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Protective effect of soybeans as protein source in the diet against cadmium-aorta redox and morphological alteration

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ABSTRACT

We investigated the effects of cadmium exposition on thoracic aorta redox status and morphology, and the putative protective effect of soybeans in the diet.
Male Wistar rats were separated into 6 groups: 3 fed with a diet containing casein and 3 containing soybeans, as protein source. Within each protein group, one was given tap water (control) and the other two tap water containing 15 and 100 ppm of Cd^{2+}, respectively, for two months.
In rats fed with casein diet, 15 ppm of Cd induced an increase of thiobarbituric acid-reactive substances (TBARS), and of the catalase (CAT) and glutathione peroxidase (GPx) activities, which were even higher with 100 ppm of Cd^{2+}, in aorta.
Also, 100 ppm Cd^{2+} exposure increased superoxide dismutase (CuZnSOD) activity; CAT, GPX, SOD, Nrf2 and metallothioneine II mRNA expressions and CAT, GPx and NOX-2 protein levels, compared with control. Aorta endothelial and cytoplasmic alterations were observed.
However, with the soybeans diet, 15 and 100 ppm of Cd^{2+} did not modify TBARS levels; CAT, GPX and Nrf2 mRNA expressions; CAT, GPx and NOX-2 protein; and the aorta morphology, compared with control.
The soybean diet attenuates the redox changes and protects against morphological alterations induced, in a dose-dependent way, by Cd in aorta.

Introduction

Cadmium (Cd) is among the most toxic pollutants, being widely distributed in the environment. Each year the EPA (Environment Protection Agency) lists a number of inorganic substances of high environmental impact. This list is led by metals such as lead (CASRN 7439-92-1), arsenic (CASRN 7440-43-9) and cadmium (CASRN 7440-43-9). High level exposure to Cd is usually the result of environmental contamination from human activities, such as mining, smelting, fossil fuel combustion and industrial use (Nordberg, 1972). In addition, it has been well established that Cd is one of the major contaminants in tobacco smoke (Li et al., 2000).

Gastrointestinal ingestion of Cd, through food and drinking water, is a major route of intake in non-smoking and non-occupationally exposed populations (ATSDR, 1999). It has been demonstrated, in humans and animals, that soluble Cd^{2+} salts accumulate and injure several tissues, including kidney (Renugadevi and Prabu, 2008), liver (Larregle et al., 2008), brain, lung (Luchese et al., 2007), adenohypophysis (Calderoni et al., 2010), prostate (Alvarez et al., 2006), and heart (Manna et al., 2008; Soares et al., 2007), depending on exposure time and dose. Some studies have attempted to correlate environmental Cd exposure with lung (Nawrot et al., 2006), kidney and prostate cancer (Järup, 2003; Satoh et al., 2002; Waalkes, 2003). Furthermore, the vascular endothelium has been suggested as a critical target of Cd toxicity, leading to many cardiovascular complications such as hypertension, atherosclerosis and cardiomyopathy (Prozialeck et al., 2006). Considerable evidence suggests that the hypertensive effect of Cd exposure results from complex actions on both, the vascular endothelium and vascular smooth muscle cells (VSMCs) (Prozialeck et al., 2008). Cd-fed ApoE −/− mice has been shown to exhibit a substantial increase of aortic plaque surface area (Knoflach et al., 2011). Blood and urinary Cd has been associated with peripheral arterial disease in a representative sample of the U.S. population (Selvin and Erlinger, 2004; Navas-Acien et al., 2005). Cadmium exposure has also been associated with future peripheral artery disease, supporting the concept that Cd exposure in the population has proatherogenic effects (Fagerberg et al., 2012).

It is known that Cd increases oxidative stress, affecting antioxidant enzyme activities (Ognjanović et al., 2008; Sinha et al., 2008). Cadmium could replace iron and copper in a number of cytoplasmic and membrane proteins, like ferritin, which in turn releases and increases the concentration of unbound iron or copper ions. These free ions participate in

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the Fenton reaction, generating reactive oxygen species (ROS) (Flora et al., 2008). The production of ROS and reactive nitrogen species (RNS), appears to be a relevant mechanism for Cd toxic effects in many tissues and organs, including cardiovascular system (Beyersmann and Hartwig, 2008; Stoths and Bagchi, 1995; Waisberg et al., 2003), which can be diminished by the presence of oxygen radical scavengers. The increased ROS production induced by Cd, can trigger lipid peroxidation, DNA damage and oxidative modifications of proteins, which can eventually lead to cellular dysfunction and necrotic cell death (Thévenod, 2009; Valko et al., 2007).

NADPH oxidase (NOX), is a multisubunit enzyme that catalyzes the reduction of molecular oxygen to form superoxide ($O_2^{\bullet -}$). Gp91phox, also known as NOX2, is the NADPH oxidase prototype and emerges as a major source of $O_2^{\bullet -}$ in vascular cells and myocytes (Griendling et al., 2000; Kim et al., 2005). The first line of defenses towards $O_2^{\bullet -}$ and H_2O_2 mediated injury, are antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Helmut, 1997). These enzymes, among others, are regulated by NF-E2-related nuclear factors (Nrf1 and Nrf2), which bind to the antioxidant response element (ARE) and regulate ARE-mediated gene expression and induction (Leonard et al., 2006; Thimmulappa et al., 2002).

Increased oxidative stress and endothelial dysfunction have been suggested to be risk factors for hypertension and atherosclerosis (Förstermann, 2008). Several autopsy studies have found association between tissue lead or Cd levels and atherosclerotic lesions (Aalbers and Houtman, 1985; Voors et al., 1982). Also, it has been proposed that Cd may partially mediate the effect of smoking on peripheral arterial disease in the general US population (Navas-Acien et al., 2004). However, the biochemical and molecular bases underlying Cd intoxication in the mammalian arteries have not been fully elucidated.

Moreover, soy proteins are becoming increasingly important in the human diet. Among the beneficial health effects, they have been described to: lower cholesterol and LDL cholesterol (Borodin et al., 2009), prevent heart disease, reduce weight in obesity (von Post-Skagegard et al., 2006; Zemel et al., 2010) and protect against breast and prostate cancer (Friedman and Brandon, 2001). Evidence suggests that a diet rich in soy protein and your isoflavone, has a scavenging activity and antioxidant properties, and can inhibit lipoprotein oxidation and reduce the incidence of coronary heart disease (Anderson et al., 1995; Clarkson, 2002; Lissin and Cooke, 2000; Tikkanen and Adlercreutz, 2000).

