormalities and cleft palate (Holt and Webb 1987). Several investigations confirmed its transport across the placenta and accumulation in foetuses (Kuriwaki et al. 2005; Kippler et al. 2010; Nakamura et al. 2012).

A growing body of works addresses the toxicity of this metal on reproductive parameters both in humans and animals (National Toxicology Program 2007). For this purpose, several experimental models have been employed including cellular systems as primary cell lines (human trophoblast cells Jar choriocarcinoma, Powlin et al. 1997), primary Sertoli cell-gonocyte coculture (Yu et al. 2005) and animal models such as mice, rabbits, hamsters, pigs, sheep and rats (Carter 2007). The rat is still preferred as a model system in studies on embryology and reproduction toxicology (Ain et al. 2006; Soares et al. 2012). Both rat placenta and human placenta are haemochorial and share some unique features regarding uterine trophoblast invasion and spiral artery remodelling (Ain et al. 2006; Soares et al. 2012).

Investigations assessing the toxic effect of Cd administered at different stages of pregnancy are scarce. Consequently, this study was undertaken to analyse the effect of a single Cd dose administered at 4, 7, 10 or 15 days of gestation on maternal organs and the offspring of pregnant rats sacrificed at 20 days of gestation.

Materials and Methods

Animals

Animal care was in compliance with the guidelines by the Bioethics Committee of the Veterinary Sciences School (U.N.C.P.B.A; Universidad Nacional del Centro de la Provincia de Buenos Aires).

Four-month-old virgin female Wistar rats (242.8 ± 22.3 g body weight) from the *bioterium* of the Veterinary Sciences School were used. Rats were kept under standardized conditions (room temperature 22 ± 2°C, 50–60% humidity and 12 : 12 h light–dark cycle) and were supplied pelleted feed and water *ad libitum* until

euthanasia. The general health status of the dams was monitored daily for signs of adverse effects (weight loss, decreased activity and decreased food or water intake).

Reproductive management

Females were mated with 6-month-old males (2 : 1). The presence of sperm in the vaginal lavage was considered day 0 of gestation (Ain et al. 2006).

Chemicals and treatments

Cadmium (II) chloride ($CdCI_2$) anhydrous powder was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Solutions were freshly prepared in sterile distilled water so that each dose resulted in 10 mg Cd^{+2}/kg body weight. The dose of treatment was based on previous reports (Padmanabhan and Hameed 1990; Zhao et al. 1997; Salvatori et al. 2004) and confirmed in our laboratory.

Cd administration was performed by a single subcutaneous (SC) injection for it renders higher systemic concentrations compared to oral administration (IARC 1993; Zalups and Ahmad 2003). Subcutaneous mode of administration was used in our present study as an intraperitoneal route of injections is not recommended during pregnancy (Nampoothiri and Gupta 2008).

Dams were randomly divided into four groups:

Group 4/20 (six animals) received a $CdCl_2$ SC injection on day 4 of gestation.
Group 7/20 (six animals) received a $CdCl_2$ SC injection on day 7 of gestation.
Group 10/20 (six animals) received a $CdCl_2$ SC injection on day 10 of gestation.
Group 15/20 (six animals) received a $CdCl_2$ SC injection on day 15 of gestation.

Correspondingly, control animals received a proportionate volume of sterile normal saline by the same route on days 4, 7, 10 and 15 of gestation.

Macroscopic examination and sample collection

At day 20, prior to euthanasia, dams were anaesthetized with 1.4% isoflurane in 100% oxygen and later euthanatised according to the protocol approved by the Bioethics Committee of the Veterinary Sciences School.

Maternal organs

Uteri, livers, kidneys and lungs were examined at necropsy. Additionally, samples of these maternal organs were collected for histopathologic examination and determination of Cd levels. After hysterectomy, the number of implantations and resorptions per dam were scored; results were expressed as mean ± SE.

