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Effects of a Subchronic Intoxication with Cadmium in the Hippocampus of Rats Fed a Soybean-Based Diet

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Abstract

Experimental and population studies have shown that sub chronic exposure to cadmium (Cd) causes severe toxic effects in several organs including in the brain, but information on the effects in the hippocampus (Hp) is rare. Soybean is an important source of protein and isoflavones. Herein we hypothesized that a sub chronic cadmium-intoxication throughout the drinking water can cause oxidative/inflammation changes and apoptosis in the Hp; and that a soybean-based diet (SBD) can modulate these effects. To accomplish this goal, we fed 4 groups of female Wistar-rats for 60 days as follows: casein-based diet + tap water (CBD); CBD + tap water with 15 ppm Cd (CBD + Cd); soybean-based diet + tap water (SBD); and SBD + tap water with 15 ppm Cd (SBD + Cd). After the treatment, molecular markers of oxidative stress, inflammation, and apoptosis were measured in serum and Hp tissue. Compared to the Hp of CBD fed rats, the Hp of CBD-Cd showed an apoptotic profile (high p53 and high Bax / Bcl-2 ratio). However, an SBD caused a pro-apoptosis pattern, *i.e,* increased p53 and Bax expression, reduced Bcl-2 expression and increased Bax / Bcl-2 ratio. Regardless increased antioxidant enzymes, Cd exposure in SBD fed rats had a synergistic effect in lipid peroxidation and protein oxidation in the Hp. Taking together our data suggest that an SBD and Cd-exposure have a synergistic effect on oxidative stress/inflammation and apoptosis in Hp. Caution may be taken on using soybean as a source of protein in patients exposed to Cd.

Keywords

Soy-based diet;
Cadmium sub chronic intoxication;
Hippocampus;
Oxidative stress;
Inflammation;
Apoptosis

ABBREVIATIONS

ABTS: 2,2'-Azino-Bis-3-Ethylbenzthiazoline-6-Sulphonic Acid; CBD: Casein-Based Diet; Cd: Cadmium; CAT, Catalase; CNS: Central Nervous System; eNOS: Endothelial Nitric Oxide Synthase; ELISA: Enzyme-Linked Immune-Sorbent Assay; GSH: Reduced Glutathione; GPX: Glutathione Peroxidase; Hp: Hp; iNOS: Inducible Nitric oxide Synthase; LPO: Lipid peroxidation; MPO: Myeloperoxidase; NF-κB nuclear factor-κB; NO: Nitric oxide; PCR: Polymerase-chain-reaction; PON-1: Paraoxonase-1; ROS: Reactive Oxygen Species; SBD: Soybean-Based Diet; SOD: Superoxide Dismutase; TAC: Total Antioxidant Capacity, TBARS: Thiobarbituric Acid Reactive Substances; TNF-α: Tumor Necrosis Factor-α.

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INTRODUCTION

Cadmium (Cd, CASRN 7440-43-9) is among the most toxic pollutant being widely distributed in the environment. Each year the Environment Protection Agency lists a number of inorganic substances of high environmental impact [1]. This list is led by metals such as lead, arsenic, and cadmium. Dangerous exposure to Cd is usually the result of environmental contamination from human activities, such as mining, smelting, fossil fuel combustion and industrial usage [2]. Gastrointestinal ingestion of Cd, through the food and drinking water, is a major route of intake in non-smoking and non-occupationally exposed populations [3].

It has been demonstrated that depending on the exposure time and the dose, in humans and animals, that soluble Cd^{2+} salts accumulate and cause oxidative injury of several tissues, including kidney [3], liver [4], lung [5], adenohypophysis [6], prostate [7], aorta [8] and heart [9]. Drinking water containing 15 ppm of Cd^{2+} for 2 months has previously been used in our laboratory to intoxicate rats and attain serum Cd^{2+} concentrations up to the World Health Organization (WHO) toxic limit. It has been shown that exposure to higher doses of Cd (i.e, 50 ppm or 200 ppm in drinking water for 3 months) modified the vascular reactivity of an isolated and perfused rat mesenteric bed [10] suggesting an effect of cadmium in the synthesis or availability of nitric oxide (•NO) in vascular physiology.