Drinking water containing 15 ppm of Cd^{2+} for 2 months, has been previously used in our laboratory (Calderoni et al., 2010; Larregle et al., 2008) to intoxicate rats and attain serum Cd^{2+} concentrations up to the World Health Organization (WHO) toxic limit. Furthermore, it has been shown that higher Cd doses (50 ppm or 200 ppm in drinking water for 3 months), modified the vascular reactivity of an isolated and perfused rat mesenteric bed (Skoczynska and Martynowicz, 2005). Thus, the purpose of this study was to chronically expose rats, via drinking water, to two different Cd doses (15 and 100 ppm of Cd^{2+}) for two months, to evaluate in the aorta: (1) the prooxidant effects of Cd on the activity and expression of antioxidant enzymes, (2) the relation between possible aorta structural alterations and Cd serum levels and, (3) to investigate the protective role of soybeans, as the diet protein source, against the possible Cd toxic effects.

Materials and methods

Diet and experimental design. Adult male Wistar rats, weighing 180–200 g at the onset of treatment, were used. They were bred in our animal facilities (National University of San Luis, San Luis, Argentina). The experimental protocols were approved by the Committee for Care and Use of Laboratory Animals of the National University of San Luis, and were in accordance with the Rat Care and Treatment Recommended Guidelines (U.S. Public Health Service, 1985). Animals were handled under standard laboratory conditions of 12 h light/12 h dark cycles in a temperature and humidity controlled room.

Rats were given free access to food and water throughout the entire experimental period. Rats were randomly divided into 6 groups of 6 animals each. Regarding the protein source, 3 groups were fed with a diet containing casein and the other 3 with a diet containing soybeans as the dietary protein.

Within the groups fed with each protein, one was given tap water (control) and the other two tap water containing 15 and 100 ppm of Cd^{2+}, (as $CdCl_2$), respectively. Rats were euthanized after 2 months of treatment.

Since ingestion is the most important route of Cd human exposure, drinking water was chosen as the exposure carrier. The Cd concentrations and exposure time were consistent with previous studies (Calderoni et al., 2010; Larregle et al., 2008; Skoczynska and Martynowicz, 2005; Thijssen et al., 2007). Furthermore, exposure to 100 ppm of Cd^{2+} was reported as environmentally realistic (Thijssen et al., 2007).

Casein and soybean diets were prepared according to AIN-93M-CAS and AIN-3M-SOY (Reeves, 1996) for laboratory rodents, respectively. No Cd was detected in the control rat drinking water. After treatment, rats were fasted overnight and euthanized by decapitation at 09:00 h. Immediately after, trunk blood samples were collected for serum separation.

The thoracic aorta was quickly excised, washed several times with ice-cold isotonic saline solution and cleaned to remove the surrounding tissue. Afterwards, aorta samples were placed in liquid nitrogen for storage. Analyses were carried out within 1–2 weeks of obtaining the samples. Additionally, enzyme determinations were performed in fresh tissues.

Serum cadmium determination. Cd in serum and aorta was determined by electrothermal atomic absorption spectrometry, using a Perkin Elmer Analyst 200 Gf, equipped with a graphite tube with a L'vov platform, with DL 0.001 μg/l and QL 0.01 μg/l (detection and quantification limits of 1 ppt and 10 ppt, respectively). A matrix modifier was used (ammonium phosphate–ammonium nitrate). The calibration curve was made with an aqueous Cd standard, to which a tensoactive agent and matrix modifier were added, in a range of 0.5 to 5 μg/l; MLD: 0.035 μg/l (Imbus, 1963). Validation was carried out on a synthetic sample (cow liver homogenate), with the addition of a standard Cd solution, traceable to standard reference material from NIST, following method 200.0 revision 1.2 4/91 protocol. Cd recovery was about 98–99%. Samples DL and QL were 0.01 and 0.1 μg/l, respectively.

Thiobarbituric acid reactive substance (TBARS) determination. Aorta lipid peroxidation levels (assessed as thiobarbituric acid reactive substances, TBARS), were measured spectrophotometrically, according to Draper and Hadley (1990). Briefly, aorta tissue was homogenized in 120 mM KCl and 30 mM phosphate buffer, pH 7.4. Proteins were precipitated with 20% tricholoroacetic acid (TCA, Sigma-Aldrich Co.) and a supernatant containing malondialdehyde (MDA), the end product of the lipid peroxidation, was incubated with a 0.7% thiobarbituric acid solution (TBA, Sigma-Aldrich Co.) to measure the TBARS content. An acid hydrolysis product of 1,1,3,3-tetramethoxy propane (TMP) was used as standard. Aorta TBARS were expressed as nmol of MDA/mg protein.

Antioxidant enzyme activities. In order to process the thoracic aorta for determination of the antioxidant enzyme activity (20 mg of wet weight), it was homogenized in 30 mM PBS buffer, with 120 mM KCl, pH 7.4, containing 1× protease inhibitors (Pepstatin A and PMSF) and 50× Triton, followed by centrifugation at 3000 rpm, for 30 min at 4 ºC. The pellet was discarded and the supernatant was used as homogenate (Gonzales-Flecha et al., 1991). The enzyme determinations were performed immediately after. CAT activity was determined by measuring the decrease in absorption, at 240 nm, of H_2O_2 decomposition (Chance et al., 1979). The results were expressed as units per milligram of protein (U/mg protein). One CAT unit is defined as the amount of enzyme required to decompose

1 mM of H_2O_2/min. GPx activity was determined following the NADPH oxidation rate at 340 nm (Flohe and Gunzler, 1984). Results were expressed as milli international units (1 IU oxidizes 1 μmol NADPH/min at pH 7.7 at 30 ºC per milligram of protein) (IU/mg protein).

Copper–zinc SOD activity was determined on the basis of its inhibitory action on the rate of superoxide-dependent reduction of cytochrome c, by xanthine–xanthine oxidase at 560 nm (McCord and Fridovich, 1969). One unit of SOD was defined as the amount of enzyme that inhibits cytochrome c reduction by 50%. Results were expressed as units per milligram of protein (U/mg protein).

All reagents were from Sigma-Aldrich Co. In all cases, total protein content was measured by the method of Lowry et al. (1951), using serum albumin as standard.