Foetuses

Once the uteri were excised, the foetuses and their respective placentas were weighed. These data were used to the calculation of litter weight as well as the placental litter weight. The foetuses were evaluated for morphometric determinations: gestational sac weight, foetal weight and length (GL: greatest length, CRL: crown-rump length; HL: head length). Results were expressed as mean ± SE. Whole foetuses were also collected for histopathologic examination, determination of the Cd levels and staining by the Alizarin red S technique.

Histological techniques

Maternal organ samples and whole foetuses were fixed in 10% buffered formalin and processed for routine histological haematoxylin and eosin staining.

Alizarin red S staining technique

To determinate skeletal malformations, the Alizarin red S staining technique was performed according to the procedure proposed by Dawson (1926). Briefly, whole foetuses of control and Cd-treated mothers were fixed in 96° ethanol for 96 h. Skin, eyes, thoracic and abdominal viscera and adipose tissue were removed from each specimen. Later, foetuses were placed in acetone for 24 h, dehydrated in 96° ethanol for 24 h and macerated in 1% potassium hydroxide. Alizarin red S staining was then performed, and foetuses were cleared in graded concentrations of glycerine. Finally, foetuses were stored in 100% glycerol. All chemicals were of analytical grade and purchased from standard commercial suppliers.

Cd levels

The determination of the control values of Cd levels was performed on samples of liver, kidney, lung, placenta and foetuses collected of 25 untreated pregnant rats sacrificed at day 20 of gestation. Tissues were prepared as described by Brown et al. (1986). Briefly, organs were weighed and placed in tubes with 2 ml of nitric acid. The samples were heated at 100°C until their volume was reduced by half. Next, 2 ml perchloric acid was added, and the samples were again heated at 100°C until their volume was reduced by half. Finally, the samples were diluted to 5 ml with deionized water. Cd concentration was determined with a GBC (Victoria, Australia) 906-AA atomic absorption spectrometer. Control values were considered within the range of arithmetic mean + 2 SD of Cd concentration found in samples of organs and foetuses of untreated dams, expressed as part per million of dry matter (ppm). Cd-treated groups were assigned in two categories whether their value outcomes were lower or higher than arithmetic mean + 2 SD.

Statistical analysis

Morphometric data were examined by Student's *t*-test and multifactorial ANOVA to establish interaction effects between time of pregnancy and time of Cd administration. Fisher's test was employed to establish differences in the relative number of implantations/number of resorptions, and for Cd concentration analysis. The

level of significance chosen was 0.05, unless indicated otherwise.

Results

Maternal organs

At necropsy, circulatory disturbances were the most frequent lesions found in all organs of treated dams; they consisted mainly in congestion and haemorrhage. No circulatory alterations were observed in control dams. The histopathologic examination of livers, kidneys and lungs confirmed congestion and haemorrhages and showed mild inflammatory infiltration, degenerative changes and necrosis (Fig. 1 a–c).

Implantations and resorptions

Table 1 summarizes the implantations and resorptions found in control and Cd-treated groups. The number of implantations ranged between 7.00 and 10.33 and 4.00–9.33 for control and Cd-treated groups, respectively.

None of the control dams showed embryonic resorptions whereas numerous resorptions were observed in Cd-treated dams of all groups, and they ranged between 1 and 13 per dam. The comparison of the number of resorptions between control and experimental groups revealed statistical significant differences (Table 1).

The microscopic evaluation of resorptions showed calcification areas, trophoblastic cellular degeneration and inflammatory cells; maternal placental debris, placental necrotic tissue and smooth muscle fibres intermingled with placental tissue. Also abundant cell death areas in the spongiotrophoblast, placental normal tissue accompanied by necrotic areas and embryonic remainings containing disorganized tissues and vascular congestion were found.

Morphometric and macroscopic evaluation of foetuses

Results of the morphometric evaluation of foetuses are presented in Table 2. The comparison to control dams showed that metal exposure did not affect the gestational sac weight, and the size and weight of foetuses of Cd-treated dams.

