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Cadmium-induced oxidative stress and histological damage in the myocardium. Effects of a soy-based diet

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ABSTRACT

Cd exposure has been associated to an augmented risk for cardiovascular disease. We investigated the effects of 15 and 100 ppm of Cd on redox status as well as histological changes in the rat heart and the putative protective effect of a soy-based diet. Male Wistar rats were separated into 6 groups and treated during 60 days as follows: groups (1), (2) and (3) were fed a casein-based diet; groups (4), (5) and (6), a soy-based diet; (1) and (4) were given tap water; (2) and (5) tap water containing 15 ppm of Cd^{2+}; and (3) and (6) tap water containing 100 ppm of Cd^{2+}. Serum lipid peroxides increased and PON-1 activity decreased in group (3). Lipoperoxidation also increased in the heart of all intoxicated groups; however protein oxidation only augmented in (3) and reduced glutathione levels diminished in (2) and (3). Catalase activity increased in groups (3) and (6) while superoxide dismutase activity increased only in (6). Glutathione peroxidase activity decreased in groups (3) and (6). Nrf2 expression was higher in groups (3) and (6), and MTI expression augmented in (3). Histological examination of the heart tissue showed the development of hypertrophic and fusion of cardiomyocytes along with foci of myocardial fiber necrosis. The transmission electron microscopy analysis showed profound ultra-structural damages. No protection against tissue degeneration was observed in animals fed the soy-based diet. Our findings indicate that even though the intake of a soy-based diet is capable of ameliorating Cd induced oxidative stress, it failed in preventing cardiac damage.

Introduction

It is broadly recognized that metal wastes are widely distributed over the soils and waters of the earth's surface, exerting detrimental effects on the environment, living beings and, particularly, human health. Given its broad usage in various industrial applications, cadmium (Cd) is one of the most abundant non-essential elements (Page et al., 1986). Since this metal cannot be degraded, the risk of environmental exposure is constantly increasing due to its accumulation via the food chain (ATSDR, 2005). In non-occupationally exposed individuals, food and smoking are the primary sources of exposure (ATSDR, 2008).

At cellular level, Cd induces physiological damage to different organs such as kidneys, liver, lung, pancreas, testes, placenta, bone and heart (Cuypers et al., 2010). There is an increasing body of evidence indicating an association between Cd exposure and an augmented risk for cardiovascular disease (Everett and Frithsen, 2008; Peters et al., 2010; Schutte et al., 2008). Cd may be deposited in the heart muscle and produce cardiotoxicity at as low as 0.1 μM concentration (Limaye and Shaikh, 1999).

Most of Cd in the body is bound to a small protein called metallothionein (MT) (Klaassen et al., 1999; Nordberg, 2004). It has been observed that Cd intoxication increases MT levels in several animal tissues (Bobillier-Chaumont et al., 2006). Binding of Cd to MTs protects cells against this heavy metal toxicity.

Cadmium ion (Cd^{2+}) exerts toxic effects through several different mechanisms which involve inactivation of –SH groups, substitution of Ca^{2+} and/or Zn^{2+} and interaction with cell surface receptors (Beyersmann and Hechtenberg, 1997). Several studies have shown that Cd stimulates reactive oxygen species (ROS) production resulting in oxidative deterioration of lipids, proteins and DNA in various target tissues such as lung, liver and kidneys, as well as testis, bone and heart (Ferramola et al., 2011; Nemmiche et al., 2007; Waisberg et al., 2003). Antioxidant enzymes such as glutathione peroxidase (GPx), copper/zinc superoxide dismutase (SOD) and catalase (CAT) are considered to be the first line of cellular defense that prevents cellular components from oxidative damages. These enzymes are generally considered as sensitive biomarkers of an organism's antioxidant responses (Lei et al., 2011). Recently, we demonstrated that chronic oral exposure to 15 ppm of Cd decreases GPx activity while increases

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CAT activity in the rat heart (Ferramola et al., 2011). It is known that the expression and coordinated induction of antioxidant enzymes are mediated by antioxidant response elements (ARE), representing a critical mechanism for protection against chemically induced oxidative/electrophilic stress. NF-E2-related nuclear factor 2 (Nrf2) binds to ARE sites and regulate antioxidant-mediated gene expression and induction (Jaiswal, 2004). GPx, SOD and CAT have been recently identified as Nrf2-regulated antioxidant enzymes (Hu et al., 2006; Kwak et al., 2003; Leonard et al., 2006; Thimmulappa et al., 2002).

Induction of oxidative stress appears to be a relevant mechanism for Cd genotoxicity which can be diminished by the presence of oxygen radical scavengers (Beyersmann and Hartwig, 2008). Soy is a unique dietary source of the isoflavones genistein and daidzein. It has been part of the Southeast Asian diet for nearly five millennia. Heavy consumption of soy in Southeast Asian populations is associated with reduction in the rates of several cancers and cardiovascular disease (Barnes, 1998). The protective effects of flavonoids have been attributed mainly to their antioxidant properties. It has been reported that flavonoids scavenge free radicals, chelate redox-active metal ions and increase MT expression (Kameoka et al., 1999; Morel et al., 1993; Sichel et al., 1991).

Taking into account all the above observations, the present study was aimed to investigate the putative protective effect of soy protein against oxidative stress and histoarchitectural changes induced in the rat myocardium after chronic exposure to 15 and 100 ppm Cd^{2+} concentrations.

Materials and methods

Animals and experimental design. The study was carried out on 8–10 week-old male Wistar rats, with an initial weight of 200–220 g. Animals were housed under conventional conditions at 22–25 °C, with a 12 h/12 h light/dark cycle. They had unlimited access to drinking water and food. Water and food intake was registered daily and no differences were observed between groups. Rats were divided into six experimental groups, with n = 6 per group, as follows: 1 — *Control casein group*: animals received tap water and were fed with a casein-based diet. 2 — *Cd 15 ppm group*: animals received tap water containing 15 ppm of Cd^{2+}, as $CdCl_2$, and were fed with a casein-based diet. 3 — *Cd 100 ppm group*: animals received tap water containing 100 ppm of Cd^{2+}, as $CdCl_2$, and were fed with a casein-based diet. 4 — *Control soy group*: animals received tap water and were fed with a soy protein-based diet. 5 — *Cd 15 ppm + soy group*: animals received tap water containing 15 ppm of Cd^{2+}, as $CdCl_2$ and were fed with a soy protein-based diet. 6 — *Cd 100 ppm + soy group*: animals received tap water containing 100 ppm of Cd^{2+}, as $CdCl_2$ and were fed with a soy protein-based diet. Rats were exposed to drinking water containing 15 ppm of Cd^{2+} in order to reach a serum Cd concentration of around 5 ppb, the level established as toxic limit in humans by the WHO (1992). On the other hand, exposure to 100 ppm of Cd^{2+} was selected following a previous study where this concentration was reported as environmentally realistic (Thijssen et al., 2007). Casein, and soy, protein-based diets were prepared in accordance to Reeves (1996), in order to fully satisfy the animals' nutritional requirements. The soy based diet was prepared using soybeans. Briefly, soybeans were cooked and subsequently dried. Finally they were grinded and sifted in a 0.42 mm pore sieve, in order to obtain the soy flour used in the soy-based diet. Following Reeves' recommendation (1996), the amount of soy flour added per kg of diet was adjusted to achieve a 12% of protein, as in the casein-based diet. The quantities of the remaining macro- and micro-nutrients were also adjusted in order to obtain isocaloric casein- and soy-based diets (Reeves, 1996). After 60 days of treatment, rats were killed by decapitation under light ether anesthesia, between 9 and 10 a.m. Serum samples were obtained from trunk blood. Left ventricles were dissected; samples were immediately frozen in liquid nitrogen and kept at −70 °C until processed for biochemical determinations. For histology and ultrastructural studies, samples were fixed in Bouin's or 4% glutaraldehyde in 0.1% sodium phosphate (pH 7.4). Animal treatment protocols were previously approved by the local ethics committee, and were in accordance with the Rats Care and Treatment Recommended Guidelines (U.S. Public Health Service, 1985).