RNA isolation and semi-quantitative RT-PCR analysis. CAT, SOD, GPX, Nrf2 and MET II mRNA levels were determined by RT-PCR. Total RNA was isolated from frozen aorta samples using TRIzol (Invitrogen Corporation), within 1–2 weeks of obtaining the samples. All RNA isolations were performed as indicated by the manufacturers. 3 μg of total RNA was reverse transcribed with 200 U of M-MLV reverse transcriptase (RT; Promega Inc.) using random primer hexamers (Biodynamics, SRL) in a 26 μl reaction mixture at 37 ºC for 1 h. Aliquots of the reverse transcription reaction mix complementary DNA (cDNA), corresponding to 0.5 μg of cDNA for each reaction, were amplified with specific primers for the rat (Table 1). The reaction samples were heated to 95 °C for 5 min followed by 40 temperature cycles; each cycle consisted of: 94 °C for 60 s, 60 ºC for 60 s, and 72 °C for 60 s. Afterwards, the extension reaction (72 °C) was continued for another 5 min. Bands were resolved in a 2% agarose gel, containing GelRed (Genbiotech) to visualize the bands; and their intensities were quantified using NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative amounts of mRNA were expressed as the ratio of band intensity for the target genes relative to that for β-actin.

Western blot analysis for CAT, GPx and NOX-2. Aorta samples were homogenized as described previously. Protein content was measured by the method of Lowry et al. (1951), using bovine serum albumin as standard. 40 μg of protein was mixed with 10 μl of sample buffer (250 mM Tris–HCl, 4% SDS, 4% β-mercaptoethanol, 0.002% bromophenol blue and 40% glycerol), boiled for 2–3 min, and loaded into a 12% SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. Separated proteins were transferred to PVDF membranes (Immobilon-P Transfer Membrane, Millipore, USA), using a blot transfer system (Bio-Rad Laboratories, Inc., Hercules, CA). After being blocked with 5% milk in TBS 1× (20 mM Tris, 0.9% NaCl, pH 7.4) for 4 h at room temperature, membranes were incubated overnight with a primary rabbit anti-CAT, anti-GPx and anti-NOX-2 polyclonal antibody solution (Santa Cruz Biotechnology, Inc.) (1:500 dilution), at 8 °C. After washing three times with TTBS (0.1% Tween 20, 100 mM Tris–HCl, pH 7.5, 150 mM NaCl), membranes were incubated with a biotinylated anti-rabbit IgG secondary antibody, obtained from goat (1:5000 dilution), for 2 h at room temperature. Membranes were washed and incubated with avidin/biotynilated peroxidase complex (Vectastain ABC-detection system, Vector Laboratories Inc.) for 1 h. The color was developed using a DAB-peroxidase substrate kit (Vector Laboratories Inc.). β-actin expression was determined as an internal control using an anti-actin antibody (Santa Cruz Biotechnology, Inc.).

Table 1
Oligonucleotide primers sequences for RT-PRC.

	Primer sequences (5′–3′)a	Fragment size	GenBank accession number
β-Actin	F: CGTGGGCCGCCCAGGCACCA R: TTGGCCTTAGGGTTCAGAGGG	243 bp	NM_031144
CAT	F: CGACCGAGGGATTCCAGATG R: ATCCGGGTCTTCCTGTGCAA	175 bp	NM_012520
SOD-2	F: AGCTGCACCACAGCAAGCAC R: TCCACCACCCTTAGGGCTCA	191 bp	NM_017051
GPx-1	F: CGGTTTCCCGTGCAATCAGT R: ACACCGGGGACCAAATGATG	245 bp	NM_030826
Nrf2	F: CGGCATTTCACTGAACACAAGT R: TGGCTGTGCTTTAGGTCCATT	160 bp	NM_031789
MT II	F: TCCTGTGCCACAGATGGATC R: GTCCGAAGCCTCTTTGCAGA	150 bp	NM_001137564

Histological studies. Light microscopy. For the light microscopy studies, the thoracic aortas were extracted and fixed in Bouin's fluid. The samples were dehydrated in increasing ethanol series, cleared in xylene and embedded in paraffin. All sections were obtained from the same thoracic artery section. Sections of 5 μm in thickness were obtained using a Microm HM 325 rotation microtome and stained with hematoxylin–eosin (H–E). These sections were examined using an Olympus BX-40 light microscope. Images were captured by a Sony SSC-DC5OA camera and processed with Image Pro Plus 5.0 software.

Statistical analysis

Statistical analysis was performed using two-way ANOVA, followed by the Bonferroni post test. All results are expressed as the mean ± S.E.M. Differences between means were considered significant at $p < 0.05$ level.

Results

Regardless of the diet, Cd levels were significantly higher in serum of treated animals, in comparison to controls. Animals exposed to 100 ppm of Cd showed a higher amount of Cd in serum, compared with those exposed to 15 ppm of Cd. No significant differences were observed in the serum Cd levels among the dietary groups (Table 2).

The initial rats' body weight in Cd-treated and control groups was the same. At the time of their being euthanized, no significant effect of Cd-treatment or protein source in the diet was observed on the body weight gain, compared to the respective control rats. Also, throughout the experiment, the consumption of food and water in all groups treated with Cd was similar to that in the control groups (data not shown), in accord with other authors (Larregle et al., 2008; Thijssen et al., 2007).

Aorta TBARS content

Shown in Fig. 1, are the aorta TBARS contents of the studied animal groups. In animals fed with the diet containing casein as the protein source, aorta TBARS content increased in animals intoxicated with 15 ppm, and even more with 100 ppm of Cd in drinking water ($p < 0.05$ and $p < 0.01$, respectively), in comparison to the control group. However, no significant changes were observed in the TBARS levels among the different soybean groups.

It can also be observed that using soybeans instead of casein in the diet, results in a significant decrease of the lipid peroxides levels in all groups ($p < 0.001$ for control and Cd-100 ppm, and $p < 0.005$ for Cd-15 ppm).

Antioxidant enzyme activities

Different effects of Cd intoxication on the activity of antioxidant enzymes in the aorta were observed. CAT activity in rats receiving casein in the diet, increased in the aorta when exposed to 15 ppm ($p < 0.005$) and even more when exposed to 100 ppm of Cd ($p < 0.001$), compared with the control group (Fig. 2A). In rats fed with the soybean diet, exposure to 15 ppm of Cd did not modify the aorta CAT activity, while exposure to 100 ppm resulted in an increase, compared to control ($p < 0.05$). Using soybeans instead of casein in the diet, resulted in a higher CAT activity in the 15 ppm Cd-treated group ($p < 0.05$), without

Table 2
Cadmium concentration in serum of rats exposed to CdCl2.

Groups	Cd concentration in serum (ppb)
Casein control	0.587 ± 0.037
Casein Cd 15 ppm	6.9 ± 0.37[a]
Casein Cd 100 ppm	15.37 ± 0.17[a]
Soybeans control	0.60 ± 0.020
Soybeans Cd 15 ppm	6.49 ± 0.28[a]
Soybeans Cd 100 ppm	16.14 ± 0.16[a]

Data values are the mean ± SEM from six animals per group. Cd 15 ppm: group intoxicated with tap water containing 15 ppm of Cd^{2+}; Cd 100 ppm: group intoxicated with tap water containing 100 ppm of Cd^{2+}.
[a] pm < 0.001 vs. the respective control group (two-way ANOVA/Bonferroni).

a discernible change in the control and the 100 ppm Cd-treated groups (Fig. 2A).