Numerous experimental studies in animals exposed to Cd have demonstrated behavioral disorders, morphological and biochemical changes in the central nervous system (CNS) [11-13]. Despite these studies, the mechanisms of Cd^{2+} neurotoxicity are still not completely understood. One of the effects induced by Cd is an enhancement in lipid peroxidation (LPO), which is dependent on the generation of reactive oxygen species (ROS) [12,14]. Cd penetrates the blood-brain-barrier and accumulates into the brain. Indeed, the brain is among the most susceptible organs to cadmium-induced LPO [14].

Cd-induced oxidative stress is not well understood, reactive biochemical species overproduction may result from indirect interactions of Cd at critical cellular sites or as a consequence of protective mechanisms inhibition [15]. The injury to the central nervous system (CNS) produced by Cd appears to be linked to a large loss of oxidative phosphorylation, together with a variety of conditions that produce CNS damage after inhibition of oxidative phosphorylation, all of which selectively damage the brain white matter [11,16,17].

Transcriptional and traditional toxicological examinations confirmed the systemic immune response and CNS inflammation in rats exposed to Cd Tellurite quantum dots QDs [18]. One of the most likely effects of cadmium on Hp is apoptosis. To investigate the underlying mechanism of neurotoxicity of cadmium, Madavi et al [19] examined the effects of intraperitoneal injection of cadmium during 7 days on Bcl-2 (B-cell lymphoma 2) and Bax (Bcl2-associated x) gene expression and caspase-3/7 activation (a marker of mitochondrial pathway of apoptosis) in rat Hp and frontal cortex. This study showed that Cd reduced the mRNA level of Bcl-2 in the control group at intraperitoneal doses of 1, 2 and 4 mg/kg in rat Hp and cortex cells. The mRNA level of Bax and caspase-3/7 activity increased in a dose-dependent fashion in the rat Hp.

Soybean proteins are becoming increasingly important in the human nutrition. Among the beneficial health effects, they have been described to lower cholesterol and LDL cholesterol [20], prevent heart disease [21], reduce weight in obesity [22,23], and protect against breast and prostate cancer [24]. Evidence suggests that a diet rich in soybean proteins and its isoflavones has a scavenging activity and antioxidant properties, and can inhibit lipoprotein oxidation and reduce the incidence of coronary heart disease [24-26].

Herein we tested the hypothesis that a soybean-based diet can ameliorate the deleterious effects of 60 days of intoxication with cadmium in the redox/inflammatory profile and apoptosis in the Hp of rats. To test this hypothesis, we used a rat model of subchronic intoxication with cadmium and measured the systemic effects, as well as the effects in the redox/inflammation profile and apoptosis in Hp.

MATERIALS AND METHODS

Animals and experimental design

Female Wistar-rats (8-10 weeks-old) weighing between 200 and 220 g were used in this study. Animals were housed under conventional conditions at 22-25ºC, with a 12 h/12 h light/dark cycle. They had unlimited access to drinking water and food. Water and food intake were registered daily. Diet composition is shown in (Table 1). Rats were randomly divided into 4 experimental groups, with n=6 per group, as follows: Casein-based diet (CBD) group: rats received tap water and were fed with CBD; CBD-Cd group: animals received tap water containing 15 ppm of Cd^{2+}, as $CdCl_2$, and were fed with a CBD. Soybean-based diet (SBD) group: animals received tap water and were fed with a diet supplemented with soybean flour. SBD-Cd group: animals received tap water containing 15 ppm of Cd^{2+}, as $CdCl_2$ and were fed with the SB. 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 a toxic limit in humans by the World Health Organization [27]. Purified CBD and SBD were prepared according to the American Institute of Nutrition protocol AIN-93M-CAS (CBD, and AIN-3M-SOY (SBD) for laboratory rodents, respectively. Both diets were isocaloric and is nitrogenated. The final composition value of both diets is shown in (Table 1). No Cd was detected in the drinking water or diets as assessed by electrochemical atomic absorption (see Cd determination section).

