Journal of Trace Elements in Medicine and Biology 52 (2019) 239–246

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Toxicology

A soybean-based diet modulates cadmium-induced vascular apoptosis

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ARTICLE INFO

Keywords:
Soybean-based diet
Cadmium
Aorta
Nitric oxide
Inflammation
Apoptosis

ABSTRACT

Cadmium (Cd) exposure has been associated with an increased risk of cardiovascular diseases. The diet is a modifiable source of protecting or damaging factors that may affect this risk. Herein we tested the hypothesis that a soybean-based diet (SBD) protects the vascular wall of the aorta against Cd-induced pro-inflammatory and pro-apoptotic effects. To test this hypothesis, we fed male Wistar rats for 60 days with a casein-based diet (CBD) or an SBD. These animals were also exposed to tap-water without (CBD-Co/SBD-Co) or with 15(CBD-15Cd/SBD-15Cd) or 100 (CBD-100Cd/SBD-100Cd) ppm of Cd. Inflammatory parameters (mRNAs and/or proteins) were measured in thoracic aorta tissue. These included inducible and endothelial nitric oxide synthases, cyclooxygenase-2, intracellular-adhesion molecule-1, and vascular cell-adhesion molecule-1. As pro-apoptotic parameters, we measured Bax and Bcl-2 mRNA/protein, as well as TUNEL positive cells in the aorta tissue. Compared to CBD-Co, inflammatory and apoptosis markers increased in the aorta with the concentration of Cd in the drinking water. These effects were not observed in either SBD-15Cd or SBD-100Cd, which were similar to CBD-Co. Cd content in serum and in aortas from animals fed CBD-Co/SBD-15Cd or CBD-Co/SBD-100Cd were similar suggesting that, if any, the effect of SBD is not due to changes in Cd bioaccumulation, but due to secondary effects linked to the composition of the dietary soybean flour. Our findings are consistent with a protective effect of an SBD against Cd-induced inflammation and apoptosis in the thoracic aorta in a rat model.

1. Introduction

Epidemiological studies have shown that exposure to heavy metals is associated with high prevalence and incidence of cardiovascular diseases [1]. Cadmium (Cd) is among the most toxic pollutants that are widely distributed in the environment. The most significant sources of occupational cadmium exposure include the nickel-cadmium battery industry, fume inhalation, electroplating and paint pigments [2]. Oral exposure to Cd occurs by consumption of contaminated food and/or water which are the major pathways of exposure to the metal in non-smoking and non-occupationally exposed populations [2]. Indeed, Cd exposure has been reported to be an independent risk factor for early atherosclerotic vascular-wall thickening in experimental models [3,4] and newly diagnosed patients [5,6].

Cross-sectional and prospective studies have shown that Cd exposure is associated with an increased incidence of death due to myocardial infarction, stroke and peripheral artery disease [7,8]. Experimental and epidemiological studies have shown that soluble Cd^{2+} can be accumulated and affect organ physiology [5,6]. Cd exposure can cause pro-atherogenic effects [5]. Indeed, the vascular endothelium has been suggested as a critical target of Cd toxicity, leading to several cardiovascular complications such as hypertension, atherosclerosis, and cardiomyopathy [7,9]. Disruption of the endothelium caused by Cd is thought to be due to oxidative stress, inflammation as underlying mechanisms of cell damage [10–13]. Excessive reactive-biochemical species production can induce an inflammatory response [14]. Among the enzymes involved in the inflammatory process, the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) are

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https://doi.org/10.1016/j.jtemb.2019.01.007
Received 13 August 2018; Received in revised form 21 December 2018; Accepted 11 January 2019

induced upon Cd exposure in macrophages [15–17]. At inflammation sites, these enzymes produce a large amount of nitric oxide radical (NO) and prostaglandin E, respectively. Furthermore, the cell-adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are essential components in the inflammation process, by contributing to homing of inflammatory cells at damaging sites [18]. Interestingly, Cd is known to cause inflammation by overriding the activation of the nuclear factor (NF)-κB [19–21]. NF-κB is the master regulator of the inflammatory process that controls the expression of iNOS, COX-2, proapoptotic and anti-apoptotic genes, ICAM-1 and VCAM-1, among other genes [22]. The exposition to Cd is frequently accompanied by infiltration of inflammatory cells in a number of tissues [23]. We have previously reported the effects of a subchronic intoxication with Cd in the drinking water for 15, 30 and 60 days on the rat myocardium [24]. In that study, we found that Cd concentration in the heart comes to saturation more likely due to the absence of metallothionein induction, but induction of nuclear factor erythroid 2–related factor 2 and NADPH oxide reductase-2 (NOX-2) expression in the heart tissue after the 60 days of treatment resulting in the highest oxidative damage to lipids and proteins [24].

A number of proteins regulate the apoptosis process, among them the Bcl-2 family [25]. These are a group of proteins that act to regulate the activation of pro-caspases. Some members of this family inhibit apoptosis, such as B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL), while other members promote the activation of pro-caspases; and thus cause cell death, as Bcl-2-associated death promoter (Bad) and Bcl-2-associated X protein (Bax) [26]. The pro-apoptotic effect of cadmium has been observed in a variety of cell types and tissues [27], such as the vascular endothelium [28]. However, the biochemical and molecular basis of inflammation and apoptosis underlying Cd-induced alteration in mammalian arteries have not been fully elucidated.

Moreover, the consumption of soybean is becoming increasingly important in human nutrition as a source of vegetal protein replacing animal dietary proteins, such as casein [29,30]. Among their beneficial health effects, soybean has been reported that have a number of health benefits, such as: lowering cholesterol and low-density lipopotein (LDL)-cholesterol, thus preventing heart disease, reducing weight in obesity and protecting against breast and prostate cancer [31]. Isoflavones contained in soybean act as antioxidants [29,32], inhibit lipoprotein oxidation and reduce the incidence of coronary heart disease [33,34]. We have previously found that replacing casein for soybean as a dietary protein reduces oxidative damage in the heart, but not histological changes induced intoxication with 100 ppm Cd in the drinking water for 60 days [35].