Fig. 2B shows that the SOD activity, in rats receiving casein as well as soybeans in the diet, did not change after 15 ppm Cd exposure; however, an increase was observed when treated with 100 ppm of Cd, compared with the respective control group ($p < 0.05$ and $p < 0.001$, respectively). In addition, the use of soybeans instead of casein did not modify the SOD activity.

In rats fed with the casein diet, exposure to 15 and 100 ppm of Cd increased the aorta GPx activity compared with the control group ($p < 0.05$). In rats fed with the soybean diet, an increase of GPx activity was observed only with 15 ppm Cd-treatment, compared to the control group ($p < 0.05$). Additionally, the use of soybeans instead of casein showed an increase of the GPx activity in control and 15 ppm Cd-treated groups ($p < 0.05$ and $p < 0.0005$, respectively), while no change was observed when treated with 100 ppm of Cd (Fig. 2C).

CAT, SOD, GPx, Nrf2 and MTII mRNA levels

As shown in Fig. 3A, the expression of CAT mRNA in rats receiving casein in the diet, increased in the aorta of rats exposed to 100 ppm of Cd, compared with the respective control group ($p < 0.0001$). No significant changes were observed in the expression of CAT mRNA among the different soybean groups. Employing soybeans instead of casein in the diet, results in lower levels of aorta CAT mRNA in all groups ($p < 0.05$ for control and Cd-15 ppm, and $p < 0.005$ for Cd-100 ppm).

In rats receiving casein in the diet, the aorta mRNA levels of SOD were decreased with 15 ppm, and increased with 100 ppm Cd-treatment, compared with the control group ($p < 0.001$ and $p < 0.01$, respectively).

In rats fed with the soybean diet, a decrease of mRNA levels of SOD was observed with 15 ppm Cd-treatment, without a discernible change when exposed to 100 ppm of Cd, compared to the control group

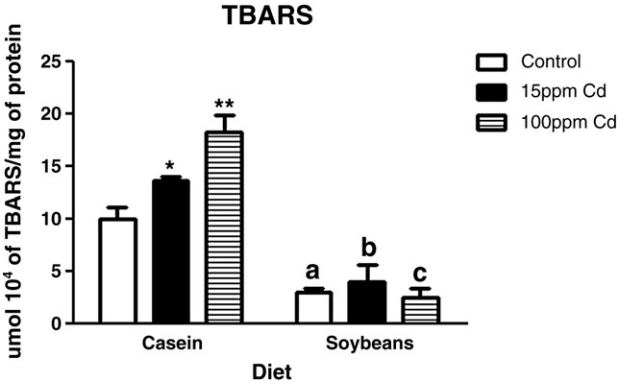

Fig. 1. Effect of cadmium intoxication on the TBARS levels in aorta. Values represent mean ± SEM for n = 6/group. *p < 0.05 and **p < 0.01 vs. casein control group. [a,c]p < 0.001 and [b]p < 0.005 vs. the respective casein group.

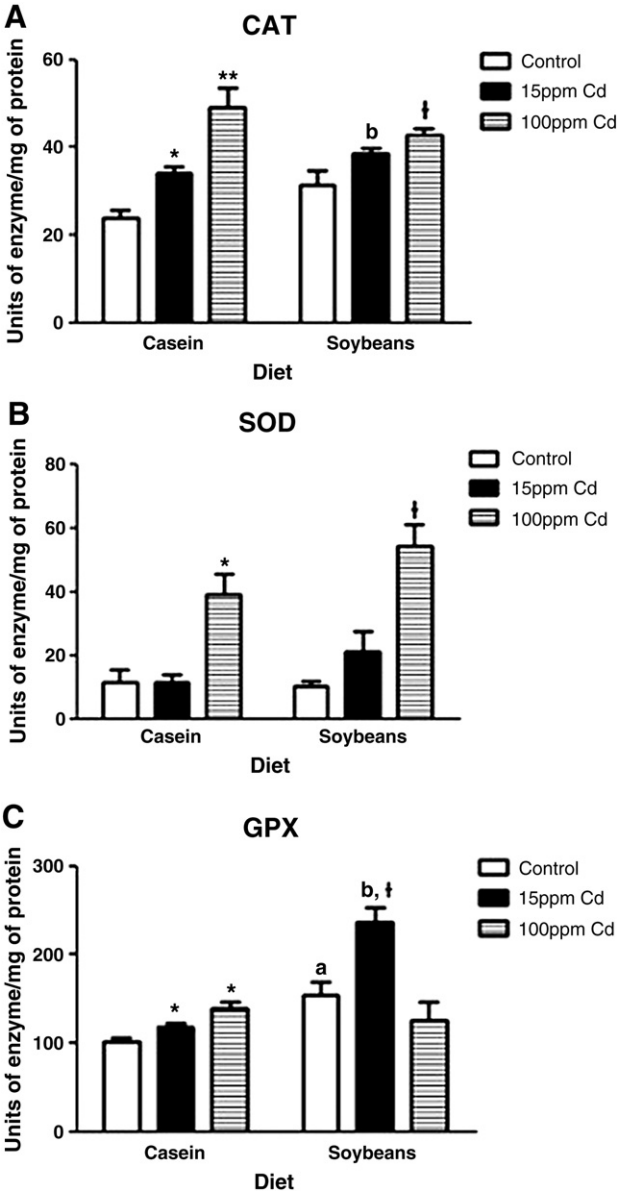

Fig. 2. Effect of cadmium intoxication on the antioxidant enzyme activities in aorta. Values represent mean ± SEM for n = 6/group. A) Catalase (CAT) *p < 0.005 and **p < 0.001 vs. casein control group; p < 0.05 vs. soybean control group; [b]p < 0.05 vs. respective casein group. B) Superoxide dismutase (SOD) *p < 0.05 vs. casein control group; p < 0.001 vs. soybeans control group. C) Glutathione peroxidase (GPx) *p < 0.05 vs. casein control group; p < 0.05 vs. soybeans control group; [a]p < 0.05 and [b]p < 0.0005 vs. respective casein group.

(p < 0.001) (Fig. 3B). In addition, using soybean instead of casein in the diet did not modify the SOD mRNA expression.

The GPx mRNA expression in the aorta of rats receiving casein in the diet, decreased with 15 ppm and increased with 100 ppm of Cd-treatment, compared with the control group ($p < 0.005$ and $p < 0.05$, respectively). No discernible changes were observed in the GPx mRNA levels among the different soybean groups, being similar to that of the casein control group.