After treatment, rats were fasted overnight and euthanized by decapitation between 9 am. and 10 am. Serum samples were obtained from trunk blood and Hp was isolated and immediately frozen in liquid nitrogen. Serum and Hp samples were kept at −20ºC and at −80ºC, respectively, until being processed. All experiments were performed according to a protocol approved by the Institutional Committee for Use of Animals in Research of the National University of San Luis (Protocol# B97/15) and following the guidelines of the Guide for Care and Use of Laboratory Animals in Research (USNIH).

Weight gain and calorie consumption

Food (g/day/rat) and water (mL/day/rat) consumption were measured every day during the 60 days that lasted the feeding. Body weight gain (g) was recorded for each animal from each group once a week during the entire feeding period (60 days).

Determination of cadmium by electrochemical atomic absorption

The concentration of Cd in food, water, serum and

Table 1. Diet composition.[1]grams/Kg of diet. [2]Vitamin mix (g/Kg mix): trans-retinyl palmitate (500.000 UI/g) 0,8 g, colecalciferol (400.000 UI/g) 0,25 g, tocoferol acetate (500UI/g) 15 g, nicotinic acid 3 g, Ca pantotenate 1,6 g, piridoxin.HCl 0,7 g, tiamine.HCl 0,6 g, riboflavin 0,6 g, folic acid 0,2 g, fitoquinone 0,075 g, cianocobalamin (0,1 % en manitol) 2,5g, D-biotin 0,02 g. [3]Mineral mix (g/Kg mix): NaCl 74 g, KH_2PO_4 250 g, MgO 24 g, $CaCO_3$ 357 g, $K_3(C_6H_5O_7)H_2O$ 28 g, K_2SO_4 46,60 g, $Fe(C_6H_5O_7)$ 6,06 g, $MnCO_3$ 0,63 g, $CuCO_3$. 0,30 g, KIO_3 0,01 g, Na_2SeO_4 0,.01025 g, $(NH_4).6Mo_7O_{24}.4H_2O$ 0,000795 g, $KCr(SO_4)_2.12H_2O$ 0,275 g, NaF 0,0635 g, $NiCO_3$ 0,0318 g, NH_4VO_3 0,0066 g, $Na_2O_3Si.9H_2O$ 1,45 g, LiCl 0,0174 g, H_3BO_3 0,0815 g. [4]Protein source: Milk casein (80% p/p, Milkaut, Santa Fe, Argentina) was added to CBD, whereas Soybean flour (Ricedal Alimentos, S.A., Lote 2-Partida 1380812) was added to SBD. [5]ra, required amount.

Diet Component (g/Kg of Diet)	CBD	SBD
Ascorbic Acid	0,10[1]	0,10
Choline	2,50	2,50
L-Cistine	1,80	1,80
Vitamin Mix[2]	10,00	10,00
Minerals Mix[3]	35,00	35,00
Fibers	50,00	50,00
Sucrose	100,00	100,00
Protein source[4]	119,00	119,00
Starch	465,70	465,70
Dextrine	ra[5]	ra
Soybean oil	40,00	40,00

Hp tissue was measured by electrochemical atomic absorption using a Perkin Elmer Analyst 200 GF spectrometer equipped with graphite tube and a Lvov Platform, with LD 0.001 g/L and LQ 0.01 g/L (detection and quantification limits of 1 ppt and 10 ppt), respectively. Calibration plots were prepared with an aqueous cadmium-standard solution with a tensoactive agent and matrix modifier, in a range of 0.5-5 g/L; MLD: 0.035 g/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 the 200.9 method revision 1.2 4/91 protocol. Cd recovery was about 98-99%. Sample's detection and quantification limits were 0.01g/L and 0.1g/L, respectively.

Redox profile in serum and Hp

Hippocampus (approximate weight 30 µg) were homogenized in 30 mM phosphate buffer containing 120mM KCl and 1× protease inhibitors (Pepstatin A and PMSF), pH 7.4, followed by centrifugation at 800 × g, for 15min at 4ºC. Serum and Hp homogenate's supernatants was used for subsequent determinations. In all cases, total protein content was measured by the Lowry reaction [28] using bovine serum albumin to prepare the calibration curve. Lipid peroxidation in serum and homogenates were assessed spectrophotometrically by measuring thiobarbituric acid reactive substances (TBARS) at 535 nm following a previously published procedure [29]. Protein carbonyl concentration was measured by enzyme-linked immunosorbent assay (ELISA) [30].