The macroscopic examination allowed the determination of a high number of malformations in the foetuses of Cd-treated dams (Table 3). Malformations included amelia, brachygnathia, omphalocele, cauda, and anotia. These anomalies were found regardless of the day of treatment. Foetuses of control mothers showed no evidence of macroscopic malformations. The analysis of Alizarin-stained experimental foetuses confirmed severe anomalies mainly in head, axial skeleton and paws (Fig. 2). Brachygnathia, and absence or incomplete development of some skull bones were the most frequent head malformations. Some vertebrae, ribs and tail bones were absent in the axial skeleton. In paws, metacarpal and metatarsal bones, absence and lower number of phalanges were the most common features found. All these congenital malformations were found in foetuses of Cd-treated mothers of all experimental groups.

The microscopic examination of haematoxylin–eosin sections of foetuses from Cd-treated dams showed, excluding malformations, no relevant pathological alterations.

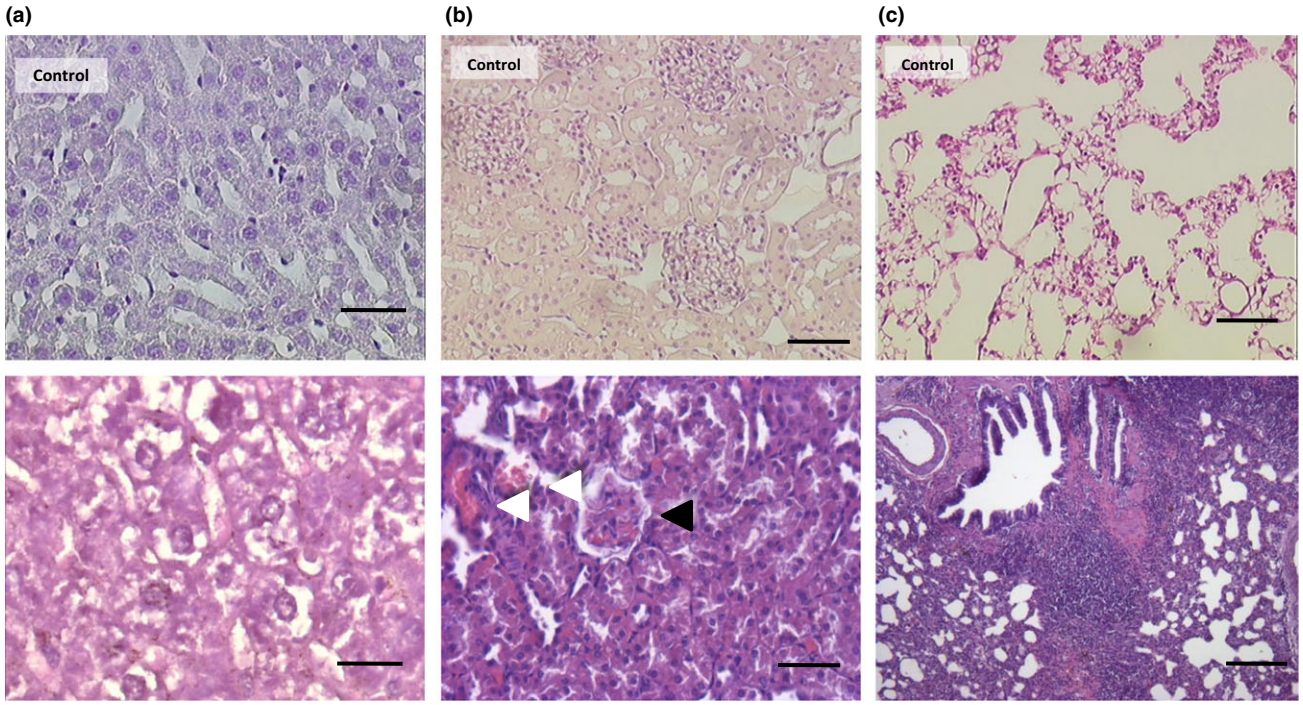

Fig. 1. Pathological lesions induced in maternal organs by Cd acute exposure. Haematoxilyn & eosin staining. (a) *Liver*. Section of the liver of control dam (20×, scale bar = 40 μm). Hidropic degeneration in the liver of a group 10/20 dam. 40×. Scale bar = 20 μm. (b) *Kidney*. Section of the kidney of control dam (20×, scale bar = 40 μm). Corpuscle necrosis (black arrow head) and vascular congestion (white arrow head) in the kidney of a 10/20 group dam. 20×. Scale bar = 40 μm. (c) *Lung*. Section of the lung of control dam (10×, scale bar = 80 μm). Inflammatory infiltrate, emphysemic and atelectasy in a lung of a group 10/20 dam. 4×. Scale bar = 200 μm.