Cadmium levels. Serum and heart Cd levels were determined by an electrothermal atomic absorption spectrometry system, using a Perkin Elmer Analyst 200 GF equipped with graphite tube and a Lvov Platform, with LD 0.001 μg/L and LQ 0.01 μg/L (detection and quantification limits of 1 ppt and 10 ppt), respectively. A matrix modifier was used (ammonium phosphate-ammonium nitrate). Calibration plot was made with an aqueous cadmium standard added with tensoactive agent and matrix modifier, in a range of 0.5–5 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 SRM from NIST, following method 200.9 revision 1.2 4/91 protocol. Cadmium recovery was about 98–99%. Sample's detection and quantification limits were 0.01 μg/L and 0.1 μg/L respectively.

Lipid peroxidation and paraoxonase-1 activity in serum. Lipid peroxidation levels were assessed as thiobarbituric acid reactive substances (TBARS) and measured spectrophotometrically according to Draper and Hadley (1990). Results were expressed as nmol of TBARS/mL of serum. Paraoxonase-1 (PON-1) activity was determined using paraoxon as substrate (Sampson et al., 2005). Results were expressed as enzyme International Units/mL of serum. All reagents were from Sigma-Aldrich Co.

Lipid peroxidation, GSH levels, and protein carbonyl-group content in the heart. Lipid peroxidation levels were determined spectrophotometrically in the heart by measuring TBARS, according to Draper and Hadley (1990).

In order to assess non-proteic thiol-group concentration in the heart, reduced GSH (rGSH) values were measured as described in Ferramola et al. (2011), following Akerboom and Sies (1981). rGSH values were expressed as nmol/g of tissue. All reagents were from Sigma-Aldrich Co. Protein carbonyl-groups were determined following a protocol described in Seminotti et al. (2008). Results were calculated using the extinction molar coefficient $E = 22000\ M^{-1}\ cm^{-1}$, and expressed as nmol of carbonyl groups/mg of proteins. Total protein content was measured by the Biuret reaction (Layne, 1957).

Antioxidant enzyme activity in the heart. The supernatants of heart homogenates were used for the determination of antioxidant enzyme activity. In all cases, tissue was homogenized in 30 mM PBS buffer, with 120 mM KCl, pH 7.4, containing 1× protease inhibitors (Pepstatin A and PMSF) followed by centrifugation at 800 ×*g*, for 15 min at 4 °C.

Catalase activity was assayed spectrophotometrically according to Aebi (1984). Briefly, decomposition of H_2O_2 was monitored at 240 nm, after the addition of heart homogenate supernatant. Enzymatic activity was expressed as International Units (IU)/mg of protein (1 IU decomposes 1 μmol H_2O_2/min at pH 7, at 25 °C). Glutathione peroxidase total activity was measured following NADPH oxidation rate, according to Flohe and Gunzler (1984) and expressed as IU/mg of protein (1 IU oxidizes 1 μmol NADPH/min at pH 7.7, at 30 °C). Superoxide dismutase total 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, according to McCord and Fridovich (1969). Enzymatic activity was expressed as IU/mg of protein (1 IU was defined as the amount of enzyme that inhibits cytochrome c reduction by 50%, at pH 7.8, at 25 °C). All reagents were from Sigma-Aldrich Co.

In all cases, total protein content was measured by the Biuret reaction (Layne, 1957).

RNA isolation and semi-quantitative RT-PCR analysis. Nrf2 and MTI mRNA levels were determined by RT-PCR. First, total RNA was isolated from heart samples using the Trizol® reagent (Invitrogen), following the manufacturer's indications. Second, 3 μg of total RNA was reversed transcribed, using random primer hexamers (Biodynamics, SRL) and M-MLV reverse transcriptase (Promega), according to the manufacturer's instructions. PCR amplification was carried out using specific oligonucleotide primers shown in Table 1. A cDNA aliquot (1/10 of the RT reaction product) was amplified with a PCR master mix, using Taq DNA polymerase (Invitrogen). PCR products were analyzed on 2% agarose gels, containing GelRed (Genbiotech) to visualize the bands. Band 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.

Histopathological analysis. Left ventricles were immersed and fixed in Bouin's solution and embedded in paraffin for light microscopy studies. Sections of 4 μm thickness (in an interval of 100 μm per animal) were stained with hematoxylin & eosin (H-E) for general histology assessment. Sirius red (S-R) staining was performed for the detection of collagen using the reagent Direct red 80 (Sigma, USA), in accordance with a standard protocol. Ten randomly selected fields from two slides of each rat (n = 3–4 rats in each group) were evaluated by 2 investigators blinded to the origin of the slides.

For transmission-electron-microscopy examination, heart samples were cut into small pieces and pre-fixed in 4% glutaraldehyde in 0.1% sodium phosphate (pH 7.4). Afterwards, specimens were washed twice in phosphate buffer and post-fixed in 1% osmium tetroxide in the same buffer at 4 °C overnight. Samples were dehydrated in an ethanol series and embedded in Spurr resin. Sectioning was carried out with a Potter Blum MT1 ultramicrotome. Slices were stained with lead citrate and uranyl acetate. Preparations were examined with a Zeiss EM electron microscope.

Apoptosis assessment. Apoptotic cells in left ventricles were identified using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in stained sections. TUNEL assay was performed according to the manufacturer's instructions (DeadEndT Fluorometric TUNEL System, Promega). Briefly, deparaffinized slides were fixed in 4% paraformaldehyde and treated with proteinase K to permeabilize the tissues. The slides were incubated with a labeling reaction mix using fluorescein as fluorophore, during 1 h at 37 °C. The reaction was stopped by the addition of 2× SSC and 3 washes in PBS were made before tissue was mounted in a fluorescence mounting medium (DAKO Corp., CA, USA). Nuclei were stained with DAPI.

