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# Maternal exposure to cadmium during gestation perturbs the vascular system of the adult rat offspring

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ABSTRACT

Several cardiovascular diseases (CVD) observed in adulthood have been associated with environmental influences during fetal growth. Here, we show that maternal exposure to cadmium, a ubiquitously distributed heavy metal and main component of cigarette smoke is able to induce cardiovascular morpho-functional changes in the offspring at adult age. Heart morphology and vascular reactivity were evaluated in the adult offspring of rats exposed to 30 ppm of cadmium during pregnancy. Echocardiographic examination shows altered heart morphology characterized by a concentric left ventricular hypertrophy. Also, we observed a reduced endothelium-dependent reactivity in isolated aortic rings of adult offspring, while endothelium-independent reactivity remained unaltered. These effects were associated with an increase of hem-oxygenase 1 (HO-1) expression in the aortas of adult offspring. The expression of HO-1 was higher in females than males, a finding likely related to the sex-dependent expression of the vascular cell adhesion molecule 1 (VCAM-1), which was lower in the adult female. All these long-term consequences were observed along with normal birth weights and absence of detectable levels of cadmium in fetal and adult tissues of the offspring. In placental tissues however, cadmium levels were detected and correlated with increased NF-κB expression – a transcription factor sensitive to inflammation and oxidative stress – suggesting a placentary mechanism that affect genes related to the development of the cardiovascular system.
Our results provide, for the first time, direct experimental evidence supporting that exposure to cadmium during pregnancy reprograms cardiovascular development of the offspring which in turn may conduce to a long term increased risk of CVD.



## Introduction

Evidence from both human and animal studies suggests that many diseases manifested in adulthood are associated with environmental factors during fetal life, as consequence of the metabolic plasticity of organisms. (Barker et al., 2002; Louey and Thornburg, 2005) Low birth weight, childhood growth, and subsequent disease in adulthood have all been linked to several adverse environmental influences during early development (Bateson et al., 2004; McMillen and Robinson, 2005; Gluckman et al., 2007). In the adult, lifestyle, nutrition, and physical activity are known factors that significantly affect the risk for cardiovascular disease (CVD). In addition, a number of studies suggest an association between risk of CVD and abnormal intrauterine growth, despite normal birth weight (Hoet and Hanson, 1999; Hawkins et al., 2000). Although these phenotypic consequences have been defined based on experimental studies mainly focused on maternal nutrition perturbations, little attention has been given to the role of increasing air pollutants, contaminants, and toxicants emerging from industrial development in CVD risk.

Cadmium ($Cd^{2+}$) is a heavy metal extensively used in industrial processes and consumer products. Although widely dispersed in the environment, tobacco smoke may be the main source of $Cd^{2+}$ exposure affecting the general population (Bhattacharyya et al., 2000; Henson and Chedrese, 2004). It has been shown that direct $Cd^{2+}$ exposure may be involved in CVD including hypertension, atherosclerosis, and myocardial infarction, probably due to endothelial dysfunction (Wolf and Baynes, 2007; Messner et al., 2009; Gallagher and Meliker, 2010; Peters et al., 2010). Recent reports highlighting the relationships of $Cd^{2+}$ exposure and vascular diseases indicate that the vascular endothelial cells are potential targets for $Cd^{2+}$ toxicity (Prozialeck et al., 2006, 2008). The function and integrity of the vascular endothelium, lined with endothelial cells (EC), play a critical role in the mechanisms of blood flow, maintenance of the vessel wall structure, and circulatory function (Galley and

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Webster, 2004). Endothelial cells are dynamic, having both metabolic and synthetic functions, with significant autocrine, paracrine, and endocrine actions that influence smooth muscle cells, platelets, and peripheral leucocytes (Galley and Webster, 2004). Endothelial cells express several adhesion molecules such as the vascular endothelium molecule (VCAM), which is minimally expressed on resting EC; its expression, however, can be increased by cytokines and toxicants including $Cd^{2+}$ as recently reported (Park et al., 2009).

$Cd^{2+}$ is a bivalent cation whose molecular mechanisms of toxicity are not fully understood. It has been reported that $Cd^{2+}$ is unable to generate free radicals, neither in vitro nor under physiological conditions (Cuypers et al., 2010). Nevertheless, $Cd^{2+}$-mediated oxidative stress effects have been previously described, and represents a potential mechanism for its toxicity (Eneman et al., 2000). In this respect, lipid oxidation has been linked to cardiac disease and atherosclerosis, the primary cause of heart disease (Kovacs et al., 1997; Kummerow et al., 2000). Another potential mechanism of $Cd^{2+}$ toxicity may be associated with the activation of specific genes as a defensive reaction against metal-induced oxidative stress. It has been shown that $Cd^{2+}$ can induce the expression of heme oxygenase-1 (HO-1), a gene induced by agents that cause oxidative stress, as a mechanism to protect against aortic endothelial dysfunction (Chen et al., 2008). Also, transcription factors sensitive to oxidative stress, such as AP-1 and NF-κB, are activated when animals and cultured cells are exposed to $Cd^{2+}$ (Yang et al., 2007).

Since most toxicological reports have been focused on studying the effects of direct heavy metals exposure, little is currently known about the potential effects of prenatal exposure and its consequences in CVD during adulthood. In this study, we investigated whether prenatal exposure to $Cd^{2+}$ (30 ppm) affects fetal growth, and endothelial and cardiovascular function. To determine whether endothelial vascular function and heart morphology changes were associated with inflammation or oxidative stress mechanisms, the expression of *VCAM-* and, *HO-1* genes was quantified in aortas of adult animals previously exposed to $Cd^{2+}$ during their intrauterine life. Additionally, in order to evaluate whether the effects are induced by a direct exposure of fetuses to $Cd^{2+}$ either through transference by the placenta or through breast-feeding, we measured $Cd^{2+}$ levels in fetuses, maternal milk and offspring tissues at different postnatal periods. Some of these responses were evaluated in females and males, since heavy metal toxicity has gender dimorphism (Vahter et al., 2007). All experiments were conducted by feeding animals with a diet containing n-3 fatty acids to protect the cardiovascular (CV) system, thus avoiding possible effects that may have interfered with diet (Galli and Risé, 2009).