Table 1. Number of implantations and resorptions in control and Cd-treated dams[a]

	Control	Group 4/20	Control	Group 7/20	Control	Group 10/20	Control	Group 15/20
Implantations per dam	10.33	4.00**	7.00	9.33**	7.00	5.17**	9.33	6.67**
Total implantations	62.00	24.00**	42.00	56.00*	42.00	31.00**	56.00	40.00**
Resorptions per dam	0.00	3.17**	0.00	1.33*	0.00	3.50**	0.00	4.17**
Total resorptions	0.00	19.00**	0.00	8.00**	0.00	21.00**	0.00	25.00**

[a]Results are expressed as arithmetic means.
**$p < 0.01$; *$p < 0.02$.

Table 2. Morphometric characteristics of the concepti[a]

Group	Gestational sac weight (g)	Weight (g)	GL (cm)	CRL (cm)	HL (cm)
Control	4.94 ± 0.28	3.96 ± 0.12	3.19 ± 0.65	2.60 ± 0.77	1.07 ± 0.55
4/20	5.02 ± 0.42	3.89 ± 0.10	3.37 ± 0.39	2.69 ± 0.29	1.35 ± 0.10
Control	5.02 ± 0.42	3.77 ± 0.08	3.34 ± 0.20	2.60 ± 0.11	1.37 ± 0.04
7/20	4.95 ± 0.45	3.85 ± 0.30	3.62 ± 0.19	2.92 ± 0.20	1.48 ± 0.19
Control	4.40 ± 0.46	3.36 ± 0.53	3.18 ± 0.29	2.51 ± 0.20	1.32 ± 0.10
10/20	4.71 ± 1.91	3.70 ± 1.66	3.27 ± 0.77	2.65 ± 0.65	1.30 ± 0.27
Control	4.41 ± 1.61	3.24 ± 1.42	3.08 ± 0.82	2.45 ± 0.70	1.27 ± 0.20
14/20	5.23 ± 1.53	4.22 ± 1.39	3.59 ± 0.45	2.86 ± 0.45	1.36 ± 0.10

GL, greatest lenght; CRL, crump rump lenght; HL, head length.
[a]Results expressed as mean ± SE. Differences were not statistically significant.

Cd levels

Table 4 summarizes the results of Cd levels for maternal organs, placentas and foetuses. Cd levels of all organs and foetuses from the totality of the experimental groups increased and were highly significant when compared to controls ($p < 0.001$) with the exception of lung in 7/20 group, which was significant at $p < 0.014$. However, lungs of dams intoxicated on days 4 and 7 and foetuses from groups 10/20 and 15/20 resulted in the minimal percentage of elevated Cd concentrations.

Discussion

In the present study, the deleterious effect of a single dose of Cd administered to pregnant rats on days 4, 7, 10 or 15 of gestation was evaluated regarding the characteristics of the offspring and several features of the embryonic morphogenesis. The outcomes revealed a clear embryotoxic and a teratogenic effect of this heavy metal. Although Cd-treated dams showed no clinical signs of intoxication, the histopathologic findings in all evaluated maternal organs verified the systemic toxicity achieved by Cd administration, which was additionally confirmed by high Cd levels. These results are in agreement with previous works by Rubio et al. (1998), Tzirogiannis et al. (2004), Koyu et al. (2006) and Edwards and Prozialeck (2009), who reported similar lesions for lungs, kidneys and liver.

In contrast to the investigation of Baranski et al. (1982) who reported either a teratogenic or a foetotoxic effect depending on the administered dose, we found both effects under the experimental design employed rendering embryonic resorptions and skeletal defects.