In order to investigate a possible modulating effect of a soybean-based diet (SBD) on Cd-induced pro-inflammatory/pro-apoptotic effects in the thoracic aorta, we used an experimental model of rats subchronically intoxicated with Cd and fed a casein (CBD)- or a soybean (SBD)-based diet for 60 days. Data gathered from this study will provide evidence about the possible beneficial effects of an SBD against vascular inflammatory and apoptotic damage induced by Cd-exposure.

2. Materials and methods

2.1. Diet and experimental design

Adult male Wistar rats weighing 180–200 g were used. The experimental protocol was approved by the Committee for Care and Use of Laboratory Animals of the National University of San Luis (San Luis, Argentina) in accordance with the Guide for the Care and Use of Laboratory Animals). Animals were kept with 12h:12 h light: dark cycles in a temperature and humidity controlled room. Animals were given free access to food and water for 60 days. Animals were randomly fed either a casein-(CBD) or a soybean-(SBD) based diet and were exposed to drinking water without or with 15 or 100 ppm of Cd^{2+} (as $CdCl_2$) for 60 days. This protocol resulted in the following six experimental groups: CBD-0 (or CBD-control), CBD-15Cd, CBD-100Cd, SBD-0 (or SBD-control), SBD-15Cd and SBD-100Cd. Since ingestion is the most important route of human exposure to Cd, drinking water was chosen as the exposure vehicle [36]. No Cd was detected in the tap water used as a vehicle. Moreover, a dose of Cd of 100 ppm as the highest used in our study has been reported as environmentally feasible [37]. The Cd exposure protocol was similar to that previously used in studies carried out in our laboratory [24,35,38].

CBD and SBD were prepared according to AIN-93M-CAS and AIN-3M-SOY diets for laboratory rodents, respectively [38,39]. After treatment, animals were fasted overnight and sacrificed by decapitation at 09:00 am, and immediately after, trunk blood samples were collected for serum separation.

The thoracic aorta was quickly excised, rinsed three times with ice-cold isotonic saline solution and cleaned to remove the surrounding tissue. Afterward, aorta samples were kept in liquid nitrogen until analyses or paraffin-embedded for histology analyses. Analyses were carried out within 1–2 weeks of obtaining the samples. Additionally, enzymes determinations were performed in fresh tissues.

2.2. Determination of Cd in serum and thoracic aorta tissue

Cd concentration in serum and thoracic aorta tissue was determined by electrothermal atomic absorption spectrometry using a Perkin Elmer Analyst 200 Gf equipped with a graphite tube with an L´vov platform. The detection limit was of 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 tensioactive agent and matrix modifier were added, in a range of 0.5 to 5 μg/l; MLD: 0.035 μg/l [40]. 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. Cd concentration in serum and aorta tissue is shown as μg/L serum and μg Cd/g aorta tissue, respectively.

2.3. Nitrites determination in thoracic aorta explants culture

For determining NO release from explants of the thoracic aorta, the slices of aorta were cut and incubated in phosphate buffered saline (PBS), pH 7.4 at 37 °C. After 2 h of incubation with stirring, the reaction was stopped by addition of 5% phosphoric acid. Supernatants were used for NO measuring. NO was indirectly measured by assaying nitrite, one of the most stable products of NO oxidation in aqueous solution. Nitrite was assessed using the Griess reagent and absorbance was read at 540 nm. Sodium nitrite was used as standard [41]. The results are shown as μmoles $NO_2^{\downarrow}$/mg of tissue protein.

2.4. RNA isolation and semi-quantitative RT-PCR analysis

VCAM-1, ICAM-1, Bax and Bcl-2 mRNA expression in thoracic aorta tissue were determined by RT-PCR. Total RNA was isolated from frozen thoracic aorta samples using TRIzol as indicated by the manufacturer (Invitrogen) within 1–2 weeks of obtaining the samples. 3 μg of total RNA were reverse transcribed with 200 IU of M-MLV reverse transcriptase (RT; Promega Inc.) using random primer hexamers (Biodynamics, SRL) in a 26 μl reaction mixture at 37 °C for 1 h. Aliquots of complementary DNA (cDNA, 0.5 μg) were amplified with rat-specific primers (Table 1). The reaction samples were heated to 95 °C for 5 min followed by 40 temperature cycles; each cycle included: 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s. Afterward, 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

Table 1
Primer sequences.

	Primers sequences (5'–3')[a]	Fragment size	Gene Bank accession number
β-actin	F: CGTGGGCCGCCCAGGCACCA R: TTGGCCTTAGGGTTCAGAGGG	243 bp	NM 031144
VCAM-1	F: CTACAAGTCTACACCTCCCCC R: GAACAACGGAATCCCCAACC	349 bp	NM_012889.1
ICAM-1	F: CTTTGCCCTGGTCCTCCAAT R: CGCTCTGGGAACGAATACACA	362 bp	NM_012967.1
Bax	F: GAGCTGCAGAGGATGATTGCT R: GTGTCCAGCCCATGATGGTT	199 bp	NM_017059.2
Bcl-2	F: GGATCCAGGATAACGGAGGC R: ATGCACCCAGAGTGATGCAG	141 bp	NM_016993.1

their intensities were quantified using NIH ImageJ software (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.