## Materials and methods

*General.* All procedures were performed according to the guidelines of the American Veterinary Medical Association (AVMA) (Report of the AVMA, Panel on Euthanasia, 2001) and approved by our local Bioethics Committee for Animal Experimentation at the Institute of Nutrition and Food Technology (INTA), University of Chile, Santiago, Chile.

*Animals and treatments.* Virgin female Wistar rats (200–250 g weight) were placed in animal cages with mature male breeders and mating was confirmed by the presence of a vaginal copulation plug (day 0 of gestation). Once females with vaginal plugs were identified, they were housed separately and considered pregnant (gestational day 0). Pregnant rats were singly housed and maintained 12 h light: 12 h dark with free access to pure distilled water (control: C, $n=16$) or with $CdCl_2$ (treated: T, 30 ppm of $Cd^{2+}$, $n=16$) given from day 0. The treatment with $Cd^{2+}$ continued throughout pregnancy. Animals were feeding *ad libitum* with a diet (Champion, Co, Santiago, Chile) composed of 12.7% protein, 33.4% total fat (n-3 fatty acids: 3·% DHA, and 3% EPA) and 522 Kcal per 100 g of food.

*Measurements and sample extraction.* Maternal weight, water and food consumption were monitored throughout pregnancy. One day before delivery (day 20 of pregnancy), half of the pregnant rats ($n=8$ per group) were anesthetized and placentas and fetuses were extracted. A total of 192 offspring were weighed and sized. Placentas were weighed and frozen in liquid nitrogen and then kept at −80 °C until analysis of $Cd^{2+}$ levels and molecular analysis. The rest of pregnant rats ($n=8$ per group) continued until delivery (day 21 of pregnancy) and litters were randomly culled to 10 offspring (5 males and 5 females) which were maintained with their mothers until weaning (day 21 postnatal). The fifth day after delivery and during lactation, milk samples of 6 different $Cd^{2+}$-treated rats were collected for $Cd^{2+}$ measurements. After weaning, offspring of the treated group were randomly selected and sacrificed, and blood and organs were extracted for $Cd^{2+}$ measurements. The remaining offspring were separated by gender and maintained in cages with distilled water and food *ad libitum* until 60—70 days old, when echocardiographic studies were conducted. Afterwards, animals were sacrificed by exposure to an atmosphere of 100% carbonic anhydride to induce cerebral death. Aortas were extracted for vascular reactivity determinations and molecular analysis. To minimize litter to litter variation, two adult males and two females' offspring of each litter were randomly chosen; for statistical analyses, we used the mean of the offspring values belonging to each litter ($n=8$ litters per treatment).

*Determination of cadmium concentrations.* Cadmium was determined in fetuses, placentas, and tissues from 21 days old offspring (blood, liver, kidney and aortas), maternal milk (extracted during the whole lactation period and pooled), and aortas at 60 days old, by inductively coupled plasma with mass spectrometry ICPMS (ICPMS Agilent 7500 with a $Cd^{2+}$ program) with a detection limit (LOD) of 15 ng/ml (blood and milk) and 30 ng/g of dry tissue. Plasma and milk samples (1 ml) were extracted with nitric acid (1 ml) and $H_2O_2$ (0.5 ml) for 1 h at 150 °C to dryness. This extraction protocol was repeated three times. Then, 0.2 ml of nitric acid was added and samples were diluted to 10 ml. Diluted samples were then measured with an internal standard. Similar protocol was applied to lyophilized samples of tissues and fetuses. Placentas, fetuses and tissues were lyophilized and stored at −20 °C until $Cd^{2+}$ measurements.

*Extraction of aortas and determination of vascular reactivity.* The thoracic aortas were rapidly removed and carefully cleaned of all fat and connective tissue, taking special care to avoid endothelial damage. Aortic rings (2 - 3 mm) were mounted immediately on two L-shaped stainless steel hooks in a 30 ml organ bath containing a modified Krebs–Henseleit solution maintained at 37 °C and bubbled with a 95% $O_2$ and 5% $CO_2$ gas mixture, as previously described (Pinardi et al., 1992). One of the hooks was attached through an FT-03 force-displacement transducer to a screw gauge and a model 7 Grass polygraph (Grass Instruments, Quincy, Mass, USA) to record changes in vessel wall tension, while the other was fixed to the bottom of the bath. The resting tensions of the arterial rings were set to 1.5 g by means of the screw gauge. The rings were allowed to equilibrate for 60 min, changing the solution at 15 min intervals to prevent metabolite accumulation. After the stabilization period and before the experiment, a maximal muscle tension was induced by a 70 mM KCl depolarizing solution, as an internal control. The rings were challenged twice until the response reached a plateau, followed by a complete return to the baseline after thoroughly washing to avoid any residual effect of this solution. Following re-equilibration, norepinephrine (NA; $10^{-7}$ M) was added to the bath and the contractile response was allowed to reach a plateau. Acetylcholine (ACh; $10^{-8}$ to $10^{-4}$ M) was then added in a cumulative fashion to the bath in ½ $\log_{10}$ increment. The relaxation response was allowed to reach a plateau before adding the next ACh

concentration. After washing the rings several times to completely wash out ACh and to attain baseline tension, NA ($10^{-7}$ M) was again added and the relaxation induced by sodium nitroprussiate (NP; $10^{-8}$ to $10^{-6}$ M) was recorded. The maximal relaxation induced with NP was achieved with a unique high dose of 100 μM. After the experiment, the wet weight of each ring was registered. Developed muscular tension was expressed as mg tension/mg wet weight. All changes were expressed as percent of the maximal response achieved by NA in each ring (Pinardi et al., 1992).