Table 3. Congenital malformations induced by maternal Cd administration[a]

Malformations	Group 4/20	Group 7/20	Group 10/20	Group 15/20
Head				
Brachygnathia	3 foetuses	4 foetuses	2 foetuses	
Skull bones	Supraoccipital bone agenesia	Supraoccipital bone agenesia	Skull bones agenesia	Skull bones agenesia
	2 foetuses	2 foetuses	2 foetuses	2 foetuses
	Parietal bone agenesia	Incomplete interparietal bone		
	1 foetus	1 foetuses		
Axial skeleton				
Lower number of vertebrae	1 foetus	1 foetus	2 foetuses	3 foetuses
Tail vertebrae agenesia	2 foetuses	2 foetuses	2 foetuses	2 foetuses
Lower number of ribs	2 foetuses	2 foetuses		
Paws				
Metacarpal bones agenesia	4 foetuses	2 foetuses	2 foetuses	2 foetuses
Metatarsal bones agenesia	4 foetuses	1 foetus	2 foetuses	2 foetuses
Lower number of phalanges	1 foetus	1 foetus	1 foetus	1 foetus

[a]No malformations were observed in foetuses of control dams.

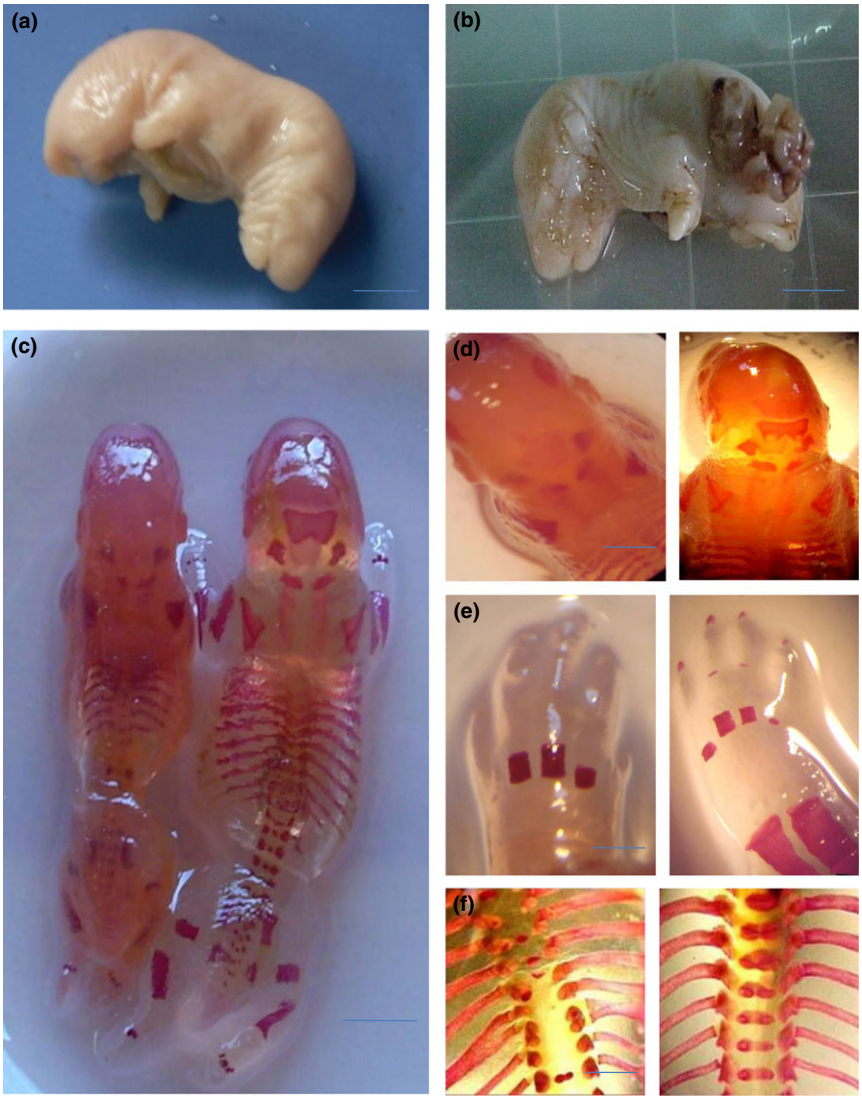

Fig. 2. Malformations induced by cadmium. Alizarin red S technique. (a) Foetus showing anotia, hind limb amelia, and cauda. Scale bar = 5.0 mm. (b) Foetus affected by omphalocele. Scale bar = 5.0 mm. (c) Skeletal alterations in a foetus of group 15/20 (left); control foetus (right). Scale bar = 5.0 mm. (d) Skull malformations in a foetus of group 15/20 (left); control foetus (right). Scale bar = 2.5 mm. (e) Metacarpals absence in a foetus of group 10/20 (left); control foetus (right). Scale bar = 0.6 mm. (f) Ribs and vertebrae alterations in a foetus of group 10/20 (left); control foetus (right). Scale bar = 2.5 mm.

Table 4. Porcentage of maternal organs, placentas, and foetuses with Cd concentration values of arithmetic mean + 2 SD or higher

	Reference value[a]	Group 4/20[b]	Group 7/20[b]	Group 10/20[b]	Group 15/20[b]
Liver	36.66	86%**	100%**	100%**	100%**
Kidneys	31.73	100%**	71%**	75%**	100%**
Lungs	5.50	37%**	67%*	78%**	80%**
Placenta	4.05	100%**	80%**	88%**	100%**
Foetuses	2.39	100%**	80%**	28%**	25%**

[a]Cd-concentration values expressed as ppm (parts per million).
[b]Values of all organs, placentas, and foetuses of control groups were lower than reference value.
**$p < 0.001$, *$p < 0.014$, Fisher's test.

Additionally, the results of the present study confirm preliminary findings, namely malformations in skull bones, vertebrae and thoracic, and pelvian limbs in the litters of pregnant rats treated with a single dose of $CdCl_2$ (Díaz et al. 2006).

Under our experimental design, the teratogenic effects of a single dose of Cd were not dependant of the time of administration. We found similar effects in foetuses of dams treated on different times of their pregnancy. Previous investigations have demonstrated that Cd is transported across the rat placenta at different times of gestation (Holt and Webb 1987; Nakamura et al. 2012). In regard to the pregnant rats that received a single Cd dose prior to the onset of placenta and embryo organogenesis (groups 4/20, 7/20 and 10/20), the metal accumulated in maternal liver might be released into systemic circulation by damaged hepatocytes (Zalups and Ahmad 2003) and thus would reach foetal organs several days after treatment as a result of its recirculation into blood stream.