Total antioxidant capacity (TAC) in serum and Hp supernatant was measured by an improved method that measures the quenching of the 2,2′-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) by both lipophilic and hydrophilic antioxidants present in serum or Hp homogenate [31]. Paraoxonase-1 (PON-1) activity in serum was determined using paraoxon and phenylacetate as substrates [32].

Catalase (CAT) specific activity was measured using a spectrophotometric assay as described by Aebi [33]. Total superoxide dismutase (SOD) specific activity was expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%, which was equal to 1 IU per milligram of the protein [34]). Total glutathione peroxidase (GPX) specific activity was assessed in Hp homogenate by following the NADPH oxidation rate, according to Flohe and Gunzler [35].

Determination of molecular markers of inflammation, DNA damage, and apoptosis in Hp

Tumor necrosis factor alpha (TNF-α) was measured in the supernatant of Hp homogenate by using an ELISA kit (BioVision, Catalog # DY410). The procedure was carried out according to the manufacturer's guidelines.

Other molecular markers of inflammation, DNA damage and apoptosis were determined in Hp tissue using semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). These molecular markers included: inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), transcription factors p53 and Nrf-2, Fas and Fas-L, Bax and Bcl-2. Briefly, total RNA was isolated from Hp samples using Trizol reagent (Invitrogen) and following the manufacturer indications. A total of 3µg of total RNA was reversed transcribed at 37ºC during 1 h, using random primer hexamers (Biodynamics, SRL) and M- MLV reverse transcriptase (Promega). PCR amplification was performed using specific oligonucleotide primers (Table 2). An aliquot of the produced cDNA 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. Bands intensities were quantified using NIH ImageJ software (Image Processing and Analysis in Java (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 28 S rRNA. As an indicator of apoptosis Bax/Bcl-2 ratio is determined.

Nitrotyrosine content-a marker of nitration was measured in Hp homogenate by ELISA. BSA-nitrotyrosine was used to produce the calibration curve [36].

Statistics-Data are shown as mean values ± SEM. Statistical analysis was performed using one-way ANOVA followed by the Tukey test. A $p<0.05$ was considered statistically significant.

Table 2. Primer sequences for RT-PCR*. [1]Abbreviations: bp, base pars; F, Forward primer; R, Reverse primer.

Gen name	Fragment size (bp)[1]	Sequence (5'-3')	Genbank accession number
eNOS	164	F: GAGATATCTTCAGTCCCAAGC R: GTGGATTGCTGCTCTGTAGG	NM 021838.2
iNOS	219	F:GCATGGACCAGTATAAGGCAAGCA R:GCTTCTGGTCGATGTCATGAGCAA	S71597
Nrf2	160	F: GGCATTTCACTGAACACAAGT R: TGGCTGTGCTTTAGGTCCATT	NM 031789.2
COX-2	282	F: CTGTATCCCGCCCTGCTGGTG R:ACTTGCGTTGATGGTGGCTGTCTT	U03389.1
FasL	178	F: TGCTGGTGGCTCTGGTTGGAA R: GTGGGCCACACTCCTTGGCTT	NM 012908.1.2
p53	107	F:TCGAGATGTTCCGAGAGCTGAATG R: CTTCTTGGTCTTCGGGTAGCTG	NM_030989.3
Bax	199	F: GAGCTGCAGAGGATGATTGCT R: GTGTCCAGCCCATGATGGTT	NM 017059.2
Bcl-2	349	F: CACCCCTGGCATCTTCTCCT R: GTTGACGCTCCCCACACACA	L14680
S28	290	F:GTGAAAGCGGGGCCTCACGATCC R:GTACTGAGCAGGATTACCATGGC	NR046239.1

RESULTS

Subchronic neurotoxicity of Cd in Hp and potential modulating effects of a diet supplemented with soybean flour was studied in a rat model. Cadmium intoxication in rats fed a CBD increased food consumption. Compared to SBD, a CBD increased food consumption similar to CBD-Cd. Food consumption did not change in SBD-Cd when compared to CBD (Figure 1A).