*Quantitative expression of VCAM, HO-1 and NF-κB mRNA.* NF-κB mRNA was determined in placentas and VCAM-1 and HO-1 mRNA were determined in aortas of offspring of both control and $Cd^{2+}$-treated mothers by real time PCR. Placentas or aortic tissue of 60–70 days old offspring were frozen in liquid $N_2$ and then at −80 °C until RNA extraction. Total RNA was extracted from whole placentas or 50 mg of aortic tissue using TRI Reagent kit (Ambion Austin, TX) according to the manufacturer's instructions. Total RNA was determined by spectrophotometry, measuring the absorbance ratio at 260/280 nm to evaluate the integrity, and the absorbance at 260 nm to determine its concentration. Single strand cDNA (ss-cDNA) was synthesized in a standard reverse transcription reaction using 1 μg of total RNA previously treated with DNAse, 200U M-MLV reverse transcriptase (Promega Madison, WI), and 0.5 μg oligo$(dT)_{15}$ (Promega). This ss-cDNA was used as template for quantitative PCR (qPCR) assays using water instead cDNA as negative control. Specific primers were designed using the premier Program v 5.00 (PREMIER Biosoft International Palo Alto, CA, USA). For HO-1, (Acc bank nº 61098187) fw: 5′-CCAGCATATACCCGCTACCT-3′, rev 5′-TCTGTCACCCTGTGCTTGAC-3′; for VCAM-1 (Acc bank no. 6981699) fw: 5′-TGCCAGCGAGGGTCTACCA-3′, rev:5′-CTCAACACCCACAGGGCTCA-3′; for NF-κB (*Rattus norvegicus* nuclear factor of kappa light chain gene enhancer in B-cells 1, p105, Nfkb1 cDNA; GenBank acc. no XM-342346) fw: 5′-GGCAGCACTCCTTATCAACC-3′; rv: 5′-GAGGTGTCG-TCCCATCGTAG-3′. Actin (Actb) was used as housekeeping gene (Accession no. 42475962); fw: 5′-CCGTAAAGACCTCTATGCCA-3′; rev 5′-AAGAAAGGGTGTAAAACGCA-3 having a product size of 352 pb. Gene transcript levels of all genes were quantified separately using the LightCycler® FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) and a program with an activation step at 94 °C for 10 min followed by an amplification step with 40 cycles of 5 sec at 94 °C, 8 sec at 64 °C and 20 sec at 72 °C for VCAM-1; 5 sec at 94 °C, 8 sec at 62 °C and 20 sec at 72 °C for HO-1 and 5 sec at 94 °C, 6 sec at 60 °C and 12 sec at 72 °C for NF-κB. The mRNA in each RNA sample was quantified by the relative standard curve method and the relative amount of the specific genes was obtained and normalized to the values of *β-actin*. Data represent the averaged of two experimental replicates from six independent biological samples.

*Determination of HO-1 by Western blot.* Frozen aortas from female adult offspring, control and exposed to $Cd^{2+}$ during gestation, were thawed and homogenized, using ice-cold radio-immuno precipitation assay buffer (RIPA)(Thermo Scientific, Rockford, USA) to extract proteins. Homogenates were centrifuged at 12,000 g for 10 min to remove nuclei and cellular debris, and supernatants were used for Western blot analysis. Total protein content was determined with an assay kit (BioRad, Hercules, Calif., USA). All steps were carried out at 4 °C unless stated otherwise. Aliquots of lysates were separated using 10% polyacrylamide gel (PAGE) containing sodium dodecyl sulfate (SDS) along with a pre-stained, broad-range, molecular weight marker (New England Biolabs Inc., MA, USA). The separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Thermo Scientific, Rockford, USA) overnight at 4 °C. The membranes were then blocked with 5% non-fat milk in tris buffered saline with 1% Tween-20 (TBS-T) for 1 h and then incubated with specific primary antibodies against HO-1 (Abcam, Cambridge, UK) at a dilution of 5 μg/mL in buffer TBS-T containing 5% non-fat milk overnight at 4 °C. For internal control, membranes were incubated with primary antibody against actin (Abcam, Cambridge, UK) at a dilution of 1:7000. The membranes were washed 4× with TBS-T for 5 min each and incubated for 1 h with goat anti-mouse secondary antibody–horseradish peroxidase conjugates (Chemicon, MA, USA) at 1:1500 dilution. After the membranes had been washed thoroughly, specific bands were developed using an enhanced chemiluminescence (ECL) Western blotting detection kit (Lightning Plus-ECL, Perkin Elmer, MA, USA) and exposing the membranes to film (Amersham hyperfilm™ ECL, Gen. Electric Health Care Limited, Buckinghamshire, UK) to obtain a signal. Bands were measured using densitometry with software (Image J).

*Echocardiographic analysis.* Female offspring (60–70 days old) of control and $Cd^{2+}$-treated rats were anesthetized with mixed Ketamine/Xylazine (40 mg/kg/3 mg/kg) given IP. Images were obtained using Sonosite 180 plus echocardiograph equipped with an electronic 10-MHz linear-array transducer. Following parameters were measured: aortic diameter (AD), left ventricular end-systolic cavity (LVESC), LV end-diastolic cavity (LVEDC), anterior left ventricular-diastolic wall thickness (ALVDWT), anterior left ventricular-systolic wall thickness (ALVSWT), posterior left ventricular-diastolic wall thickness (PLVDWT), posterior left ventricular-systolic wall thickness (PLVSWT), aorta wall thickness diameter (AWT), LV ejection volume (LVEV), LV fractional shortening (LVFS). Fractional shortening % (FS) was calculated according to the formula $FS = [(LVEDC - LVESC)/LVEDC] \times 100(\%)$, (Yoon et al., 2005).

*Statistical analysis.* Data were expressed as means ± standard error (SEM). For maternal and fetal characteristics the non-parametric Mann–Whitney test for independent groups was used. For the vascular reactivity, the non-parametric Kolmogorov–Smirnov test to compare the curves of treated with control conditions was used. The echocardiographic differences between both groups were analyzed by the non-parametric Mann–Whitney test. Real time PCR results were analyzed by Mann–Whitney test (NF-κB) or 2 ways ANOVA (HO-1 and VCAM-1). Results were considered significant at $p \leq 0.05$.

## Results

### General characteristics

Maternal and fetal characteristics are described in Table 1. As shown, no differences in initial and final weights of pregnant mothers were observed. Placental weight and food consumption were similar in both groups. The litter size and the offspring birth weight and body length were not affected by a prenatal exposure dose of 30 ppm of $Cd^{2+}$. Currently, the birth weight is not considered a marker that can predict the future development of cardiovascular disease. Therefore, following experiments were performed in the offspring at adult age (60–70 days old) to determine whether prenatal exposure to $Cd^{2+}$ affects the vascular and cardiac function.