Statistical analysis. Data are expressed as mean ± S.D. Statistical analysis was performed using one-way ANOVA followed by Tukey test. Values of $p<0.05$ were considered statistically significant.

Table 1
Oligonucleotide primers sequences for RT-PCR.

Gene name	GenBank accession number	Primers sequences (5′-3′)[a]	Fragment size
β-actin	NM_031144	F: CGTGGGCCGCCCAGGCACCA R: TTGGCCTTAGGGTTCAGAGGG	243 bp
Nrf2	NM_031789	F: CGGCATTTCACTGAACACAAGT R: TGGCTGTGCTTTAGGTCCATT	160 bp
MT I	NM_138826.4	F: ACTGCCTTCTTGTCGCTTA R: TGGAGGTGTACGGCAAGACT	312 bp

[a] F, forward primer; R, reverse primer.

Results

Cadmium levels in the serum and heart

Cadmium levels were significantly higher in the serum and heart of intoxicated animals, in comparison to controls. As shown in Table 2, such increase was dose-dependent. No significant differences were found between animals fed the casein- or the soy-based diet (Table 2).

Lipid peroxidation and PON-1 activity in serum

Cadmium exposure caused an increase in serum lipid peroxides in the Cd 100 ppm group ($p<0.01$); however, no significant change was observed in the serum TBARS in Cd 15 ppm group (Table 3). No differences were found between casein-control and soy-control groups either, nor between serum lipoperoxidation in Cd 100 ppm + soy and soy-control groups (Table 3). Consistently, we observed a significant decrease in the enzymatic activity of serum PON-1 only in Cd 100 ppm animals ($p<0.001$). No significant changes were found between controls and/or the soy-fed groups (Table 3).

Antioxidant/pro-oxidant imbalance in the heart

In animals fed either the casein- or the soy-based diet, heart lipid peroxidation showed a significant increase when they were exposed to 15 ppm and 100 ppm of Cd, in comparison to controls ($p<0.01$, Table 4). When we analyzed heart protein oxidation, we observed an increase in protein-carbonyl groups only in the heart of Cd 100 ppm animals ($p<0.001$, Table 4). Additionally, a decrease in rGSH levels in the heart of Cd 15 ppm and Cd 100 ppm rats was observed ($p<0.05$, Table 4). In our experimental conditions, the soy protein-based diet did not protect against lipoperoxidation, however it did protect against protein oxidation and the decrease in rGSH levels, in the heart of both Cd 15 ppm and Cd 100 ppm intoxicated rats (Table 4).

Interestingly, we observed differential effects of Cd intoxication on the activity of antioxidant enzymes in the rat heart. CAT activity increased significantly in the heart of Cd 100 ppm or Cd 100 ppm + soy rats, in comparison to controls ($p<0.001$ and $p<0.05$, respectively, Table 5). We also found a statistically significant augmentation in this enzyme activity in the heart of control rats fed the soy-based diet, when compared with controls given the casein-based diet ($p<0.05$, Table 5). Consistently, we observed an increase in SOD activity in Cd 100 ppm + soy animals ($p<0.01$), when compared to control S (Table 5). On the contrary, GPx activity diminished in the heart of Cd 100 ppm and Cd 100 ppm + soy rats ($p<0.001$ and $p<0.05$, respectively), in comparison to controls (Table 5). No changes were observed in GPx activity in the heart of animals intoxicated with 15 ppm of Cd.

Table 2
Cadmium concentration in the heart and serum of rats exposed to $CdCl_2$.

	Cd concentration in the serum (ppb)	Cd concentration in the heart (ppb)
Control C	0.402 ± 0.077	0.545 ± 0.037
Cd 15 ppm	5.94 ± 0.87*	2.53 ± 0.37*
Cd 100 ppm	14.89 ± 0.43*	11.14 ± 1.48*
Control S	0.634 ± 0.086	0.616 ± 0.037
Cd 15 ppm + S	7.44 ± 0.94*	2.47 ± 0.32*
Cd 100 ppm + S	15.10 ± 0.66*	11.14 ± 1.42*

Data values are the mean ± S.D. from six animals per group. C: casein; S: soy; 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+}.
* $p<0.001$ as compared to the control group (one-way ANOVA/Tukey).

Table 3
Lipid peroxidation (TBARS) and PON-1-activity assessment in serum.

	TBARS (nmol/mL of serum)	PON-1 (IU/mL of serum)
Control C	11.24 ± 1.66	312.8 ± 43.5
Cd 15 ppm	7.96 ± 0.69	317.2 ± 30.2
Cd 100 ppm	18.28 ± 3.14§	189.4 ± 30.3*
Control S	11.85 ± 3.43	226.6 ± 26.2
Cd 15 ppm + S	14.46 ± 1.86	282.9 ± 60.7
Cd 100 ppm + S	8.45 ± 0.43	249.8 ± 24.3

Data values are the mean ± S.D. from six animals per group. C: casein; S: soy; 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+}; IU: international units.
§ $p<0.01$ as compared to the control group (one-way ANOVA/Tukey).
* $p<0.001$ as compared to the control group (one-way ANOVA/Tukey).

Table 5
Antioxidant enzyme-activity assessment in the heart.

	CAT (IU/mg of protein)	SOD (IU/mg of protein)	GPX ($\times 10^{-1}$ IU/mg of protein)
Control C	12.31 ± 0.76	27.88 ± 4.62	4.47 ± 0.49
Cd 15 ppm	12.96 ± 0.96	33.88 ± 3.91	4.17 ± 0.38
Cd 100 ppm	16.39 ± 1.78*	26.07 ± 4.74	2.39 ± 0.35*
Control S	16.46 ± 0.82§	33.01 ± 5.81	3.88 ± 0.34
Cd 15 ppm + S	16.01 ± 1.33	34.39 ± 3.22	3.28 ± 0.23
Cd 100 ppm + S	19.31 ± 1.45#	44.93 ± 3.12#	2.79 ± 0.11#

Data values are the mean ± S.D. from six animals per group. C: casein; S: soy; 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+}; IU: International Units.
* $p<0.001$ as compared to the control C group (one-way ANOVA/Tukey).
# $p<0.05$ as compared to the control S group (one-way ANOVA/Tukey).
§ $p<0.05$ as compared to control C group (one-way ANOVA/Tukey).

Nrf2 and MTI expression in the heart

Fifteen ppm of Cd in the drinking water did not modify Nrf2 or MTI expression levels in the heart of rats fed either a casein- or a soy-based diet. Interestingly, Nrf2 mRNA levels increased in the heart of Cd 100 ppm and Cd 100 ppm + soy animals ($p<0.01$), in comparison to controls (Fig. 1A). An augmentation in MTI mRNA levels was also observed in the Cd 100 ppm hearts ($p<0.05$, Fig. 1B). However, no significant changes in MTI expression were observed in Cd 100 + soy hearts (Fig. 1B).