2.5. Western blot analysis for eNOS, iNOS, and COX-2

Thoracic aorta samples were homogenized as described previously. Protein content was measured by the method of Lowry [42] using bovine serum albumin as a standard. 40 µg of protein were 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 then loaded onto a 12% SDS-PAGE gel. Separated proteins were transferred onto a PVDF membrane (Immobilon-P Transfer Membrane, Millipore USA) using a blot transfer system (BioRad Laboratories, Hercules, CA). After being blocked with 5% milk in Tris-buffer saline (TBS) 1X (20 mM Tris, 0.9% NaCl, pH 7.4) for 4 h at room temperature, membranes were incubated overnight at 8 °C with a primary rabbit anti-eNOS, anti-iNOS and anti-COX-2 polyclonal antibody solution (Santa Cruz Biotechnology) were used at 1:500 dilution. After rinsing for three times with TTBS (0.1% Tween 20, 100 mM Tris-HCl, pH 7.5, 150 mM NaCl) membranes were incubated for 2 h at room temperature with a biotinylated goat anti-rabbit IgG secondary antibody (1:5000 dilution). Membranes were washed and incubated with avidin-horseradish peroxidase complex (Vectastain ABC-detection system, Vector Labs) for 1 h. The color was developed using a 3,3–diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs). β-actin was determined as an internal control using an anti-®-actin antibody (Santa Cruz, Biotechnology).

2.6. Immunohistochemistry analysis for Bax and Bcl-2

For the immunohistochemistry studies, the thoracic aortas from CBD-0, CBD-100, SBD-0, and SBD-100 were removed and fixed in Bouin's fluid. The samples were dehydrated in increasing ethanol series, cleared in xylene and embedded in paraffin. Sections of 5 µm of thickness were obtained using a Microm HM 325 rotation microtome. The slides were treated with anti-Bax and anti-Bcl-2 antibody (1:500) for immunohistochemical evaluation. Slides of small and large intestines were used as positive (with antibody) and negative (without antibody) controls. These sections were examined using an Olympus BX-40 light microscope. Images were acquired using the Sony SSC-DC5OA camera and processed using the Image-Pro Plus 5.0 software. The percentage of positivity for each protein in the different photographs were analyzed using the Media Cybernetic Image-Pro Plus 3.0.1 program.

2.7. Identification of apoptotic cells by terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL)

Apoptosis was measured in slides of thoracic aorta tissue obtained from control and 100 ppm Cd-exposed rats using the DeadEnd™ Colorimetric TUNEL System (Promega, USA), according to manufacturer's instructions. Apoptosis index (AI) = [number of TUNEL positive cells/number of cells scored] × 100 was calculated. One-hundred cells were scored in three different view-fields by two independent observers.

2.8. Statistical analysis

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

3. Results

3.1. Effect of CBD and SBD on the accumulation of Cd in the thoracic-aorta tissue

In order to test whether the different concentration of Cd in the drinking water affects its concentration in the serum and in the thoracic-aorta tissue, we measured its concentration in the serum and tissue obtained from rats exposed for 60 days to Cd at two different concentrations and fed a CBD or an SBD (Table 2). Compared to CBD-control and SBD-control, Cd concentration in the thoracic-aorta tissue of Cd-treated animals increased with the concentration of Cd in the drinking water. Indeed, in both diets, CBD and SBD, the thoracic-aorta tissue of rats exposed to 15 and 100 ppm Cd in the drinking water showed more than 10- and 30-fold higher concentrations, respectively. We found no difference in the concentration of Cd, in neither serum or aorta tissue, when CBD-control vs SBD-control, CBD-15Cd vs SBD-15Cd and CBD-100Cd vs SBD-100Cd were compared (Table 2). Moreover, food and drink consumption, as well as body weight gain were similar among the six experimental groups (data not shown).

3.2. Effect of dietary protein and Cd exposure in nitric oxide synthesis in the thoracic aorta

Because our previous studies have shown that Cd-exposure causes pro-oxidant and pro-inflammatory profile in diverse tissues [43] and cells [16], and because of the relationship between cardiovascular effects of Cd exposure we cultured explants of thoracic aorta tissue from our six experimental groups and measured nitrite accumulation in the culture medium. We also measured mRNA for the endothelial (eNOS) and the inducible (iNOS) nitric oxide synthases in the aorta tissue (Fig. 1). The nitrite accumulation in the culture of explants isolated from animals exposed to 100 ppm Cd (CBD-100Cd) was higher than the CBD-control and the CBD-15Cd groups. There was no difference in NO production between CBD and CBD-15Cd aorta explants (Fig. 1**A**). Interestingly, nitrite accumulation in the explants from SBD-100Cd was

Table 2
Cadmium concentration in serum and bioaccumulation in the thoracic aorta.

	Cd concentration	
Groups	Serum (µg/L)	Aorta (µg/g tissue)[a]
CBD Control	0.59 ± 0.04	0,34 ± 0,03
CBD + 15 ppm Cd	6.90 ± 0.37*	3,73 ± 0,26*
CBD + 100 ppm Cd	15.37 ± 0.17*	10,46 ± 1,22*
SBD Control	0.60 ± 0.02	0,32 ± 0,04
SBD + 15 ppm Cd	6.49 ± 0.28*	3,35 ± 0,28*
SBD + 100 ppm Cd	16.14 ± 0.16*	11,03 ± 1,74*

[a] Data values are shown as mean veles ± SEM from six animals per group. Rats were fed a casein (CBD)- or a soybean (SBD)-based diet and exposed them to either 15 or 100 ppm Cd^{2+} in the drinking water for 60 days. Cd was measured in the serum and in the thoracic aorta tissue. Asterisk indicates $p < 0.001$ versus the respective control group.