### Vascular reactivity studies

Fig. 1 shows the results of the endothelium-dependent and independent reactivity assessed by ACh and NP-induced relaxation respectively. Endothelium-dependent reactivity was gender sensitive, being stronger in aortic rings of male offspring than in female offspring, independently of the treatment (Fig. 1A, MC vs FC). Results show that the developmental exposure to $Cd^{2+}$ induced a decreased endothelium-dependent reactivity both in female (FC vs FT, Fig. 1B) as in males (MC vs MT, Fig. 1C). Endothelium-independent response was also gender sensitive, having the male aortas an endothelium with increased responsive capacity to NP compared to females (MC vs FC, Fig. 1D). However, no differences in the NP-induced relaxation were observed in both treated males (MC vs MT, Fig. 1E) and females (FC vs

**Table 1**
Maternal and fetal characteristics.

| | C | T |
|---|---|---|
| Initial mother weight (g)[a] | 223.7 ± 15 | 230.5 ± 16 |
| Mother weight at term (g, day 20 of pregnancy)* [a] | 390.6 ± 21 | 360.1 ± 19 |
| $Cd^{2+}$ consumption/day (mg)[b] | 0 | 0.8 ± 0.2 |
| Total $Cd^{2+}$ consumption (mg)[c] | 0 | 16.9 ± 4 |
| Litter size | 15 ± 2 | 14.3 ± 2 |
| Offspring birth weight (g)[d] | 3.57 ± 0.3 | 3.52 ± 0.2 |
| Offspring body length (cm)[d] | 3.53 ± 0.2 | 3.50 ± 0.2 |
| Placental weight (g)[d] | 0.5 ± 0.1 | 0.6 ± 0.1 |

Results are expressed as means ± SEM of control (C) and $Cd^{2+}$-treated (T) groups.
[a] $n = 8$ for control and $Cd^{2+}$-treated respectively.
[b] Results calculated in relation to daily consumed water.
[c] Total $Cd^{2+}$ consumed during the whole pregnancy period.
[d] $n = 83$ and 109 offspring for control and $Cd^{2+}$-treated respectively.

FT, Fig. 1F) compared with their corresponding controls. These results show that developmental $Cd^{2+}$ exposure induces a specific effect on endothelial tissue of the aortas of male and female offspring at adult age.

### *Echocardiographic studies*

We studied whether alterations in vascular reactivity were concomitant to changes in echocardiography parameters. As seen in Table 2, echocardiography revealed an increased aorta's wall thickness (AWT, Fig. 2C and D), and a marked increase in the anterior left ventricular wall thickness in systole and diastole (ALVSWT and ALVDWT) in adult female and male offspring of $Cd^{2+}$-treated rats, in a pattern of concentric hypertrophy. As a consequence, a reduced LVEDC was also observed (Table 2, Fig. 2A and B). At this stage, developmental $Cd^{2+}$ exposure did not induce heart failure since ventricles ejected similar blood volume in both groups (Table 2, LVEV: 0.2 ± 0.08 vs. 0.21 ± 0.03 ml/beat respectively) and the percentage of left ventricular fractional shortening (LVFS) did not change (Table 2).

### *Cadmium concentrations in offspring tissues*

To assess whether prenatal $Cd^{2+}$ exposure induced changes in vascular reactivity and cardiac morphology directly through the placenta and/or breast-feeding, or through some indirect way, we measured the $Cd^{2+}$ concentrations in placentas, maternal milk and offspring tissues at different postnatal periods. Results show that $Cd^{2+}$ levels in 6 different biological samples of whole fetuses ($n = 6$), maternal milk ($n = 6$), blood, liver, kidney and aorta of 21 days old offspring ($n = 6$) and aorta of 60 days old offspring, were below the calculated detection limit (LOD) in any of the analyzed sample, with the exception of placenta samples ($n = 6$) where a mean of 1.12 ± 0.24 μg of $Cd^{2+}$ per g of dry weight was detected. Thus, cardiovascular effects may have occurred either by direct fetal exposure to undetectable very low