Histological evaluation

Heart tissue from the control groups had normal morphological features. General histology assessment with H-E showed a slight degeneration of the cardiac tissue in Cd 15 ppm rats. The presence of hypertrophic cardiomyocytes and fusion of some of them were observed. However, some cardiomyocytes presented normal morphological features (Fig. 2). A greater alteration of the cardiac muscle showing hypertrophic cardiomyocytes and vacuolated areas with necrotic aspect was observed in the Cd 100 ppm group (Fig. 2). No protective effects were observed in Cd 15 ppm + soy and Cd 100 ppm + soy animals.

S-R staining showed dilated interstitial space and modest deposits of extracellular matrix between hypertrophic cardiomyocytes in Cd 15 ppm hearts (Fig. 3). A significant degeneration of the cardiac muscle with increased collagen deposition was observed in Cd 100 ppm hearts (Fig. 3). No protection against histological changes was found in Cd15 ppm + soy and Cd 100 + soy animals.

In order to assess ultrastructural changes in the left ventricle of rats exposed to Cd, transmission-electron-microscopy was performed. Images show degenerative changes in the structure of the cardiomyocytes in Cd 15 ppm rats. We observed the presence of intracellular edema and alterations in myofibril arrangements (Fig. 4). Cadmium increased the severity of such structural alterations in Cd 100 ppm rats (Fig. 4). Ultrastructural study also revealed that exposure to both doses of Cd produced mitochondrial damage in the left ventricle. We observed loss of mitochondrial cristae, disruption of the mitochondrial membrane and vacuolization (Fig. 4). We also showed that intoxication with Cd led to a dilation of the endomembrane–T-tubule system in Cd 15 ppm and Cd 100 ppm myocardium (Fig. 4). Finally, TEM images revealed that exposure to Cd caused modification of the normal structure of the intercalated disks and dilation of intercellular spaces with an abundant deposit of collagen fibers in Cd 15 ppm and Cd 100 ppm hearts (Fig. 5). No protection was observed in animals fed the soy-based diet.

Table 4
Lipid peroxidation (TBARS), protein carbonyl-groups and rGSH assessment in the heart.

	TBARS ($\times 10^{-2}$ nmol/mg of protein)	Protein carbonyl-groups (nmol/mg of protein)	rGSH (nmol/g of tissue)
Control C	1.36 ± 0.26	1.57 ± 0.63	501.12 ± 6.34
Cd 15 ppm	10.94 ± 2.43*	2.25 ± 0.34	363.75 ± 19.08#
Cd 100 ppm	6.71 ± 1.31*	3.84 ± 0.75*	385.43 ± 45.65#
Control S	2.41 ± 0.30	2.88 ± 0.76	367.25 ± 102.34
Cd 15 ppm + S	9.44 ± 2.63*	2.02 ± 0.32	382.37 ± 60.69
Cd 100 ppm + S	5.52 ± 1.02#	3.06 ± 0.75	377.59 ± 62.46

Data values are the mean ± S.D. from six animals per group. C: casein; S: soy; 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+}.
# $p<0.05$ as compared to the control group (one-way ANOVA/Tukey).
* $p<0.001$ as compared to the control group (one-way ANOVA/Tukey).

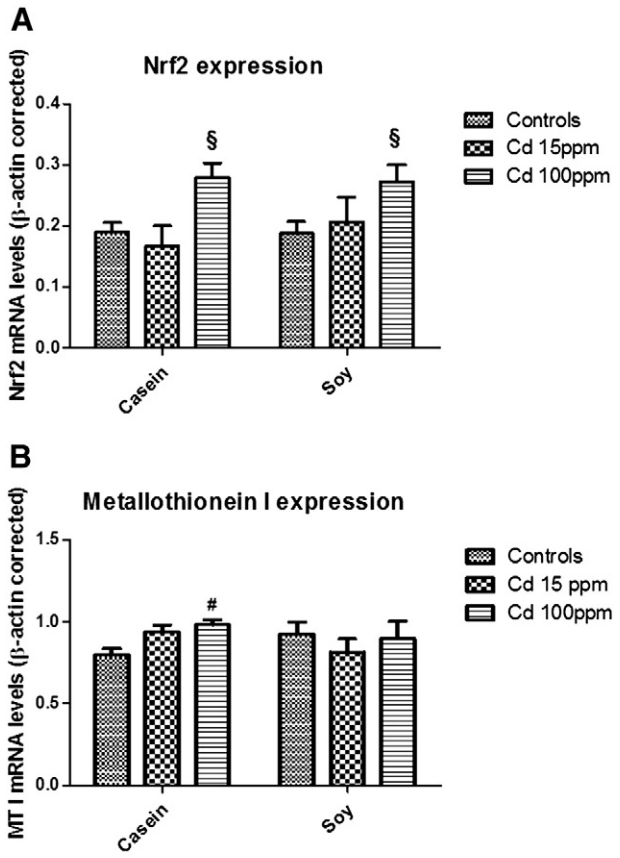


Fig. 1. Nrf2 and MT I expression in the heart of cadmium intoxicated rats. Nrf2 (A) and MT I (B) transcript levels were measured by RT-PCR and normalized against β-actin mRNA levels. Values are expressed as mean ± SD with #$p<0.05$ and §$p<0.01$, when treated groups were compared to the respective controls (one-way ANOVA/Tukey).

Apoptosis assessment

When we performed cardiomyocyte-specific TUNEL staining, it showed no apoptotic activation in Cd intoxicated animals (data not shown).

Discussion

Cadmium exposure produces oxidative stress and histological damage in the heart. A soy-based diet might improve cadmium-induced cardiac impairment in rats. It has been suggested that the ingestion of soy protein may help to protect against cardiovascular disease risk factors (Rudkowska, 2008).

As expected, in this work, Cd levels increased significantly in the rat serum and heart after 60 days of drinking tap water contaminated with 15 and 100 ppm of the metal. Similar results were recently found by others and by us (Ferramola et al., 2011; Manna et al., 2008; Thijssen et al., 2007). In the present study, Cd absorption and distribution in the animals wouldn't be altered by the ingestion of a soy-based diet, given that no differences were found in serum and heart Cd-levels between casein- and soy-fed animals.

Interestingly, we found that 100 ppm of Cd decreases antioxidant PON-1 activity and increases lipid peroxidation in the serum of rats fed the casein-based diet. Evidences of Cd-induced oxidative stress in serum have been previously reported by us (Ferramola et al., 2011). Given that PON-1 is responsible for HDL antioxidant capacity, it has an important role in preventing atherosclerosis (Tomás et al., 2004). Soy isoflavones have been demonstrated to possess antioxidant properties in biological systems (Kao and Chen, 2006). Interestingly, and for the first time in our knowledge, in this work, the soy-based diet prevented serum oxidative effects of Cd intoxication and thus, it would protect against cardiovascular disease. This is consistent with the inhibition of LDL oxidation in the presence of soy isoflavones observed by Tikkanen and Adlercreutz (2000).