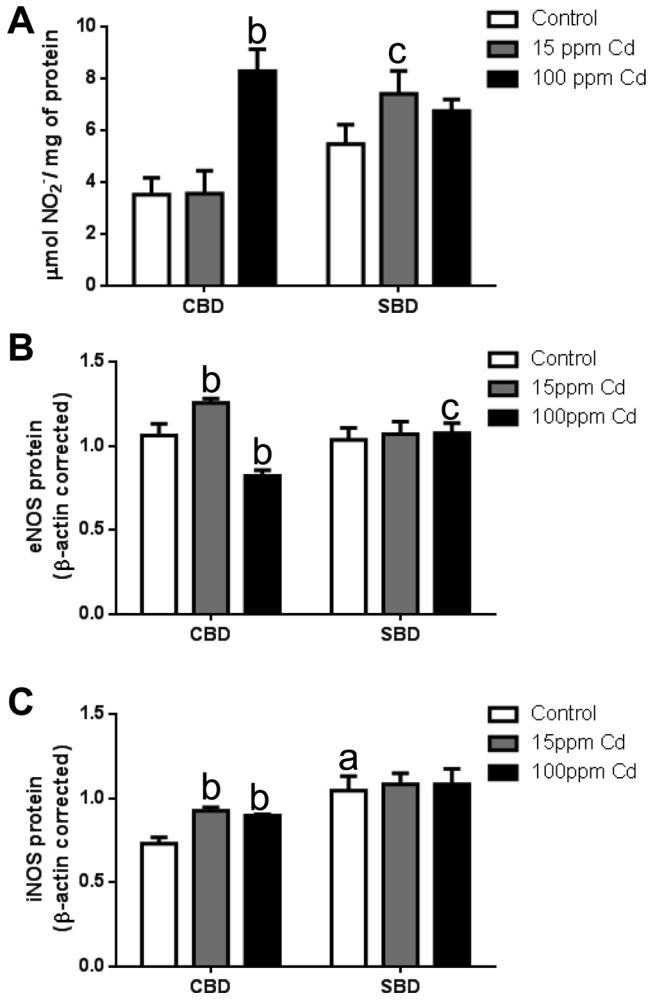

Fig. 1. Effect of a soybean-based diet and cadmium exposure on nitric oxide production in the thoracic aorta. A) Nitrite concentration in the supernatant of thoracic-aorta explants was measured using the Griess assay. **B**) eNOS and **C**) iNOS expression were measured in the aorta tissue by Western blot. Data are shown as mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). a, b and c indicate $p < 0.05$. a, Compares CBD-control vs SBD-control. b compares CBD groups with respect to CBD-control group. c, compares CBD groups respect to SBD groups.

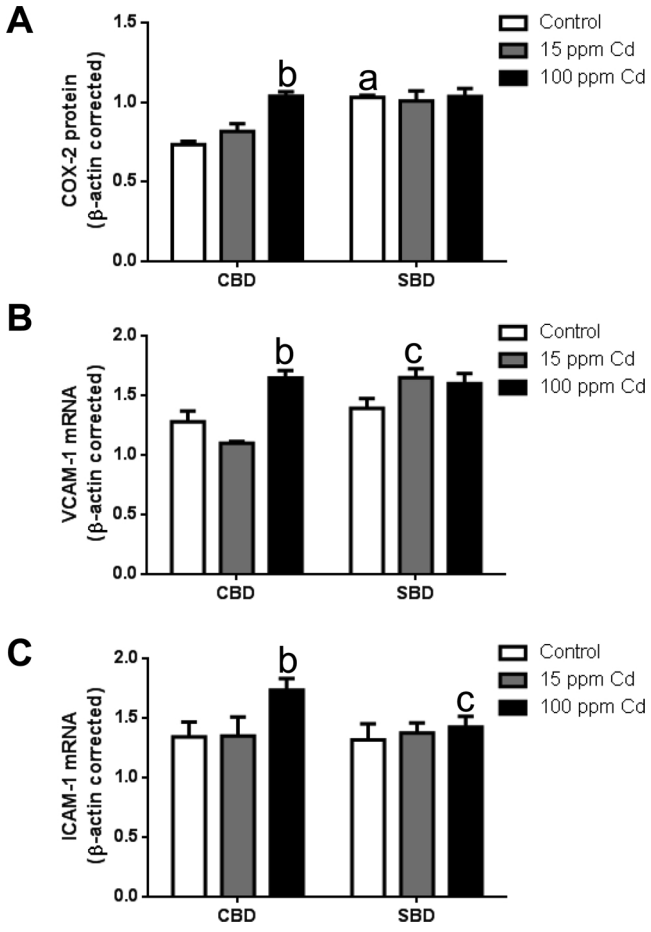

Fig. 2. Effect of an SBD and cadmium exposure on inflammation in the thoracic aorta. A) COX-2 expression was measured as mRNA using RT-PCR; **B**) as in A, but VCAM-1 expression was measured; **C**) As A, but ICAM-1 expression was measured. β-actin was used as a housekeeping gene. Data are shown as mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). a, b and c indicate $p < 0.05$. a, Compares CBD groups with the respective SBD groups. b compares CBD groups with respect to CBD-control group. c, compares CBD groups respect to SBD groups.

not different to SBD alone. Moreover, nitrite accumulation in explants cultures did not change when CBD-control and SBD-control were compared. There was a tendency to increase NO production in the aorta of SBD compared to CBD. When NO production by explants from animals fed an SBD, and with or without either 15 or 100 ppm Cd was compared, we found no differences. However, the production of form the aorta explants was higher in SBD-15Cd when compared to CBD-15Cd.

Both eNOS and iNOS are two critical enzymes in the production of NO in the vasculature [44]. Compared to the aorta of animals fed a CBD, eNOS expression increased in those animals treated with 15 ppm but decreased in animals exposed to 100 ppm (Fig. 1**B**). We found no differences in eNOS expression in the thoracic aorta when the aorta tissue from CBD versus SBD fed rats was compared. Interestingly, eNOS expression was higher in the aorta of rats fed SBD-100Cd compared to rats fed a CBD-100Cd.