After exposure to 100 ppm of Cd in rats receiving casein in the diet, the mRNA levels of Nrf2 in aorta increased, compared with control ($p < 0.05$). No changes were observed when treating with 15 ppm of Cd. In addition, no changes were observed in the Nrf2 mRNA expression among the different soybean groups. Using soybeans instead of casein in the diet shows higher levels of the Nrf2 mRNA in the 15 ppm Cd-treated group ($p < 0.01$) (Fig. 3D).

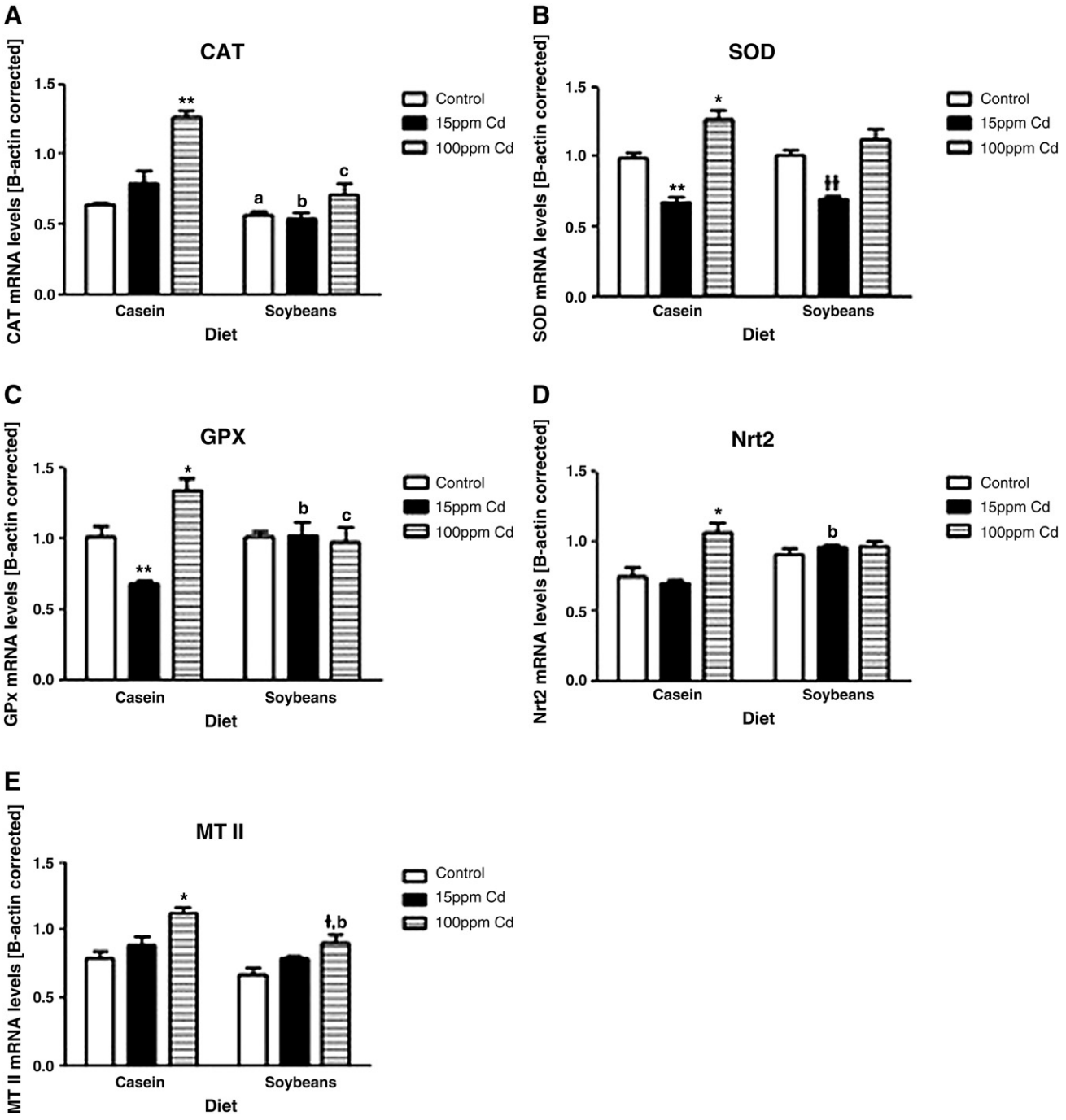

Fig. 3. Effect of cadmium intoxication on the enzyme mRNA expressions in aorta. Target mRNA was normalized by the level of beta-actin mRNA. Values represent mean ± SEM for n = 6/group. A) $^{**}p < 0.0001$ vs. casein control group; ^{a}, $^{b}p < 0.05$ and $^{c}p < 0.005$ vs. respective casein group. B) $^{**}p < 0.001$ and $^{*}p < 0.01$ vs. casein control group; $p < 0.001$ vs. soybeans control group. C) $^{**}p < 0.005$ and $^{*}p < 0.05$ vs. casein control group; ^{b},$^{c}p < 0.05$ vs. respective casein group. D) $^{*}p < 0.05$ vs. casein control group; $^{b}p < 0.01$ vs. respective casein group. E) $^{*}p < 0.05$ vs. casein control group; $p < 0,05$ vs. soybeans control group; $^{b}p < 0.05$ vs. respective casein group.

The MT II mRNA expression in rats receiving casein, as well as soybeans, in the diet, increased in the aorta of rats exposed to 100 ppm of Cd, compared with the respective control group ($p < 0.05$). Using soybeans instead of casein in the diet shows lower MT II mRNA levels in the 100 ppm Cd-exposed group ($p < 0.05$) (Fig. 3E).

CAT, GPx and NOX-2 and protein expression in aorta

In order to evaluate the consequences of Cd intoxication at transductional levels, we measured CAT, GPx and NOX-2 enzymes (the latest being involved in the superoxide production) by Western blot in aorta of Cd exposed rats.

As shown in Fig. 4, only 100 ppm Cd exposure modified the protein levels of CAT, GPx and NOX-2 in aorta of rats receiving casein in the diet, showing an increase compared with the corresponding control ($p < 0.005$, $p < 0.05$ and $p < 0.05$, respectively). No significant changes were observed in the protein expression of the three enzymes among the different soybean groups. The use of soybeans instead of casein in the diet shows lower CAT, GPx and NOX-2 protein levels in the 100 ppm Cd-treated group ($p < 0.05$), without change in the control and 15 ppm Cd- treated groups.