There is an increasing body of evidence indicating an association between Cd exposure and an augmented risk for cardiovascular disease. Tellez-Plaza et al. (2008) found a modest increase in systolic or diastolic blood pressure associated with increasing blood Cd levels. A large US study showed an association between urinary Cd and myocardial infarction in women (Everett and Frithsen, 2008). Furthermore, a study carried out in Belgium reported a relationship between urinary cadmium excretion and changes in some physiological indicators of cardiovascular function i.e. pulse wave velocity, arterial pulse pressures, and arterial compliance and distensibility, in subjects environmentally exposed to the metal (Schutte et al., 2008). Additionally, Peters et al. (2010) found that environmental exposure to cadmium is associated with significantly increased prevalence of stroke and heart failure in the US adult population.

On the other hand, increasing evidence suggests that oxidative stress may play a crucial role in different types of cardiac diseases (Chen et al., 1998; Peters et al., 2010; Wang et al., 1998). It is well known that NADPH oxidase is a major source of reactive oxygen species (ROS) in the heart. Heymes et al. (2003) found an increased NADPH oxidase activity in the end-stage failing human heart. Interestingly, in transgenic mice in which SOD is overexpressed, the infarct size is markedly reduced. This observation would demonstrate that ROS are involved in the cardiac lesion (Chen et al., 1998; Wang et al., 1998).

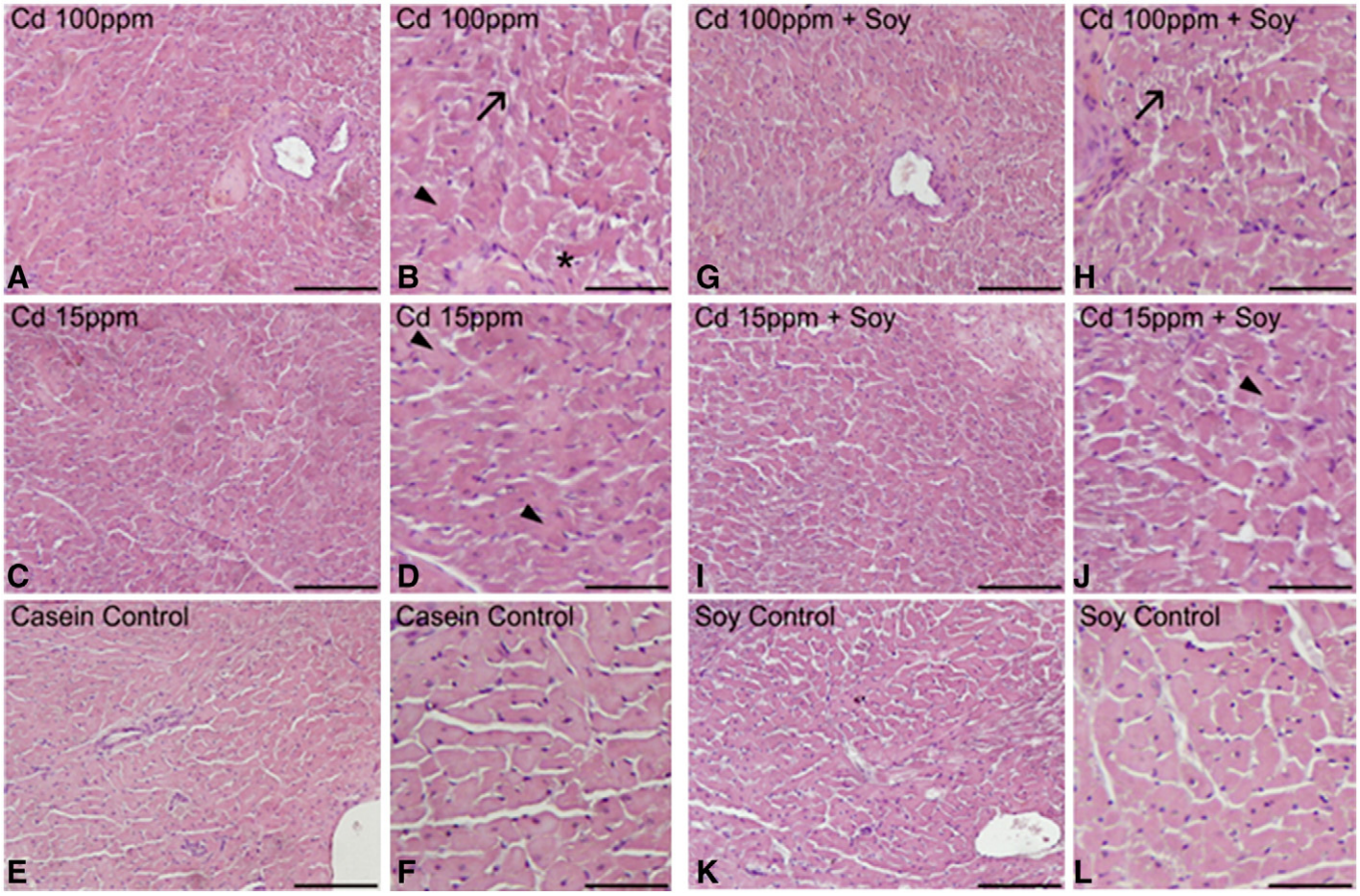


Fig. 2. Light micrographs of myocardium tissue. (A–B) Cd 100 ppm, necrosis and disarrangement of cardiac muscle fibers (arrows). (C–D) Cd 15 ppm, hypertrophic muscle fibers (arrow head). (E–F) Casein control. (G–H) Cd 100 ppm + soy. (I–J) Cd 15 ppm + soy. No significant improvements were observed in the myocardium of animals fed with a soy-based diet (G–J). (K–L) Soy control. H&E stain. Bars: A, C, E, G, I, K: 200 μm; B, D, F, H, J, L: 50 μm.