The iNOS has been shown to play a significant role in inflammatory processes such as atheroma formation and progression [44]. Most of iNOS expression occurs in innate immune cells, such as macrophages [45]. We found that the aorta tissue from rats fed an SBD-control expresses more iNOS protein than the same tissue from rats fed a CBD-control (Fig. 1**C**). Although not at the same level than SBD fed animals, the aorta of CBD animals exposed to 15 or 100 ppm Cd showed a higher iNOS expression than CBD control. Cd exposure did not change iNOS expression in the aorta of SBD-control respect to SBD exposed to Cd.

3.3. VCAM-1, ICAM-1, Bax and Bcl-2 mRNA concentration

In order to test whether SBD can modulate the inflammatory profile caused by Cd exposure, we measured the expression of COX-2, VCAM-1, and ICAM-1. Likewise iNOS, these inflammation mediators are also under the control of the master regulator of inflammation, NF-κB [46]. COX-2 expression was higher in the aorta tissue of animals fed an SBD compared to CBD fed rats (Fig. 2**A**). Comparing animals fed a CBD we found that COX-2 expression was higher in the aorta of animals exposed to 100 ppm. However, we did not find any difference in animals exposed to 15 ppm when compared to CDB-control (Fig. 2**A**). Expression of COX-2 in the aorta of CBD-100Cd was similar to SBD fed animals. Expression of COX-2 in the aorta of animals fed an SBD did not change whether animals were exposed to 15 or 100 ppm Cd. These shreds of evidence suggest that an SBD ameliorates inflammation caused by Cd-exposure in the aorta tissue.

The nature of dietary protein did not change VCAM-1 expression in aorta tissue of rats (Fig. 2**B**). However, and similar to COX-2 expression,

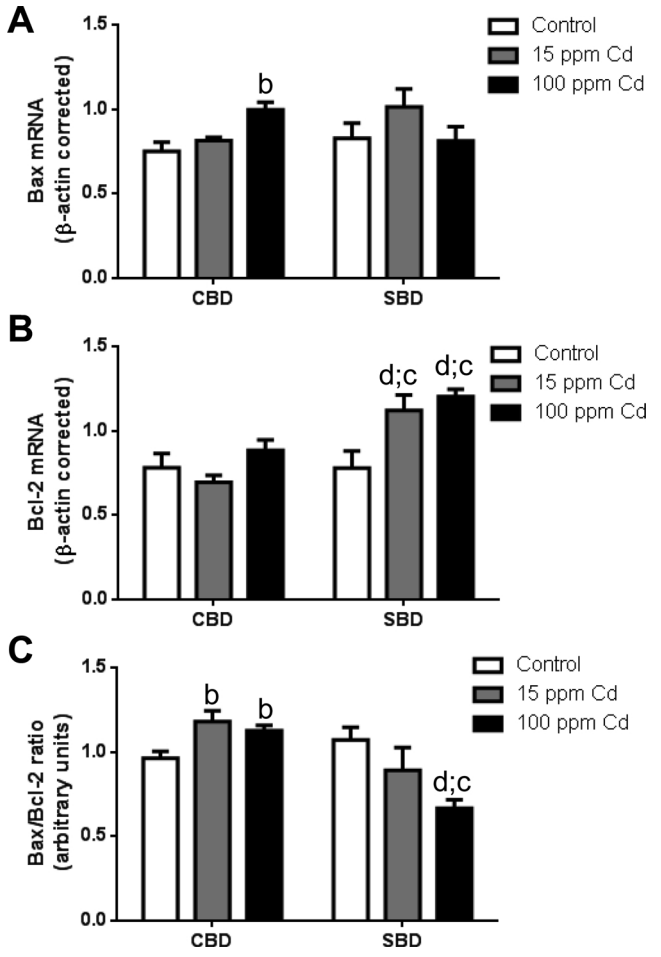

Fig. 3. Effect of an SBD on apoptosis parameters in the thoracic aorta. A) Bax and **B**) Bcl-2 expression in the aorta tissue were measured as mRNA by using RT-PCR. β-actin was used as a housekeeping gene. **C**) Bax/Bcl2 ratio. Data are shown as mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). b, c and d indicate $p < 0.05$. b compares CBD groups with respect to CBD-control group. c, compares CBD groups respect to SBD groups. d Compares SBD groups with respect to SBD-control.

100 ppm increased VCAM-1 expression in aorta tissue of rats fed a CBD, whereas 15 ppm Cd did not. VCAM-1 expression was higher in the aorta of rats from the SBD-15Cd than from the CBD-15Cd group.

ICAM-1, another marker of inflammation, did not change by the nature of the dietary protein (Fig. 2**C**). ICAM1 expression was higher in the aorta of animals fed a CBD-100Cd compared to CBD-control. In addition, ICAM-1 expression was higher in the aorta tissue from the CBD-100Cd compared to SBD-100Cd fed rats.