We did not observe any change in water consumption in any of the experimental groups (Figure 1B). In animals fed a CBD, Cd increased body weight gain starting from 30 days of feeding/ intoxication (Figure 1C). We observed no changes in body weight gain during the experimental period between animals fed either a CBD or an SBD. Weight gain was not different when CBD and CDB-Cd were compared, however, we found that starting the 10th day of diet weight gain was reduced in SBD-Cd when compared to SBD group (Figure 1C). Changes in systolic- and diastolic-blood pressure were determined every 10 days, starting at day 35 of treatment (Figure 1D-1E). In the group of animals fed with a CBD, cadmium-induced an increase in systolic blood pressure at day 45^{fh} of diet and higher diastolic blood pressure the day 35^{fh} of diet (Figure 1D).

Cd concentration in serum (ppb) were the same as was previously reported by our research team (i.e, CBD, 0.402 ± 0.077; CBD-Cd, 5.94 ± 0.87; SBD, 0.634 ± 0.086; SBD-Cd, 7.44 ± 0.94) [37]. However, using the same technology and procedures we did not detect Cd in hippocampus tissue in any of the experimental groups. This data may suggest that most of the effects we observe in the Hp are due to indirect effects afforded by Cd.

Because redox changes and oxidative stress markers have been reported in cadmium toxicity [37-39], we assessed redox parameters at systemic levels by measuring total antioxidant capacity (TAC), paraoxonase-1 (PON-1) activity, lipid peroxidation and protein oxidation in the serum of animals from the different groups after the 60 days of intoxication. Cd intoxication reduced serum TAC in animals fed a CBD (CBD vs CBD-Cd, Figure 2A). Feeding rats an SBD increased TAC compared to a CBD. This increased TAC in animals fed an SBD did not change by Cd intoxication (SBD vs SB-Cd), this resulted in almost twice serum TAC in SBD-Cd when compared to CBD-Cd (Figure 2A). Interestingly, as shown in Figure 2B, the PON- 1 activity was lower in animals fed an SBD than in SBD (CBD vs SBD), and this was not further affected by Cd intoxication (SBD vs SBD-Cd). Cd intoxication did not affect PON-