Morphological changes induced by Cd in aorta

The aorta intima layer of the control animals fed with the casein diet was composed of a continuous layer of endothelial cells. In the tunica media, several elastic fibers were seen to be lying parallel to each other, with smooth muscle cells interposed between them. Irregular luminal layers of endothelial cell linings were observed in aortas of 15 ppm Cd-treated animals, which were more evident after exposure to 100 ppm of Cd. In this last group, light microscopy images revealed structural changes in tunica intima cells, exhibiting clearer and bigger cytoplasms than control. Cells of the tunica media in close contact with the intima, also showed these morphological alterations. Aorta histological sections of control animals, as well as 15 ppm Cd-exposed-rats, fed with the soybean diet, exhibited normal characteristics of all the artery wall tunics. The 100 ppm Cd-treated rats showed only limited changes in the vascular endothelium, compared with its control, as well as with aorta from rats fed with the casein diet and intoxicated with 100 ppm of Cd (Fig. 5).

Discussion

The production of ROS and RNS induced by Cd, could be responsible for its toxic effects in many tissues and organs (Beyersmann and Hartwig, 2008; Stoths and Bagchi, 1995; Waisberg et al., 2003). This study provides experimental evidence of the effect of Cd, ingested through drinking water, on the aorta redox state and morphology, and the attenuation of those effects when casein is replaced by soybeans as the protein source of the diet.

Environmental Cd exposure has been associated with cardiovascular disease among men (Menke et al., 2009; Prozialeck et al., 2006). A study

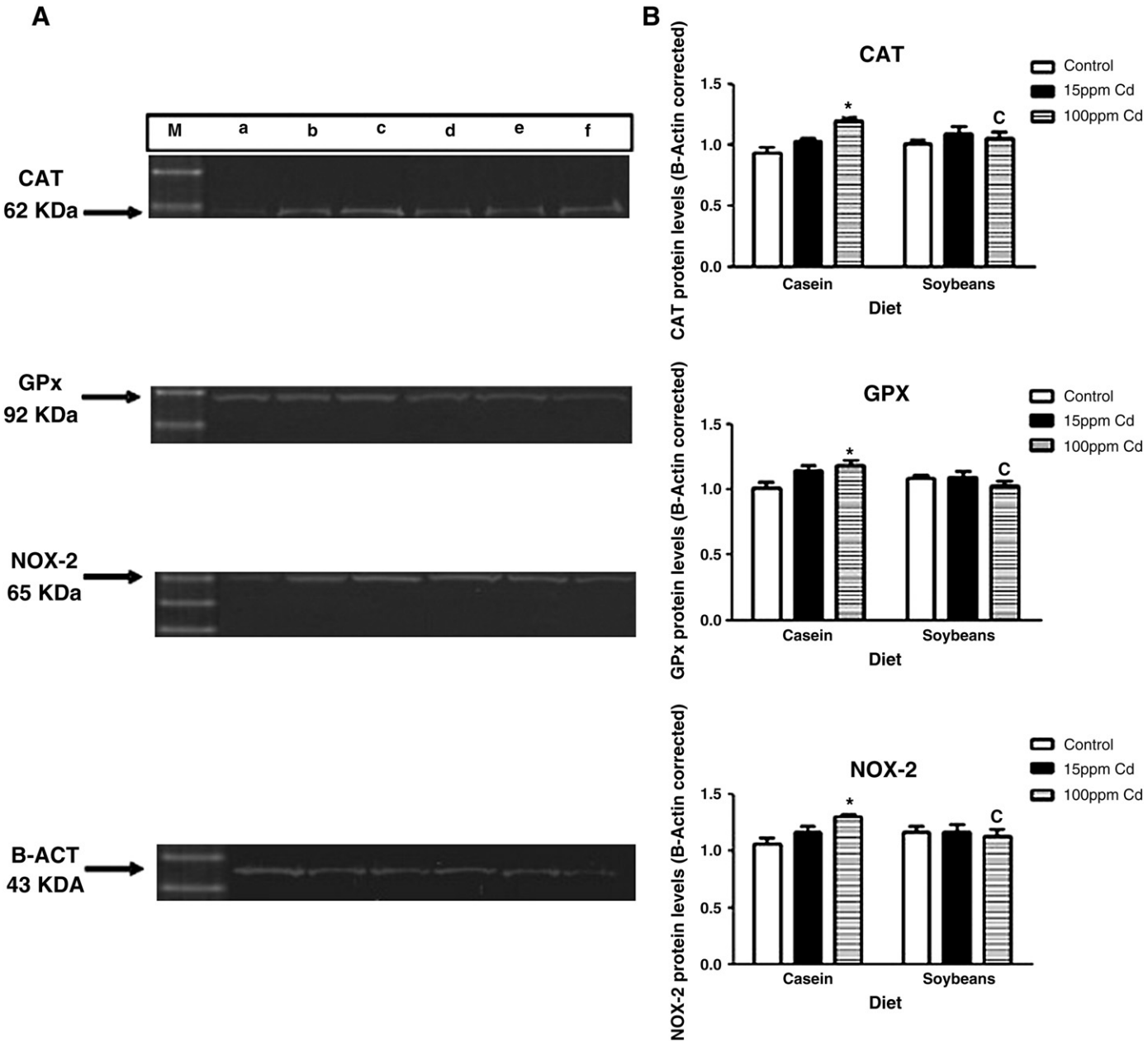

Fig. 4. Effect of cadmium intoxication on the protein expression of antioxidant enzymes and NOX-2 subunit in aorta. Representative Western blot analysis for (A) catalase (CAT), (B) glutathione peroxidase (GPx), (C) NADP(H) oxidase (NOX-2) and (D) beta-actin used as an internal control. M, molecular weight marker; 1, casein control; 2, casein 15 ppm Cd-treatment; 3, casein 100 ppm Cd-treatment; 4, soybean control; 5, soybean 15 ppm Cd-treatment; 6, soybean 100 ppm Cd-treatment. On the side, is a quantification of the intensity of the fragment bands. Identical results were obtained in six animals per group. (A) $^{*}p < 0.005$ vs. casein control group; $^{c}p < 0.05$ vs. respective casein group. (B) $^{*}p < 0.05$ vs. casein control group; $^{c}p < 0.05$ vs. respective casein group. (C) $^{*}p < 0.01$ vs. casein control group; $^{c}p < 0.05$ vs. respective casein group.

carried out in Belgium, reported a relationship between urinary Cd excretion and changes in some physiological indicators of cardiovascular function, such as pulse wave velocity, arterial pulse pressures, and arterial compliance and distensibility, in subjects subjected to environmental exposure to the metal (Schutte et al., 2008). Using drinking water contaminated with 15 ppm of Cd^{2+} (Calderoni et al., 2010; Larregle et al., 2008), plasma Cd levels in rats attained WHO (1992)-defined toxic levels, which corresponded to those observed in the plasma of alkaline battery assembly workers (Jakubowski et al., 1987). A 100 ppm Cd concentration in the drinking water is equivalent to a daily intake of 2000 μg Cd/day/rat, in accord to the daily human intake of Cd in the most heavily contaminated areas (600–2000 μg/day) (WHO, 1992). We observed that Cd levels in serum of intoxicated rats, showed a dose-dependent increase, as it was reported by other authors (Ferramola et al., 2012; Thijssen et al., 2007). It is conceivable that Cd absorption and distribution in the animals, are not modified by the diet containing soybeans instead of casein, since no differences were found in serum Cd levels between casein and the corresponding soybean group, like it has been recently reported in the heart, using the same experimental model (Ferramola et al., 2012).