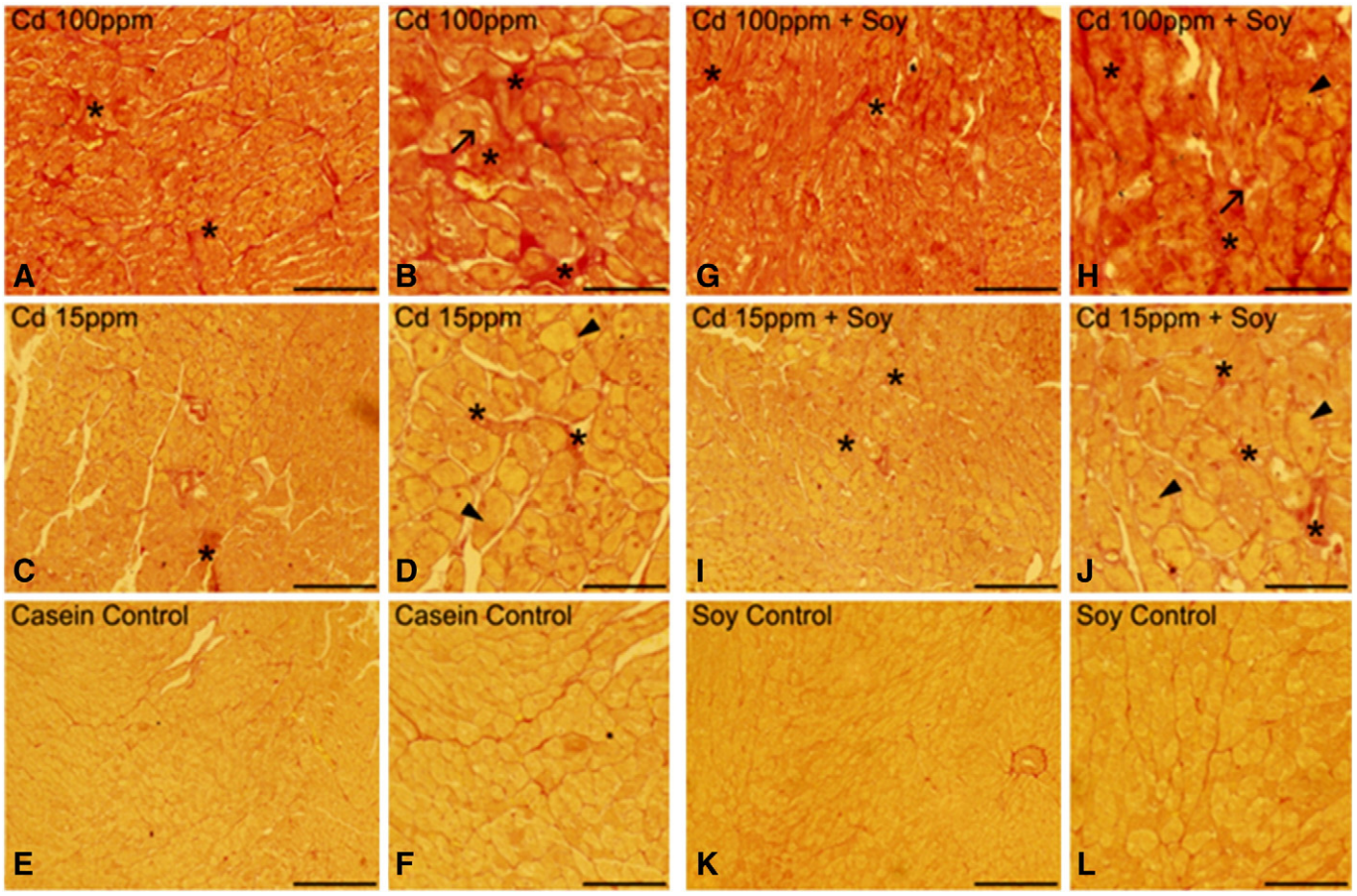
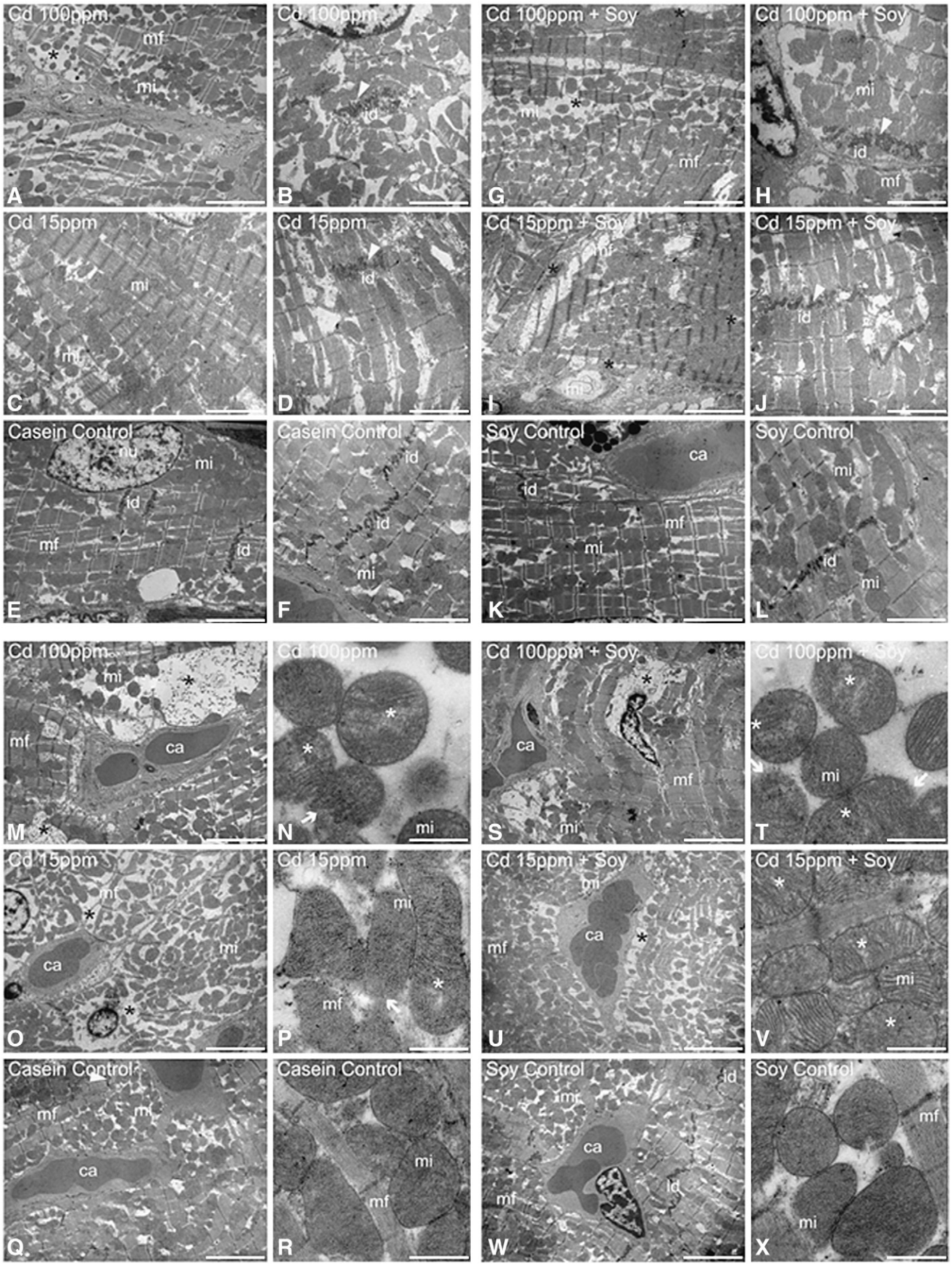


Fig. 3. Light micrographs of myocardium tissue. (A–B) Cd 100 ppm. (C–D) Cd 15 ppm. Necrosis and disarrangement of cardiac muscle fibers (arrows), hypertrophic cardiomyocytes (arrow head) and deposed extracellular material (asterisk) were observed in Cd exposed animals (A–D). (E–F) Casein control. (G–H) Cd 100 ppm + soy. (I–J) Cd 15 ppm + soy. No significant improvements were observed in the myocardium of animals fed with a soy-based diet (G–J). (K–L) Soy control. Sirius red stain. Bars: A, C, E, G, I, K: 200 μm; B, D, F, H, J, L: 50 μm.