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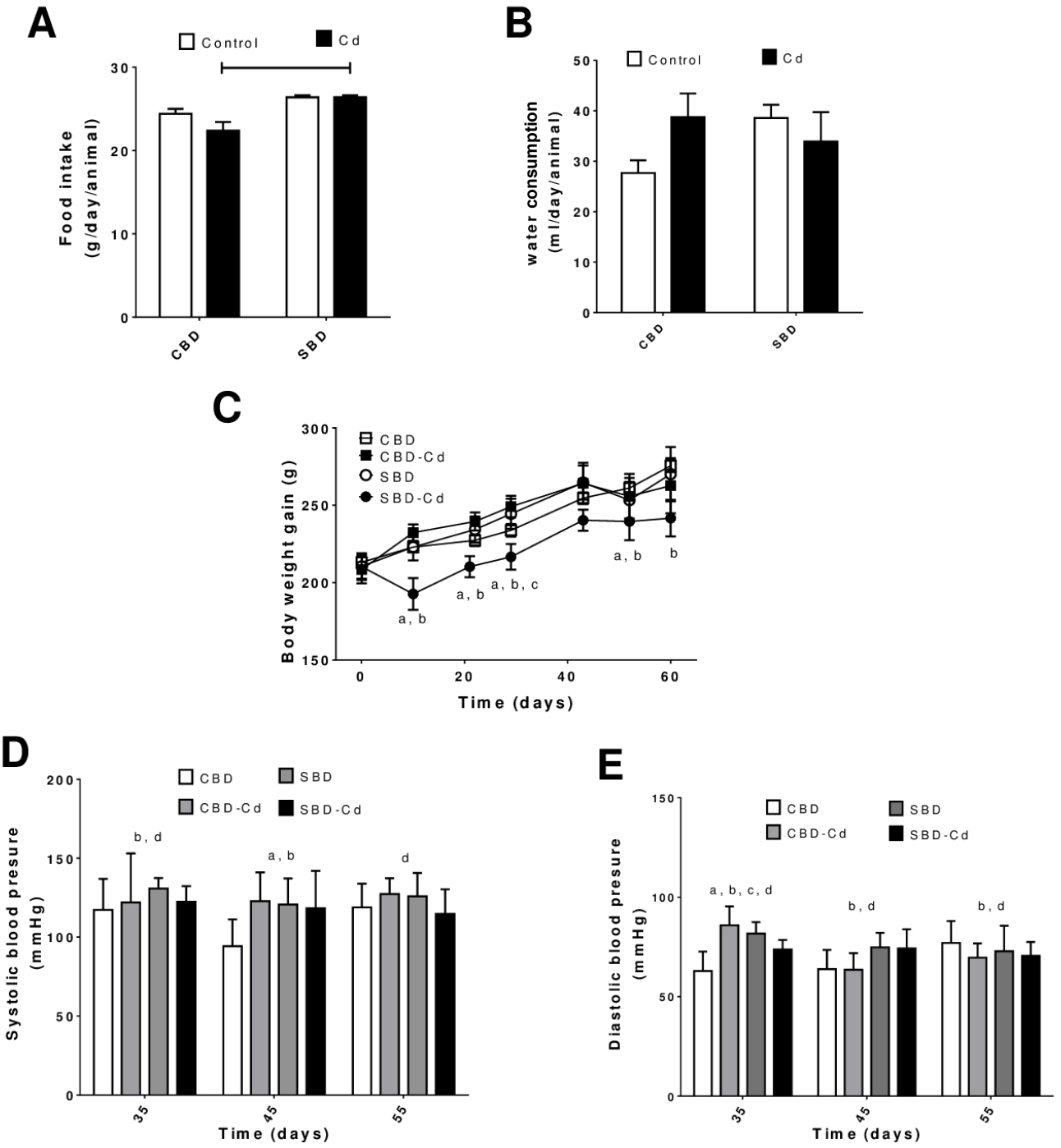

Figure 1: Effect of the diet and subchronic intoxication with Cd on food intake, water consumption, body-weight gain curve, and blood pressure. A) Food consumption, **B**) Water consumption, **C**) Body- weight gain curve, **D**) Systolic blood pressure and **E**) Diastolic blood pressure. Values are shown as mean values ± SEM for n = 6/group. The following letters indicate $p<0.05$ when groups when the groups were compared: a: CBD vs. CBD-Cd, b: CBD vs. SBD, c: SBD vs. SBD-Cd and d: CBD-Cd vs. SBD- Cd. Connecting segment indicates $p<0.05$.

1 activity when compared to non- intoxicated rats (CBD) (Figure 2B). As a marker of systemic lipid peroxidation, we measured the TBARS in serum. Changing either the protein source or Cd intoxication did not affect TARBS in serum (Figure 2C), however, Cd intoxication reduced protein oxidation in serum as