Oxidative stress has been linked to various cardiovascular diseases, such as hypertension, hypercholesterolemia, and heart failure (Charach et al., 2012; Förstermann, 2008; Inagi, 2006; Silva et al., 2012). However, to our knowledge, there are no reports of the effect of different doses of Cd (15 ppm and 100 ppm) on the redox balance in aorta, when the dietary protein source is soybeans instead of casein. Consistent with the studies mentioned above, after 2 months of 15 and 100 ppm Cd exposure, we found increased TBARS levels, which are used as a criterion for the degree of lipid peroxidation in the aorta of rats receiving casein as a source of dietary protein. The induction of lipoperoxidation was associated to an increased oxidative stress, since alterations in the activity of enzymes that constitute the first line of defense against $O_2\bullet^-$ and H_2O_2, were observed. In fact, Cd (15 ppm) increased the activity of CAT and GPx in the aorta of rats receiving casein in the diet, suggesting a high H_2O_2 availability. Since the SOD activity was unchanged, results indicate a differential effect of Cd intoxication on the activity of antioxidant enzymes in the artery.

An even higher increase of aorta CAT and GPx activities was induced when animals fed with the casein diet were exposed to 100 ppm of Cd. In this case, the high CAT and GPx activities were accompanied by an increase of the respective mRNA and protein levels, suggesting post-transcriptional and post-traductional modulation of Cd on the expression of those enzymes in the artery. Also, aorta SOD activity and mRNA levels were increased. Furthermore, consistent with a high oxidative environment, the protein levels of NOX-2 increased in the aorta of rats exposed to 100 ppm of Cd. Since NOX-2 is the major source of $O_2\bullet^-$ in the vessel wall (Griendling et al., 2000), a high $O_2\bullet^-$ production might occur with 100 ppm Cd^{2+} exposure. Also, NAD(P)H oxidase activity may provide a source of ROS, which activates Nrf2/ARE-mediated antioxidant gene expression, such as CAT, GPx and SOD, in order to maintain redox homeostasis (Mann et al., 2007a,b). Accordingly, the aorta mRNA levels of Nrf2 were increased after intoxication with 100 ppm of Cd, compared to control. These results clearly show that 100 ppm Cd^{2+} exposure provokes a redox imbalance in aorta of rats fed with the casein diet. Considering that the aorta TBARS content was increased with 15 ppm of Cd, and even more after exposure to 100 ppm of Cd in the groups fed with the casein diet, compared with control, the obtained data suggest that 100 ppm Cd treatment results in an abnormal excessive production of ROS that leads to lipid peroxidation. Therefore, the increase of antioxidant enzyme activities would not be enough to decrease TBARS levels.

Vascular endothelium and smooth muscle cells (VSMCs) have been suggested to be a critical target of Cd toxicity (Prozialeck et al., 2008), which leads to blood pressure increase (Tellez-Plaza et al., 2008) and peripheral arterial disease (Lee et al., 2011; Navas-Acien et al., 2004). Also, it has been well established, that chronic inflammation and oxidative stress play crucial roles in endothelial dysfunction (Kim et al., 2006). Therefore, the irregular luminal layers of endothelial cell linings observed in aortas of Cd-treated animals fed with the casein diet, might affect the normal endothelial barrier function. In vitro experiments on endothelial cells, have indicated that Cd concentrations well below the range currently considered toxic, induce cell death leading to increased endothelial cell permeability (Messner et al., 2009). Furthermore, 100 ppm Cd-intoxication produced cytoplasmic alterations in the aorta of rats fed with the casein diet, which could be due to edema or hydropic swelling because of the metal toxicity. After Cd exposure, mitochondrial swelling has been observed in rat's heart and kidney (Kobroob et al., 2012; Shemarova et al., 2011). Also after Cd

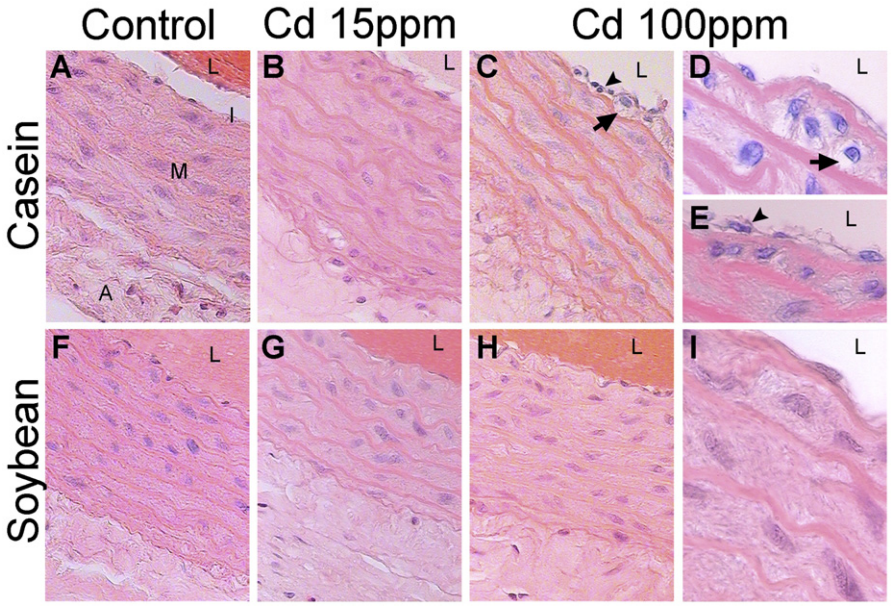

Fig. 5. Morphological analysis of the aorta sections of casein (A–E) and soybean (F–I) groups, stained with hematoxylin–eosin. The three aorta layers exhibit normal histological characteristics in control animals (A and F) and 15 ppm Cd exposed-rats fed with the soybean diet (G). The 100 ppm Cd exposed-rats fed with casein (C–E), show irregular layers of endothelial cells (arrowheads). Some cells of the tunica intima and media exhibit cytoplasmic alterations (arrows). I: tunica intima; M: tunica media; A: tunica adventitia; L: lumen. A–C and F–H: X400; D, E and I: X1000.

exposure, ultrastructural alterations have been found in the hepatocytes, including numerous large lipid droplets, mitochondrial condensation and swelling, and rough endoplasmic reticulum (RER) dilatation and vesiculation (Thophon et al., 2004). Even though a significant increase of triglyceride content was observed in aorta of 100 ppm Cd-exposure rats fed with the casein diet (see supplemental data), the H–E staining does not allow one to establish that cytoplasmic alterations are consistent with lipid droplets.