In view of the diversity of routes of administration, the doses of Cd administrated and the experimental designs employed, the finding of skeletal alterations proves that developing bone is a general target tissue for Cd. Negative effects on developing skeleton found in this study are not limited to rats. Mice foetuses on day 18 of gestation collected from Cd-treated females revealed, at gross examination, exencephaly, facial abnormalities and axial skeletal dysmorphogenesis (Padmanabhan and Hameed 1990). Additionally, Scott et al. (2005) report that exposure of mice to $CdSO_4$ at day 9.5 of gestation induces post-axial forelimb ectrodactyly. It is known that this metal alters differentially the proliferation and cell death indices in different cell types for Cd inhibits apoptosis and stimulates cell proliferation in a murine cell line but, on the other hand and depending on the concentrations, it may cause apoptosis or necrosis (Martin and Pognonec 2010; Templeton and Liu 2010) or autophagy (Yang et al. 2009). Alterations in cell proliferation and death kinetics may cause irreversible modifications in morphogenesis. Other explanations regarding bone malformations have been associated with the deleterious effects Cd has on angiogenesis Prozialek et al. (2006), and the maintenance of cell adhesion (Thompson and Bannigan 2008) as both processes are intimately associated to osteogenesis (Cancedda et al. 2000). Although the aforementioned processes might take part in the pathogenesis of Cd-induced skeletal alterations, the effect of Cd on gene expression could not be discarded. Recent investigations have demonstrated that Cd inhibits Hox genes expression in the somites of chicken (Doi et al. 2010a). While Doi et al. (2010c) associate Cd-treatment with ventral body wall defects, changes in Hox genes could also account for bone malformations (Young et al. 2009). It has also been demonstrated that Cd in chicken embryos decreases the expression of different genes of the TGF beta group, whose products are also involved in osteogenesis (Doi et al. 2010b). Another important substance for normal development of skeletal tissues is the hypoxia-induced factor alpha 1, which regulates the expression of some genes in response to changes in tissue oxygen levels and whose expression is altered by the presence of high Cd concentrations (Duval et al. 2009). It follows then, that the appearance of skeletal malformations in Cd poisoning may be the result of different mechanisms acting together.