In accordance with ours and others' previous results (Ferramola et al., 2011; Manna et al., 2008), in the present study, TBARS increased in the heart of all animals exposed to Cd. Interestingly, this augmentation was higher in Cd 15 ppm exposed animals than in those exposed to 100 ppm of Cd. Probably, this could be explained by the increased CAT activity observed in the heart of animals exposed to 100 ppm of Cd. Protein carbonyl-groups were also assessed as an extent of protein oxidation in the heart. We found an increase in protein carbonyl-groups in the heart of Cd 100 ppm rats and fed the casein-based diet. Consistently, we also observed a decrease in rGSH levels in the heart of all animals exposed to Cd and fed the casein-based diet. No changes were found in protein carbonyl-groups and rGSH levels in the heart of rats fed the soy-based diet. Cadmium-induced oxidative stress has been observed in other tissues and several species (Manna et al., 2008; Ognjanović et al., 2008; Sinha et al., 2008). Even though soy failed to prevent the increase of TBARS levels, it avoided the augmentation of protein carbonyl-groups and the decrease of rGSH levels in the heart. Similarly, Manna et al. (2008), observed a decrease in protein oxidation and rGSH depletion in the heart of Swiss albino mice exposed to Cd when the animals were treated with taurine, a cysteine derivative with antioxidant properties. Thus, in this model, the soy-based diet would protect the heart of animals exposed to Cd against protein oxidation and rGSH depletion. It is worthy of mention that Hagen et al. (2009) found an improvement in ventricular systolic and diastolic function and a decrease in myocardial oxidative stress after induction of MI, when rats were given an isolated soy protein diet.

As previously observed by us and others (Ferramola et al., 2011; Ognjanović et al., 2008; Sinha et al., 2008), we found an imbalance in the activity of antioxidant enzymes in the heart of animals intoxicated with Cd. Although GPx activity decreased and CAT activity increased in all groups of rats exposed to 100 ppm of Cd, we found an increase in SOD activity only when Cd 100 ppm animals were fed the soy-based diet. Additionally, the increase in CAT activity was significantly higher in Cd 100 ppm + soy animals in comparison to those intoxicated with Cd 100 ppm and fed a casein-based diet. Similarly, when Barbosa et al. (2011) investigated the effect of chronic ingestion of soy-protein extracts on the antioxidant status in rats, they found that soy protein increased CAT activity in liver, and SOD activity in erythrocytes. However, GPx activity did not increase in the erythrocytes after the intake of soy-protein. In our study, the increase in SOD activity in the heart of intoxicated rats fed a soy-based diet, would lead to higher levels of CAT substrate H_2O_2. The last would explain the higher increase in CAT activity observed in the heart of Cd 100 ppm animals and fed the soy-based diet. On the other hand, although a lower GPx activity might explain soy protein failing in preventing increased lipoperoxidation, increased SOD and CAT activities might protect against protein oxidation and rGSH depletion.

At this point, we wondered whether Cd intoxication would affect the expression of some antioxidant enzyme regulators. It is known that Nrf2 is a transcription factor that regulates the expression of CAT and SOD antioxidant enzymes (Hu et al., 2006; Kwak et al., 2003; Leonard et al., 2006; Thimmulappa et al., 2002). Thus, we continued to analyze the expression of Nrf2 in the heart of all groups of rats. As expected, the increased activity of antioxidant enzymes was consistent with the induction of Nrf2 expression in the heart of Cd 100 ppm and Cd 100 ppm + soy groups. On the other hand, the


Cd 100ppm
mf
mi
A
Cd 100ppm
id
B
Cd 100ppm + Soy
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G
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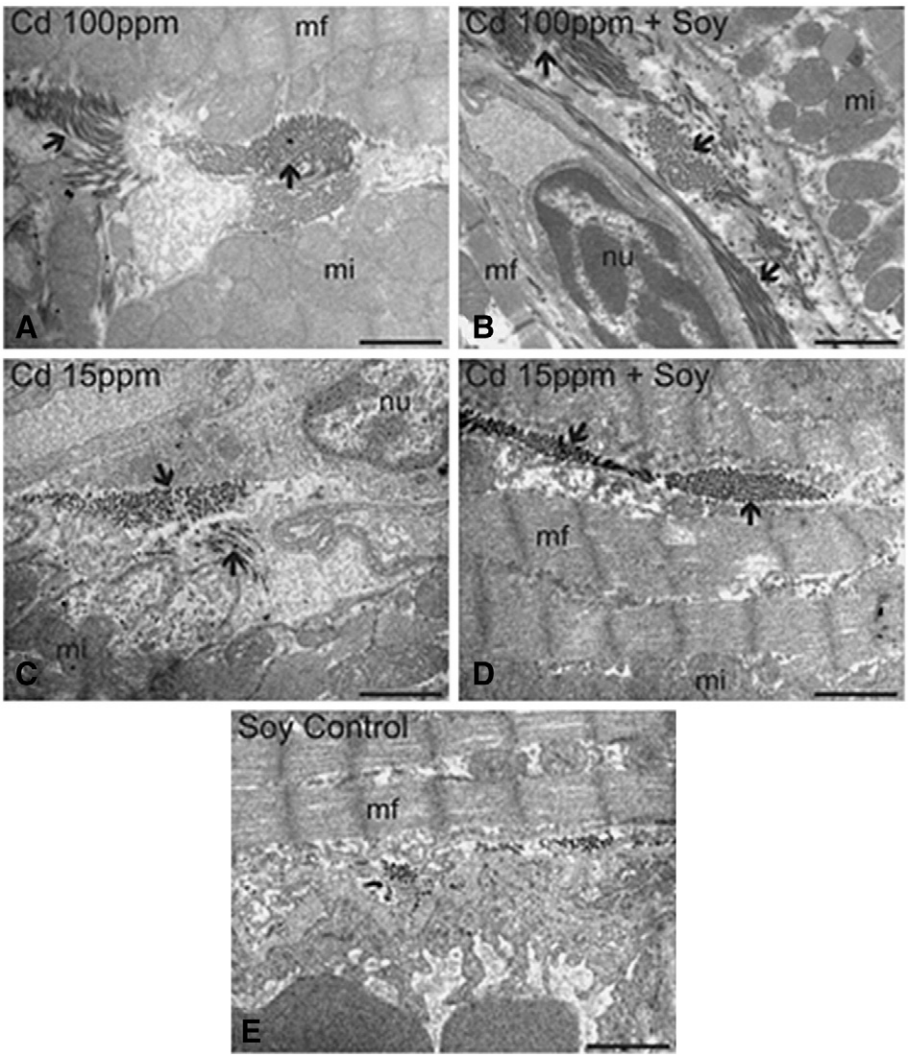

Fig. 5. TEM micrographs of heart myocardium. (A) Cd 100 ppm. (C) Cd 15 ppm. Increased intercellular spaces with abundant deposition of collagen fibrils (arrows) in Cd exposed animals (A, C). (B) Cd 100 ppm + soy. (D) Cd 15 ppm + soy. Soy treatment does not modify the alterations produced by the Cd (B, D). (E) Soy control. mf: myofibril; mi: mitochondria, nu: nucleus. Bars: A–E: 2.2 μm.