It is known that lipoperoxidation plays an important role in the degree of vascular damage. Premature onset of clinical coronary atherosclerosis has been associated with increased levels of lipid peroxidation (Fabbi et al., 2004). It has been recently observed in our laboratory that there is an increase in serum TBARS levels with a consistent decrease in circulating PON-1 (arilesterase) enzyme activity, after 60 days of 100 ppm Cd intoxication, in rats fed with a casein-based diet (Ferramola et al., 2012), which could be a risk for LDL oxidation (Tomás et al., 2004). Thus, it is conceivable that the redox imbalance and lipoperoxidation induced by 100 ppm Cd exposition can cause, at least in part, the morphological alterations observed in the aorta of rats fed with the casein diet. It cannot be discarded that atherosclerotic changes might be evident with a longer Cd exposition time.

Soybeans and soybean-derived products, represent the major sources of dietary isoflavones (Bingham et al., 2003). Soy isoflavones have been demonstrated to possess antioxidant properties in the cardiovascular system. Recently, the antihypertensive, antioxidant, and anti-inflammatory potential properties of fermented soy milk in vitro systems, have been shown (Martinez-Villaluenga et al., 2012). Furthermore, administration of soybean extracts has been shown to inhibit hyperglycemia and free radical-mediated oxidative stress, and to improve vascular functions on streptozotocin-induced diabetic rat (Lim et al., 2012).

Interestingly, in rats fed with the soybean diet, not only we found no significant changes in the aorta TBARS levels between Cd-treated and untreated groups, but those TBARS levels were markedly lower, compared to casein fed groups, suggesting an antioxidant effect of the soybean diet. In particular, in the aorta of 100 ppm Cd-intoxicated rats, where a clear redox imbalance was demonstrated when casein was the protein diet, soybeans induced a decrease of NOX-2 protein, which would be associated to a lower $O_2^{\bullet -}$ production. In addition, soybeans did not modify the CAT, SOD and GPx activities, compared with the corresponding casein group. However, these enzyme activities would be enough to inactivate a low $O_2^{\bullet -}$ level, thus, leading to a reduction of the TBARS content in aorta. In addition, in the soybeans fed groups, the mRNA Nrf2 levels, and mRNA and protein levels of CAT and GPx in aorta, were not modified by 100 ppm Cd treatment, suggesting that no post-transcriptional and post-traductional modifications were induced by Cd.

The low TBARS levels in aorta of 15 ppm Cd-exposed rats, fed with the soybean diet, were associated to a high activity of GPx and CAT, compared with the respective casein group; nevertheless, the effects at molecular level were inconsistent, since a decrease of CAT mRNA level and an increase of GPx mRNA level, were observed. However, the mRNA and protein levels of CAT and GPx were unchanged, while the enzyme activities were increased compared with its control. This suggests that 15 ppm Cd-treatment, as it was observed with 100 ppm Cd-exposure, did not induce post-transcriptional and post-transductional modifications in rats fed with the soybean diet. Since the SOD activity and mRNA levels were not modified in any of the soybean groups, compared to the respective casein groups, the antioxidant effect of soybeans is very likely not mediated by SOD.

Thus, all the above contributes to suggest that soybeans in the diet prevent or reduce further lipoperoxidation in the aorta.

On the other hand, the transcription of MT genes can be up-regulated in response to Cd (Bobillier-Chaumont et al., 2006). It has been suggested that the induction of oxidative stress may play a key role in the Cd-induced MTI expression, through the activation of ARE elements in the MTI gene promoter (Li et al., 1998). The nuclear factor Nrf2 is known to be stimulated by oxidative stress in the cardiovascular system (Mann et al., 2007a, 2007b; Ungvari et al., 2010). Nrf2 binds to the ARE, regulating ARE-mediated gene expression and induction (Leonard et al., 2006; Thimmulappa et al., 2002) and induces MTI and MTII expressions (Wu et al., 2012). The increase of MTII expression in the aorta of rats intoxicated with 100 ppm of Cd and fed with the casein diet, compared to control, was associated to the increased expression of Nrf2 mRNA. In addition, a low increase of MTII mRNA expression and no change in the Nrf2 mRNA expression, were observed in the aorta when soybeans were used instead of casein. This could be related to the lower oxidative stress observed in the aorta of rats fed with soybeans, compared to casein.

As it was expected, the aortas from soybean Cd-exposed groups showed very few morphological alterations. In fact, the aortas of control animals, as well as 15 ppm Cd exposed-rats, exhibited normal characteristics of all the artery wall tunics, and only limited changes in the vascular endothelium of the aorta from 100 ppm Cd-treated rats were observed, compared with their control. The attenuation of morphological changes in the aorta by the soybean diet was not due to a decreased Cd intestinal absorption, since no differences were found in serum Cd-levels between casein- and soy-fed animals. Thus, dietary soybeans seem to protect against vascular morphological alterations produced by Cd exposure.

The interactions among the antioxidant nutrients appear to be very important in protecting cells, since the concentration of each antioxidant alone may not be adequate to effectively protect the vascular cells against lipid peroxidation. Compared with the major antioxidants in plasma (ascorbic acid, uric acid, α-tocopherol, β-carotene, and other carotenoids), the concentration of isoflavones in plasma is relatively low and can reach concentrations comparable with those of carotenoids [1 μmol/l] (Yeum et al., 1996), after the consumption of meals containing soy products, that are high in isoflavones or isoflavone supplements (Upritchard et al., 2003; Yeum et al., 1996). The possible interaction between different antioxidants in vivo may modify the overall antioxidant status, making the evaluation of the effect of particular antioxidants hard to interpret.

Our overall results, and for the first time to our knowledge, show that alterations in the redox balance and morphology induced by Cd intoxication in the aorta, might be attenuated or prevented by the replacement of casein by soybeans in the diet. Dietary soybeans could provide some potential benefit in the prevention of arterial injury by an antioxidant action.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.07.016.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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