3.4. Effects of dietary protein on Cd-induced apoptotic changes in the thoracic aorta of rats

In order to test whether the nature of dietary protein can modulate apoptotic changes induced by Cd-exposure in the drinking water, we measured the expression of Bax and Bcl-2 genes, and also determined the apoptotic index using the TUNEL assay. The expression of Bax and Bcl-2 in animals fed either a CBD or an SBD was not different (Fig. 3**A**). When CBD fed animals were exposed to 100 ppm Cd, Bax expression was higher than CBD-control and CBD-15Cd. Bax expression in SBD fed animals was similar, whether animals were exposed to water or water containing 15 or 100 pm Cd. The expression of the anti-apoptotic gene Bcl-2 did not change in animals fed a CBD whether they were exposed or not to Cd (Fig. 3**B**). However, Bcl-2 expression in the aorta of animals fed an SBD increased when these animals were exposed to 15 and 100 ppm Cd. There was no difference in Bcl-2 expression in animals fed an SBD whether the drinking water contained 15 or 100 ppm Cd. In the aorta of animals fed an SBD exposed to 15 or 100 ppm Cd the expression of Bcl-2 was higher than in animals fed a CBD. Bax/Bcl-2 ratio was similar in CBD and SBD fed animals (Fig. 3**C**). In animals fed a CBD, the Bax/Bcl-2 ratio increased with the concentration of Cd in the drinking water. In animals fed an SBD, the exposure to 100 ppm Cd decreased this ratio. Compared to CBD-100Cd the Bax-Bcl-2 ratio was lower in SBD-100Cd. These data suggest that an SBD prevents pro-apoptotic effects of Cd in the thoracic aorta of rats.

3.5. Immuno-histochemical determination of Bax, Bcl-2 and apoptotic index in the aortic tissue

In order to corroborate RT-PCR data of Bax and Bcl-2 expression, we performed immunohistochemistry and measured Bax and Bcl-2 positive cells in aorta tissue (Fig. 4). To simplify our study we only analyzed the effect of dietary protein on animals exposed to 100 ppm Cd. As observed in Fig. 4**A** Bax positive cells in the aorta of animals fed a CBD increased when exposed to 100 ppm Cd. These effects of 100 ppm Cd were not observed in SBD fed animals exposed to the same dose of Cd (Fig. 4**A** and **B**). Bax expression induced by 100 ppm Cd was prevented in the aorta of animals fed an SBD.

We also analyzed Bcl-2 positive cells and found that Cd exposure did not change Bcl-2 positive cells in aorta tissue of animals fed a CBD (Fig. 5**A** and **B**). However, Cd exposure increased Bcl-2 positive cells in the aorta tissue of SBD fed animals. These data are consistent with our mRNA data shown in Fig. 3. An SBD increased Bcl-2 positive cells in the thoracic aorta of animals exposed to 100 ppm Cd.

To corroborate the data on expression of apoptosis markers we measured the number of TUNEL positive cells in the aorta of animals fed with either a CBD or an SBD with or without 100 ppm of Cd in the drinking water (Fig. 6). There was no difference in the number of apoptotic cells (TUNEL positive) between animals fed a CBD or an SBD. Representative image (Fig. 6**A**) and image analysis (Fig. 6**B**) show that compared to CBD, Cd exposure increased almost twice the number of TUNEL positive cells in aorta tissue. Feeding of animals with an SBD reduced almost to control the number of apoptotic cells induced by exposure to 100 ppm in the drinking water. These data are consisting with a protective effect of an SBD on Cd-induced apoptosis in the thoracic aorta of rats.

4. Discussion

Herein we tested whether replacement of casein for soy as dietary protein sources can modulate the pro-inflammatory and pro-apoptotic effects of a subchronic intoxication with Cd in the aorta tissue of rats. In agreement with the data shown herein, we have previously shown that an SBD did not affect Cd concentration in serum and tissues of rats intoxicated with either 15 or 100 ppm Cd [35,38]. These pieces of evidence suggest that changing casein for soybean as a dietary protein source does not affect Cd absorption and bioaccumulation in the aorta tissue.

Pro-inflammatory properties of cadmium have been reported to be a consequence of a high infiltration of the inflammatory cell into different tissues [12]. Induction of adhesion molecules such as ICAM-1 and VCAM-1 are key players in the homing inflammatory cells into irritated tissues [47]. We found that compared to the CBD-100Cd group, the expression of ICAM-1 mRNA was lower in the SBD-100Cd group. This may result in a reduced leukocyte accumulation into the vasculature wall. It has been shown that cadmium enhances the expression of ICAM-1 in other vasculatures, such as cerebral endothelial cells [48]. Consequently, any effect of an SBD on Cd-induced pro-inflammatory or pro-apoptotic effects in the aorta may not be due to changes in the toxicokinetics of Cd, but more likely to a component in the diet.