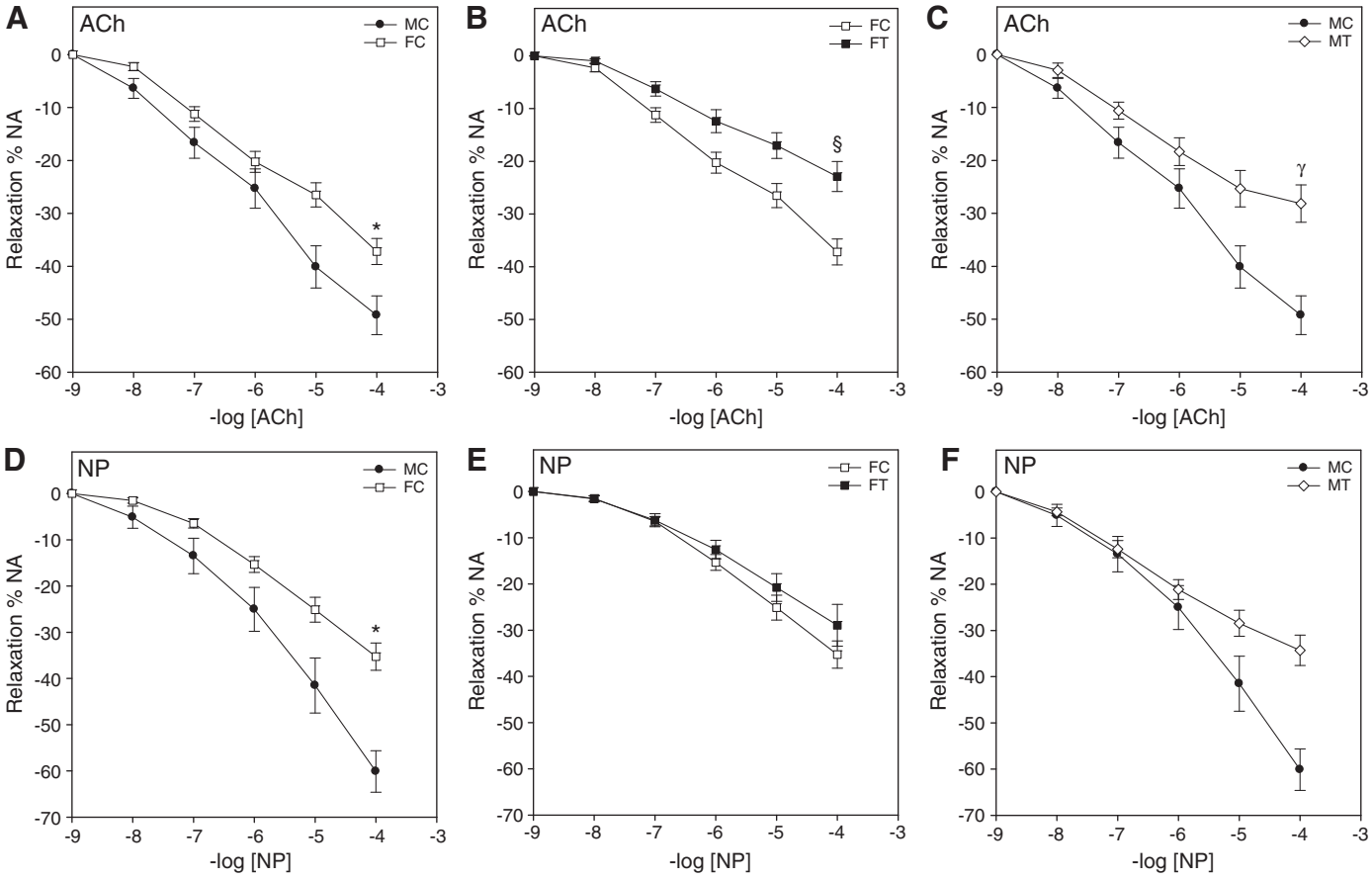


**Fig. 1.** Concentration-response curves for acetylcholine ($10^{-9}$ to $10^{-4}$)(-log ACh)-induced relaxation (A–C) determined in thoracic aortic rings of male (MC) and female control offspring (FC) at 60–70 days old compared with male and female offspring of rats exposed to 30 ppm of $Cd^{2+}$ during pregnancy (MT, FT). The developed tension of each cumulative dose is expressed as percentage of maximal contractile response achieved by norepinephrine ($10^{-4}$ M) (NA). Data are means ± SEM; *$p < 0.02$ for MC vs FC (A); §$p < 0.01$ for FC vs FT (B); γ$p < 0.02$ for MC vs MT (C). Concentration-response curves for sodium nitroprusside (NP, $10^{-9}$ to $10^{-4}$ M) evoked vasorelaxation (D–F) in thoracic aortic rings of male control (MC) and female control offspring (FC) at 60–70 days old compared with male and female offspring of rats exposed to 30 ppm of $Cd^{2+}$ during pregnancy (MT, FT). The developed tension of each cumulative dose is expressed as percentage of maximal contractile response achieved by norepinephrine (NA, $10^{-4}$ M). Data are means ± SEM; *$p < 0.02$ for MC vs FC (D).

**Table 2**
Echocardiographic parameters in adult offspring of control (C) and $Cd^{2+}$-treated (T) pregnant rats.

| Echocardiographic data | Group | |
|---|---|---|
| | C | T |
| LVESC (mm) | 3.0 ± 0.03 | 2.8 ± 0.05 |
| LVEDC (mm) | 6.0 ± 0.03 | 4.9 ± 0.03* |
| ALVSWT (mm) | 2.5 ± 0.2 | 3.5 ± 0.5** |
| ALVDWT (mm) | 1.5 ± 0.2 | 2.0 ± 0.2§ |
| PLVSWT (mm) | 2.7 ± 0.3 | 2.7 ± 0.3 |
| PLVDWT (mm) | 1.9 ± 0.3 | 1.9 ± 0.2 |
| AD (mm) | 0.2 ± 0.02 | 0.2 ± 0.01 |
| AWT (mm) | 0.45 ± 0.08 | 0.7 ± 0.07γ |
| LVEV (ml/beat) | 0.2 ± 0.08 | 0.21 ± 0.03 |
| LVFS (%) | 49.8 ± 5 | 43.4 ± 8 |

LVESC: left ventricular end-systolic cavity; LVEDC: left ventricular end-diastolic cavity; ALVSWT: anterior left ventricular-diastolic wall thickness; ALVDWT: anterior left ventricular-systolic wall thickness; PLVSWT: posterior left ventricular-systolic wall thickness; PLVDWT: posterior left ventricular-diastolic wall thickness; AD: aortic diameter; AWT: aorta wall thickness diameter; LVEV: left ventricular ejection volume; LVFS: left ventricular fractional shortening. Data are shown as mean ± SEM; *$p<0.001$; **$p<0.001$; §$p<0.002$; γ$p<0.001$ vs C respectively.

$Cd^{2+}$ levels or indirectly, caused by a disruption of the placental function and physiology induced by the $Cd^{2+}$ accumulated in placenta.

*Expression of inflammation and oxidative stress markers*

*NF-κB expression in placentas of control and $Cd^{2}$ -treated rats.* Because $Cd^{2+}$ traces were not detected in fetuses but in placentas, we hypothesized that vascular effects observed in adult offspring could have been originated in the placenta during fetal development through oxidative stress induced by the accumulated $Cd^{2}$ in this tissue. Since $Cd^{2+}$ is a redox-stable metal and therefore, radical production by $Cd^{2}$ must be mediated through some indirect mechanisms, we determined the expression of NF-κB, a transcription factor which is sensitive to oxidative stress (Liu et al., 2009). We found an increased expression of NF-κB expression in placentas of $Cd^{2+}$-treated rats compared with placentas of control animals (Fig. 3).

*HO-1and VCAM-1 expression in aortas of adult offspring.* To evaluate whether adult offspring of exposed mothers show any sign of being exposed to some level of oxidative stress, and because $Cd^{2+}$ is associated to the expression of genes related to cellular redox status, we determined the expression of HO-1 and VCAM-1, both oxidative stress targets molecules. Results show that offspring of $Cd^{2+}$-treated rats expressed higher aortic HO-1 mRNA (Fig. 4A) being the magnitude of the $Cd^{2+}$-induced effect on HO-1 expression effect higher in females than in males (FT vs FC and MC vs MT, Fig. 4A). Also, protein level of HO-1 was increased in $Cd^{2+}$-treated offspring compared to the controls (FC vs FT, Fig. 4B and C). We did not observe gender differences in the expression (mRNA and protein) of HO-1 in aortas of untreated males and females (Fig. 4). In opposite way, VCAM-1 expression in aortas was gender sensitive since it was higher expressed in males than female's offspring. No treatment effect was observed in both, females and males (Fig. 5).