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). Consistently, in the present study we observed an induction of MTI expression in Cd 100 ppm rats, the group with the highest pro-oxidant status. On the contrary, soy protein prevented the increase of MTI expression. As previously explained, oxidative stress was significantly diminished in Cd 100 ppm + soy animals, with lower levels of protein carbonyl groups, no decrease in rGSH levels and increased CAT and SOD activities. Thus, we suggest that antioxidant properties of soy would avoid Cd induction of MTI expression.

Histological examination of hematoxylin-eosin stained myocardial tissue of Cd-exposed rats showed notable changes. The tissue sections from Cd exposed rat heart showed the development of hypertrophic and fusion of cardiomyocytes along with foci of myocardial fiber necrosis. No inflammatory cell response was noticed. These changes were more evident in Cd 100 ppm and Cd 100 ppm + soy animals. Ultra-structural evaluation of the cardiac damage showed intracellular edema, alterations in myofibril arrangements, mitochondrial damage, dilation of the endomembrane–T-tubule system and dilation of intercellular spaces with an abundant collagen fiber deposit. Similar results have been reported in the heart of mammals exposed to other heavy metals, such as lead and mercury (Kline, 1960; Stoev and Lazarova, 1998).

It has been observed that ROS cause cardiomyocyte hypertrophy, interstitial fibrosis and activation and expression of MMPs, leading to cardiac remodeling (Hill and Singal, 1996; Siwik et al., 2001). Increased cardiac collagen deposition is observed in almost every cardiac disease and plays an important role in the deteriorating function of the diseased heart (de Jong et al., 2001). In the present work, ultrastructural studies and S-R staining showed a significant increase in cardiac collagen deposition in all the animals exposed to Cd. The

Fig. 4 TEM micrographs of heart myocardium. (A–B, M–N) Cd 100 ppm. (C–D, O–P) Cd 15 ppm. Changes in tissue structure with the presence of intracellular edema (black asterisk), alterations in the arrangement of the myofibrils within the cardiac muscle fiber and changes in the normal structure of the intercalated disks (white arrow head) in Cd exposed animals (A–D). Marked mitochondrial damage with loss of mitochondrial cristae (white asterisk) and mitochondrial membrane disruption (white arrow), particularly at doses of 100 ppm (N). (E–F, Q–R) Casein control. (G–H, S–T) Cd 100 ppm + soy. (I–J, U–V) Cd 15 ppm + soy. Soy treatment does not modify the alterations produced by the Cd (G–J, S–V). (K–L, W–X) Soy control. ca: capillary, id: intercalated disk; mf: myofibril; mi: mitochondria, nu: nucleus. Bars: A, C, E, G, I, K, M, O, Q, W: 3.33 μm; B, D, F, H, J, L: 2.2 μm, N, P, R, T, V, X: 1.42 μm.

myocardial degeneration was dose-dependent, with the highest collagen deposition found in Cd 100 ppm animals. Similarly, Khong et al. (2011) found a significant increase in myocardial fibrosis, as evidenced by greater proportional area of interstitial collagen I and III immunostaining, and the presence of cellular hypertrophy with increased cardiomyocyte cross-sectional area, in diabetic rats. Along with histological changes, these diabetic rats showed a significant reduction in diastolic function. Treating those animals with the synthetic flavonol DiOHF (39,49-dihydroxyflavonol) significantly attenuated the detrimental changes observed in the myocardium. In the present study, even though a soy-based diet attenuated the Cd-induced oxidative stress, it failed in preventing cardiomyocyte damage. Chemicals with antioxidant properties have been seen to avoid Cd-induced cardiac damage. Manna et al. (2008) treated Swiss albino mice with $CdCl_2$ administered orally (4 mg/kg body weight) during 6 days. This treatment induced an extensive degeneration in cardiac muscle and interstitial fibrosis. However, no histological damage was observed in the heart of Cd-intoxicated mice pre-treated with taurine. Since taurine is a cysteine rich compound, it could exert its beneficial effects by binding Cd as well as by diminishing myocardial oxidative stress. On the contrary, even though in the present study a soy-based diet was able to decrease myocardial oxidative stress, it failed in preventing Cd-induced cardiac damage; probably, due to the inability of soy components for scavenging Cd. As we mentioned in the Introduction, cadmium ion exerts toxic effects through several different mechanisms which involve inactivation of –SH groups, substitution of Ca^{2+} and/or Zn^{2+} and interaction with cell surface receptors (Beyersmann and Hechtenberg, 1997; Yazıhan et al., 2011). These mechanisms of Cd toxicity might be the ones involved in Cd-induced cardiac damage, besides the induction of oxidative stress.

Previous works from Bansal and Parle (2010) and Byun et al. (2010) report antioxidant effects of soy-based diets, after 60 days and 10 weeks of treatment, respectively. In our knowledge, this is the first study where a soy-based diet is administered to Cd-intoxicated animals. Taking into account Bansal and Parle's (2010) and Byun et al.'s (2010) works and given that our experimental model uses 60 days of Cd-intoxication in order to achieve a chronic exposure, and that Om's group (Om and Shim, 2007; Paik et al., 2003) observed significant effects after 60 days of soy isoflavone administration (daidzein and genistein) to Cd-treated mice, we decided to administer the soy-based diet to our rats for the same period of time. Interestingly, 60 days of a soy-based diet are sufficient to diminish Cd-induced oxidative stress, however it fails in preventing cardiac tissue degeneration. These observations could be explained by the fact that Cd exerts its toxic effects by several other mechanisms, besides affecting the cellular redox balance. Thus, we suggest that it is important to evaluate histological degeneration as well as oxidative stress induction when assessing the capacity of a compound in preventing Cd-induced cardiac damage.

In conclusion, our findings indicate that, Cd intoxication produces oxidative stress, as well as extracellular and ultrastructural damages in the myocardium and that soy intake may be a good nutritional strategy to reduce cardiac oxidative stress.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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