The teratogenic effects of Cd are not limited to bone tissue as reported by Salvatori et al. (2004) in a study addressing long-term effect in the offspring of rats exposed to Cd. Besides cleft palate, deformed ribs and reduced cranial ossification, these researchers found renal cavitation and visceral anomalies, that is hypoplasic lungs, deformed veins (Salvatori et al. 2004). Our findings in this study are partially consistent with outcomes reported by Salvatori et al. (2004), as we found omphalocele as a soft tissue lesion solely in foetuses of group 15/20. Such malformations induced by high levels of Cd have also been found in chicken embryos (Thompson and Bannigan 2008) and have been associated with a multiplicity of toxic effects produced by the downregulation of the expression of Hox and TGF beta genes (Doi et al. 2010a,b; respectively) or changes in adhesion molecules (Thompson and Bannigan 2008; Doi et al. 2010c).

Baranski et al. (1982) and Hazelhoff Roelfzema et al. (1989) have reported foetotoxic effects as the decrease in foetal weight showing a dose–response relationship. In contrast, in our study, we found no significant differences between foetal weights in control and experimental groups. Discrepancies among reports might be attributable to differences in the experimental design. In the present study, single SC doses of $CdCl_2$ resulted in numerous resorptions and foetal deaths in the majority of the treated dams. This is in coincidence with Padmanabhan and Hameed (1990) who administered single SC doses of $CdCl_2$ to mice from day 7–12 of gestation. As many prenatal deaths occurred in our experimental groups, most possibly the surviving foetuses were able to adapt and thus showed weights and sizes higher than foetuses from control mothers.

In accordance to the foetal alterations described, we have found, by means of the determination of Cd levels that this metal reached the foetuses regardless of the day of administration or stage of pregnancy. However, the Cd levels in embryos and foetuses resulted higher when the treatment was performed before the development of a functional chorioallantoic placenta, namely on days 4–7. In contrast, when the toxic was administered later than the onset of chorioallantoic placentation (days 10–15 according to Wooding and Burton 2008), the foetal levels of Cd decreased. Possibly, already developed placenta accumulates Cd and no such large quantities of the metal are transferred to the foetus.

Results from our study showed that a single SC–Cd dose administered to pregnant rats induced maternal and embryo toxicity and also exerted teratogenic effects revealed by maternal lesions, embryonic and foetal death and occurrence of malformations, respectively. The deleterious effects found were similar to those previously reported for other animal models suggesting a high conservation of the pathogenic mechanisms of Cd. Those effects also are in agreement to other investigation performed under different experimental designs. Additionally, many of the analysed aspects showed a slight dependence on the time of administration of the toxic that might be due to the accumulation of the metal in different organs, as we were able to demonstrate by its concentration.

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Conflict of interest

The authors declare there is no conflict of interest with any financial organization regarding the material discussed in this work.

Author contributions

MC Díaz is the main contributor of the work performed, and concerning all tasks. NV González has analised data, written the

English version of the manuscript, and revised it critically. Susana Gómez, Miguel A Quiroga, and Roberto Najle have performed multiple roles in the experimental phase of the work. CG Barbeito is the main researcher and chief of the team; he contributed in the writing and critical reading of the manuscript, both original and revised versions.

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