## Discussion

Our results demonstrate that pregnant rats exposed to 30 ppm of $Cd^{2+}$ in the drinking water during the whole pregnancy, deliver offspring showing altered endothelial function and heart morphology at the adult age. These effects may have been originated during the fetal development through a $Cd^{2+}$-induced mechanism, not completely understood, which reprograms long-term responsiveness of the offspring cardiovascular system. This finding is relevant since the observed effects were evidenced with undetectable $Cd^{2+}$ traces in the

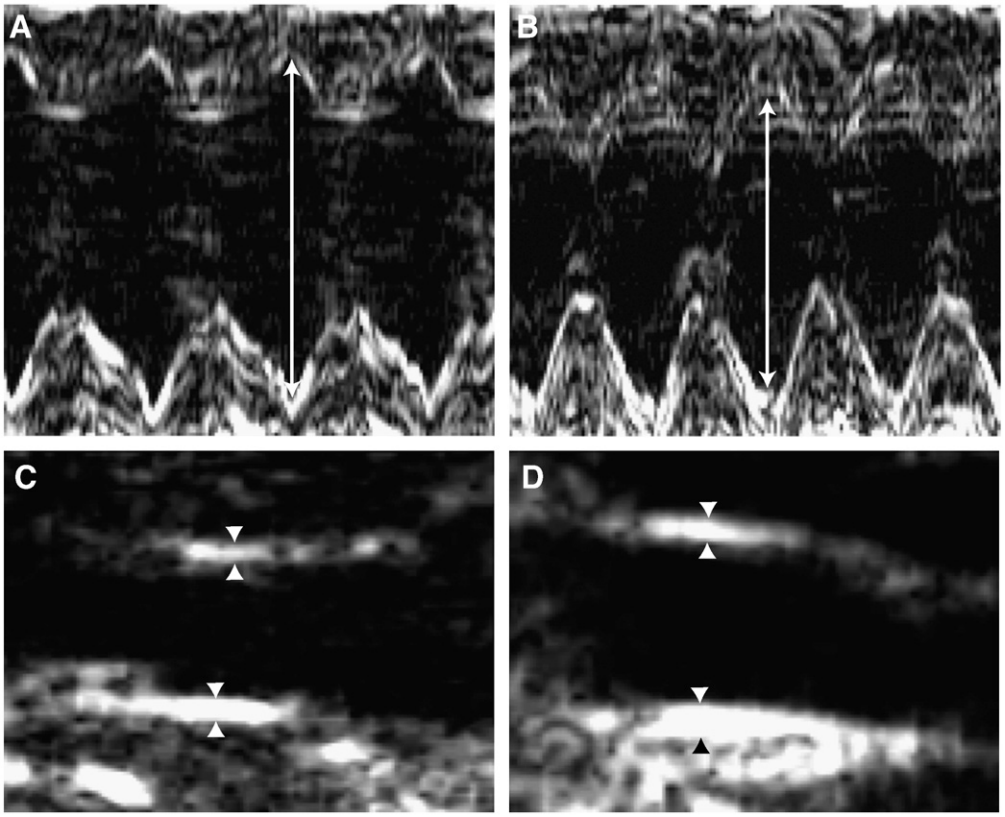


**Fig. 2.** Left ventricular-end diastolic cavity (LVEDC) of control (A) and $Cd^{2+}$-treated offspring (B) at 60–70 days old. Aortic wall thickness (AWT) of control (C) and $Cd^{2+}$-treated (D) offspring. White arrows indicate the dimension of LVEDC (A, B) and AWT (C, D) respectively.

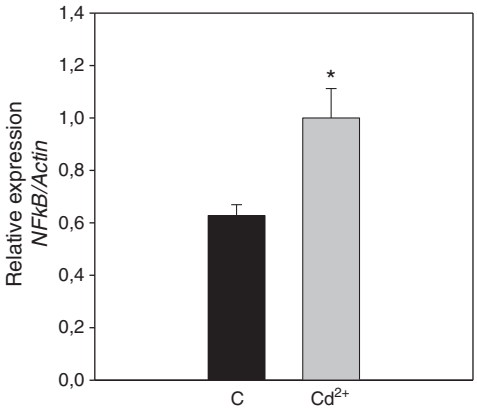


**Fig. 3.** Quantitative NF-κB mRNA of placentas. NF-κB mRNA was determined by real time PCR as described in Materials and methods. Results are expressed as means ± SEM of the NF-κB/β actin ratio of duplicate experimental determinations of six different biological samples of placentas of control (C) and $Cd^{2+}$-treated rats ($Cd^{2+}$); *$p<0.001$.

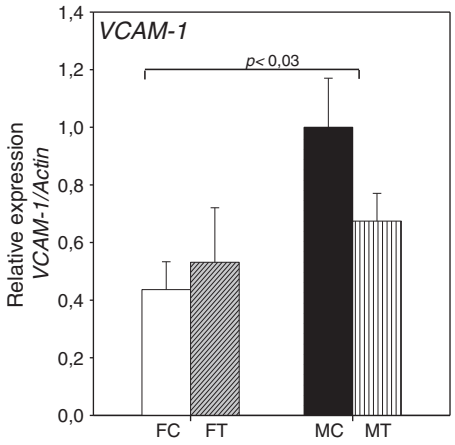


**Fig. 5.** Quantitative VCAM-1 mRNA of male and female aortas. Results are expressed as mean ± SEM of the VCAM-1/β actin ratio of duplicate experimental determinations of six different biological samples of female control (FC) and $Cd^{2+}$-treated offspring (FT) and male control (MC) and $Cd^{2+}$-treated offspring (MT).