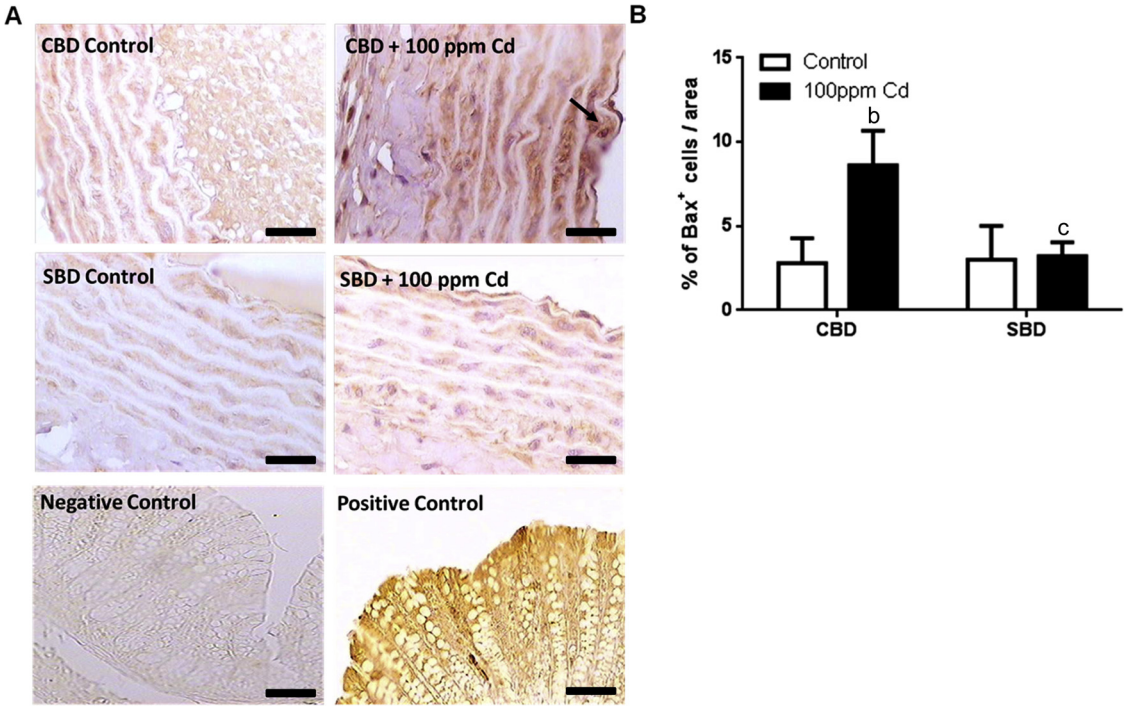

Fig. 4. Effect of an SBD and cadmium exposure on Bax expression in the thoracic aorta. A) Immunohistochemistry anti-Bax in sections of the thoracic aorta. Positive and negative controls are shown. Arrows indicate positive nuclei. **B**) Quantitative analysis of Bax positive cells. Data are shown as a representative image and mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). b and c indicate $p < 0.05$. b compares CBD-100Cd group with respect to CBD-control group. c compares CBD-100Cd group respect to SBD-100Cd group. Scale bar is 25 μm, whereas for negative and positive controls they are 100 μm.

Our data show that Cd reduces eNOS protein expression, but enhances iNOS and COX-2 expression. At sites of inflammation, eNOS activity is induced at the endothelium, whereas iNOS and COX-2 are induced in the inflammatory cells throughout NF-κB activation [44,49]. NF-κB is the master regulator of the expression of inflammatory mediators including chemokines, cytokines, enzymes and adhesion molecules [50], thus any modulation of its activation may prove to be effective in blocking inflammation. In this regard, soy phytoestrogens

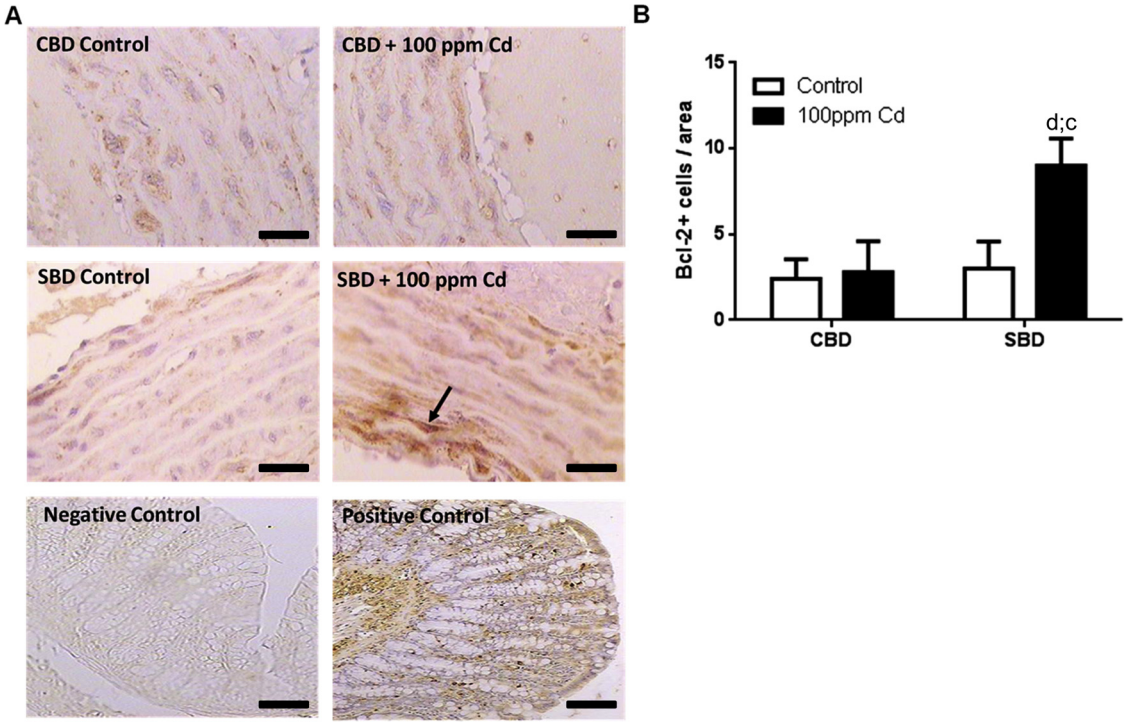

Fig. 5. Effect of an SBD and cadmium exposure on Bcl-2 expression in the thoracic aorta. A) Immunohistochemistry anti-Bcl-2 in sections of the thoracic aorta. Positive and negative controls are shown. Arrow indicates a positive nucleus. **B**) Quantitative analysis of Bcl-2 positive cells. Data are shown as a representative image and mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). c and d indicate $p < 0.05$. c compares CBD-100Cd group respect to SBD-100Cd group; d compares SBD-100Cd group with respect to SBD-control group. Scale bar is 25 μm, whereas for negative and positive controls they are 100 μm.