exposed fetuses, without changes in the birth weight and, in spite of a diet containing high levels of n-3 fatty acids, known by its protective properties on the cardiovascular system (Galli and Risé, 2009). In rats, $Cd^{2+}$ is retained in placental tissues and, apparently it is only transported and stored in the fetus when the level of $Cd^{2+}$ exposure is high (Kuriwaki et al., 2005); in humans, however, $Cd^{2+}$ traces in umbilical cord blood and maternal milk have been detected even in non-occupational exposed population (Walker et al., 2006; Röllin et al., 2009; Kippler et al., 2009). Although we cannot discard that very low, undetectable $Cd^{2+}$ levels may have been transferred to the fetuses through the placenta or milk during breast-feeding, the absence of detectable $Cd^{2+}$ traces in fetuses and milk found in our study aimed us to suggest that subsequent deleterious effects on the offspring may be related to $Cd^{2+}$-induced placental dysfunction. To explore the possibility that indirect effects could be mediated by an inflammatory and/or oxidative stress mechanism, and, assuming that $Cd^{2+}$ is unable to directly generate free radicals in vivo, we determined the expression of NF-κB in placental tissues. The increased expression of NF-κB found in placentas of exposed rats suggests that $Cd^{2+}$ accumulated in placentas may lead to placental dysfunction as a consequence of an abnormally elevated oxidative stress status. However, direct measurements of additional biomarkers of oxidative stress at placental or fetal level are needed to conclusively establish that the cardiovascular impairment observed in the early exposed adult offspring are associated with an abnormal placental oxidative stress condition (Fig. 3).

We have previously reported that in humans, $Cd^{2+}$ levels in placental tissues are correlated with birth weight, and that mothers delivering low birth weight neonates had higher levels of $Cd^{2+}$ and other heavy metals in their placentas (Ronco et al., 2005; Llanos and Ronco, 2009). In rats, prenatal exposure to 50 ppm of $Cd^{2+}$, but not to 30 ppm or less, induced significantly lower birth weight offspring when compared to offspring from control non-exposed pregnant rats (Ronco et al., 2009), stating that the relationship between $Cd^{2+}$ exposure and birth weight is directly dependent on the exposure dose. Probably, higher prenatal $Cd^{2+}$ dose (> 30 ppm) may impair even more the cardiovascular function of the adult offspring. When adult rats were treated directly (i.p.) with 1 mg/kg/day with $CdCl_2$ during 15 days, a $Cd^{2+}$-induced hypertension and cardiac hypertrophy was observed (Mollaoglu et al., 2006). These effects were associated with oxidative stress and were prevented with a co-

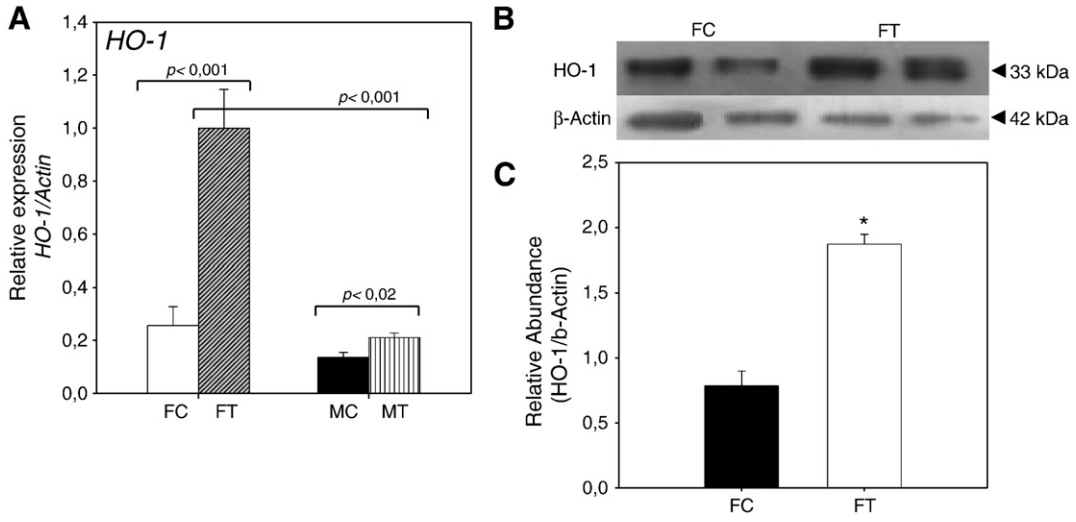


**Fig. 4.** HO-1 expression in aortas of offspring. A) HO-1 mRNA was determined by real time PCR as described in Materials and methods. Results are expressed as means ± SEM of the HO-1/β actin ratio of duplicate experimental determinations of six different biological samples of female control (FC) and $Cd^{2+}$-treated offspring (FT) and male control (MC) and $Cd^{2+}$-treated offspring (MT). B) Western blot analysis for HO-1 protein expression in the aorta tissue of adult female offspring. Lanes 1 and 2: normal control group; Lanes 3 and 4: $Cd^{2+}$-treated group. C) HO-1 western blot signals were quantified by densitometry and expressed as abundance ratios of HO-1 related to actin. Results represent the mean ± SEM of four controls (FC) and four $Cd^{2+}$-treated samples (FT). *$p<0.05$, compared with control (FC).

treatment with $Cd^{2+}$ and a flavonoid-like compound, suggesting the involvement of oxidative stress mechanisms in the $Cd^{2+}$-induced CV impairment.

Cadmium is a hazardous environmental pollutant which accumulates over 30 years in organisms playing an important role in the pathogenesis of hypertension (Satarug et al., 2005). To study the effect of $Cd^{2+}$ exposure on developmental programming of vascular reactivity, we isolated aortas from adult age offspring of rats exposed to 30 ppm of $Cd^{2+}$ throughout pregnancy to evaluate the endothelial-dependent and independent reactivity. Therefore, their respective vascular responses to ACh and NP (a nitric oxide donor) were measured. Vascular responses of aortas to ACh and NP were gender sensitive, with males having a higher vasodilatation capacity in comparison to females. These results point out that nitric oxide (NO) release is not a requirement to explain gender differences and suggest that endothelial and non-endothelial factors are operating in this observed sexual dimorphism in vascular reactivity (Robert et al., 2005). Gender differences in the sensitivity to toxic effects of metals and particularly to $Cd^{2+}$ have been previously demonstrated in humans, being females more susceptible than males (Nishijo et al., 2004; Vahter et al., 2007). The decreased relaxation to ACh but not to NP in aortas of offspring from exposed pregnant rats found in this study, are in agreement with previous studies performed with direct exposure to $Cd^{2+}$, demonstrating a specific effect on the EDR and confirming that the endothelium is a target of this element (Göcmen et al., 2000; Tzotzes et al., 2007).

Several studies have associated endothelial dysfunction to oxidative stress and NO impairment (Kolluru et al., 2006; Wolf and Baynes, 2007; Majumder et al., 2008). It has been reported that impairment of ACh-induced relaxation after $Cd^{2+}$ exposure was mediated by the reduction of eNOS expression, leading to decreased serum NO levels that may explain the hypertension caused by $Cd^{2+}$ exposure (Prozialeck et al., 2006; Yoopan et al., 2008). An insufficient relaxation through the NO system has been detected in almost all cardiovascular pathologies being the endothelial dysfunction the first manifestation of vascular damage. Our results suggest that the nitric oxide/cGMP-dependent intracellular signaling pathway in vascular smooth muscle cells was not affected because the response of aortas to NP, a nitric oxide donor, was not significantly different when comparing both groups (Kolluru et al., 2006).

Offspring's endothelial dysfunction induced by prenatal exposure to $Cd^{2+}$ found in this study was concomitant to altered echocardiographic parameters indicative of concentric ventricular hypertrophy, an adaptive response to maintain cardiac function under conditions of increased workload (Table 2, Fig. 3) (Sano et al., 2007). At the offspring ages used in this study (60–70 days old), heart failure was still not observed, since fractional shortening % (FS) and LV ejection volume (LVEV) were not altered by the prenatal $Cd^{2+}$ exposure. It has been reported that patients with hypertension often develop left ventricular (LV) hypertrophy and deterioration of the cardiac and endothelial functions as a physical response to chronic pressure overload (Isobe et al., 2002). Beta-adrenergic receptor signaling cascade as well as the renin–angiotensin–aldosterone system has been involved in the cardiac hypertrophy induction (Juric et al., 2007). Also, oxidative mechanisms have been involved in hypertension, cardiac hypertrophy, and impaired vascular function (Kopf et al., 2008).

Recently, increasing attention has been drawn to the beneficial effects of heme oxygenase-1 (HO-1) in the cardiovascular system (Loboda et al., 2008). HO-1 catalyzes oxidative degradation of heme into its breakdown products biliverdin, carbon monoxide (CO) and ferrous ions, with proven vasomodulators and anti-oxidant activities. Then, protective effects of HO-1 against endothelial dysfunction in the aorta may be due to its actions as an antioxidant and regulator of vasoactive substances (Immenschuh and Schröder, 2006). It has been demonstrated that HO-1 is induced by $CdCl_2$, NO, oxidized LDL and tobacco smoke (Favatier and Polla, 2001; Hill-Kapturczak et al., 2003). In this study, we found increased HO-1 mRNA and protein expression in aortas from the adult offspring of exposed dams. This effect was observed in both females and males although females registered a higher response. Although HO-1 induction has been reported to improve vascular relaxation, offspring of exposed rats did not show any improvement in the vascular relaxation to ACh. These results suggest that prenatal exposure to $Cd^{2+}$ could induce similar effects to those observed in angiotensin II-treated mice, where endothelial-dependent relaxation to ACh was impaired and HO-1 induction although lowered hypertension, was not able to improve vascular relaxation (Stec et al., 2008). We believe that increased HO-1 expression may be linked to the observed changes in cardiac morphology, suggestive of compensatory cardiac hypertrophy as a first adaptive response to maintain the CV function.

Dysfunctional endothelium is related to leucocytes recruiting during the atherosclerotic plaque formation; it expresses adhesion molecules such as VCAM-1, favoring contact between leucocytes and endothelium (Nyby et al., 2007). Others have previously demonstrated that VCAM-1 is fully distributed in endothelial cells of occluded arteries during accelerated atherosclerosis (Macías et al., 2003). VCAM-1 expression is increased under chronic damage associated to risk factors such as smoking, hypertension and hypercholesterolemia. In our study, the endothelial dysfunction observed in offspring from $Cd^{2+}$-exposed dams was not accompanied with changes in VCAM-1 expression in their aortas, indicating a not yet evident atherosclerosis. Male aortas of control offspring expressed higher VCAM-1 mRNA levels than females, suggesting a sex-dependent effect in the expression of that adhesion molecule. Since HO-1 modulates the expression of proinflammatory genes associated with endothelial cell (EC) activation, the higher expression (mRNA and protein) of HO-1 in females compared to males found in our study may be likely related to the lower VCAM-1 expression in females than in males (Soares et al., 2004). In this sense, sex-specific differences in the development of oxidative stress with impact on endothelial dysfunction, blood pressure and cardiovascular alterations have been reported (Kayali et al., 2007; Sartori-Valinotti et al., 2007; Rodford et al., 2008). These differences have also been associated to the development of other pathologies including asthma (Malling et al., 2010). Specifically, gestational exposure to $Cd^{2+}$ induced sex-specific effects on antioxidant status in the offspring (Pillai et al., 2009).

In summary, adult offspring from dams which were exposed to 30 ppm $Cd^{2+}$ during the whole pregnancy registered a reduced endothelium-dependent vascular reactivity and altered cardiac morphology characteristic of pressure overload. Since fetal $Cd^{2+}$ concentrations were undetectable, the magnitude of $Cd^{2+}$ amount transferred to the fetus may be considered irrelevant, and thus, cardiovascular responses observed in the offspring may be a consequence of a feto-placental unit dysfunction early developed to deal with the harmful effects of accumulated placental $Cd^{2+}$. This condition may lead to changes in gene expression, being some of them involved in appropriate adaptations to maintain cardiac function properly, that otherwise could have caused a cardiovascular failure during adulthood.

Finally, these results could have important implications to humans, since similar adverse effects could occur in adult offspring of women who were/are exposed to environmental sources of $Cd^{2+}$ during pregnancy (e.g. tobacco smoke). Importantly, in the general population, maternal smoking during pregnancy has been associated with increased cholesterol levels in the offspring (Jaddoe et al., 2008) and with other risk factors of cardiovascular disease (Power et al., 2010).

## Conflict of interest statement

The authors declare that they do not have conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2011.01.001.

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