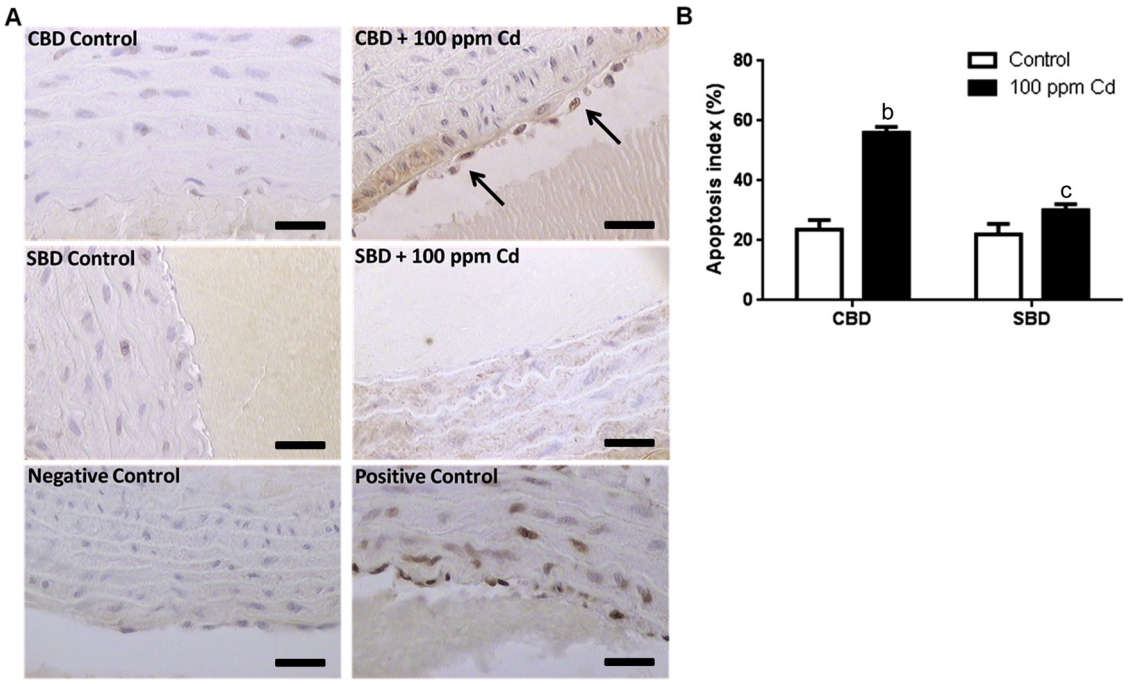

Fig. 6. Effect of an SBD and cadmium exposure on the apoptotic index in the thoracic aorta. A) TUNEL assay in sections of the thoracic aorta and negative and positive controls. Arrows indicate positive nuclei. **B**) Quantification of TUNEL positive cells in aorta tissue. Data are shown as a representative image and mean values ± S.E.M. from at least 4 independent experiments (n = 6 per group). b and c indicate $p < 0.05$. b compares CBD-control vs CBD-100Cd group; c, compares CBD-100Cd vs SBD-100Cd group. Scale bar is 25 μm, whereas for negative and positive controls they are 100 μm.

negatively modulate the expression of iNOS and COX-2 in macrophages, due to their effect on the signaling pathways involved in NF-κB activation [51]. Increased superoxide radical anion and NO production by NOX-2 and iNOS, respectively, results in the production of peroxynitrite—a powerful nitrating agent [52]. Increase iNOS and NOX-2 expression may be the mechanism by which Cd reduces the vasodilator efficiency of NO at the vasculature. In addition, protein nitration has been linked to structural and functional changes in macromolecules including the cell death process, thus affecting the tissue physiology [52]. This data and our recent publication about the effect of an SBD on Cd-induced oxidative damage in the myocardium [35] and aorta [38] highlight the protective effect of SBD components against oxidative stress. These results suggest that the soybean in the diet protects against pro-inflammatory effects of Cd.

Cadmium has been recognized as an inducer of apoptosis in a variety of tissues including the liver [53] and the vascular endothelium [54]. In animals fed a CBD, the treatment with 100 ppm of cadmium increased the expression of the pro-apoptotic protein Bax and thus increased the Bax/Bcl-2 ratio. The replacement of casein for soy as dietary protein reduced this Bax increased but increased Bcl-2 expression. This change in the diet also blocked the increase in TUNEL + cells in the aorta of rats intoxicated with 100 ppm Cd. Since the vasculature is involved in a number of physiological processes, vascular-cell apoptosis and consequent vascular dysfunction may be an initial step in a variety of pathological conditions [55,56]. It has been reported that NO produced by iNOS has a dose-dependent effect on apoptosis of vascular cells [57]. These data suggest that an SBD blocks Cd-induced apoptosis by increasing Bcl-2 expression. In this regard, it has been reported that soy isoflavones (e.g., genistein) increase endothelial cell proliferation and inhibit apoptosis induced under conditions of oxidative stress [58,59].

Altogether herein we show for the first time that dietary soy reduces inflammatory alterations and apoptosis in the aorta of rats subchronically intoxicated with Cd. Thus, an SBD can provide some potential benefit in the prevention of vascular injury induced by cadmium exposure.

Conflict of interest disclosure

Authors have no conflict of interest to disclosure

Authorship

MFFPD performed the experiments analyzed data and wrote the manuscript. MGPP performed determinations and analyzed data; VPF performed histology analyzes; FHM, performed histology studies and analyses; EJM; performed Cd determinations and data analyses; MSG, planned-supervised the overall project and wrote the manuscript; DCR, planned-supervised the experiments and wrote the manuscript.

Acknowledgments

This work was supported by research awards from the National Agency of Scientific and Technological Promotion, FONCYT, Argentina (PICT-2010-0986 to MSG and PICT-2014-3369 to DCR); National Bureau of Scientific and Technical Investigations, CONICET, Argentina (PIP-2257); and National University of San Luis, Argentina (Project 8104 to MSG and PROICO 02-3418 to DCR).

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