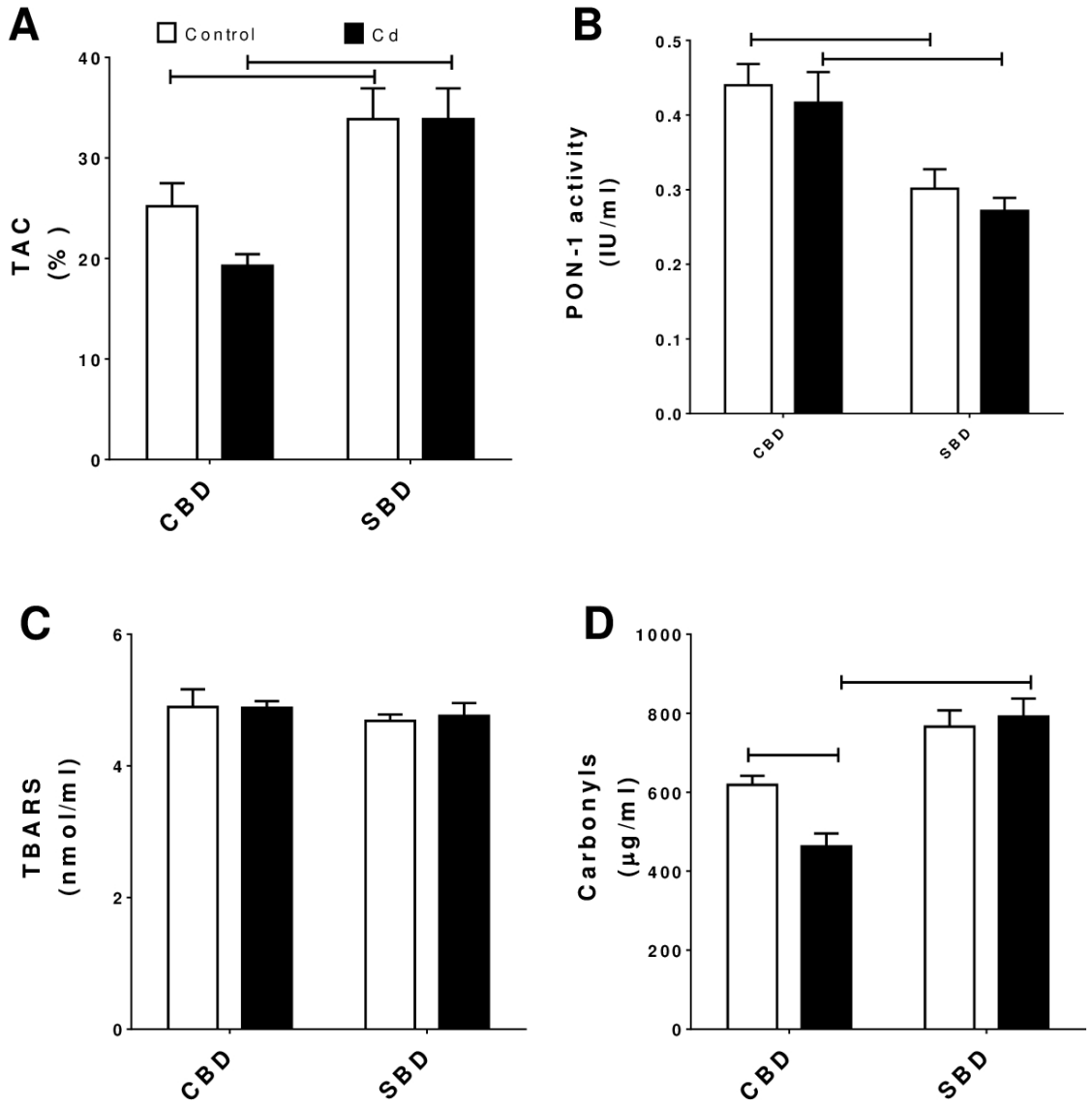

Figure 2: Oxidative stress parameters in serum: A) Total antioxidant capacity as % ABTS•+ bleaching, **B**) paraoxonase-1 specific activity (PON-1), **C**) lipid peroxidation as TBARS concentration, and **D)** protein oxidation as protein carbonyls. Assays were performed as described in Materials and Methods. Values are shown as mean values ± SEM for n = 6/group. Segments connecting group-bars indicate $p<0.05$.

assessed by protein carbonyls, in rats fed a CBD (CBD vs CBD-Cd). Intriguingly, an SBD increased protein carbonyl in serum when compared to a CBD (CBD vs SBD). When rats were fed an SBD the effect of Cd intoxication on protein oxidation was not observed (Figure 2D).

Then we determined the effect of the diets and Cd intoxication in the histomorphology of the Hp after 60 days period. Slides of Hp tissues were stained with H&E and then the images were acquired at 2x, 10x and 40x in order to show these effects (Figure 3). In rats fed a CBD, Cd intoxication increased cytoplasmic eosinophilia, nucleus fusion and pignosis in the Hp (CBD vs CBD-Cd). This effect of Cd intoxication was enhanced when casein was replaced by soybean in the diet (CDB-Cd vs SBD-Cd). When the Hp of animals fed a CBD were compared to animals fed an SBD, we observed increased eosinophilia, nucleus pignosis, and fusion. These effects were most evident in the SBD in relation to the other experimental groups (Figure 3).

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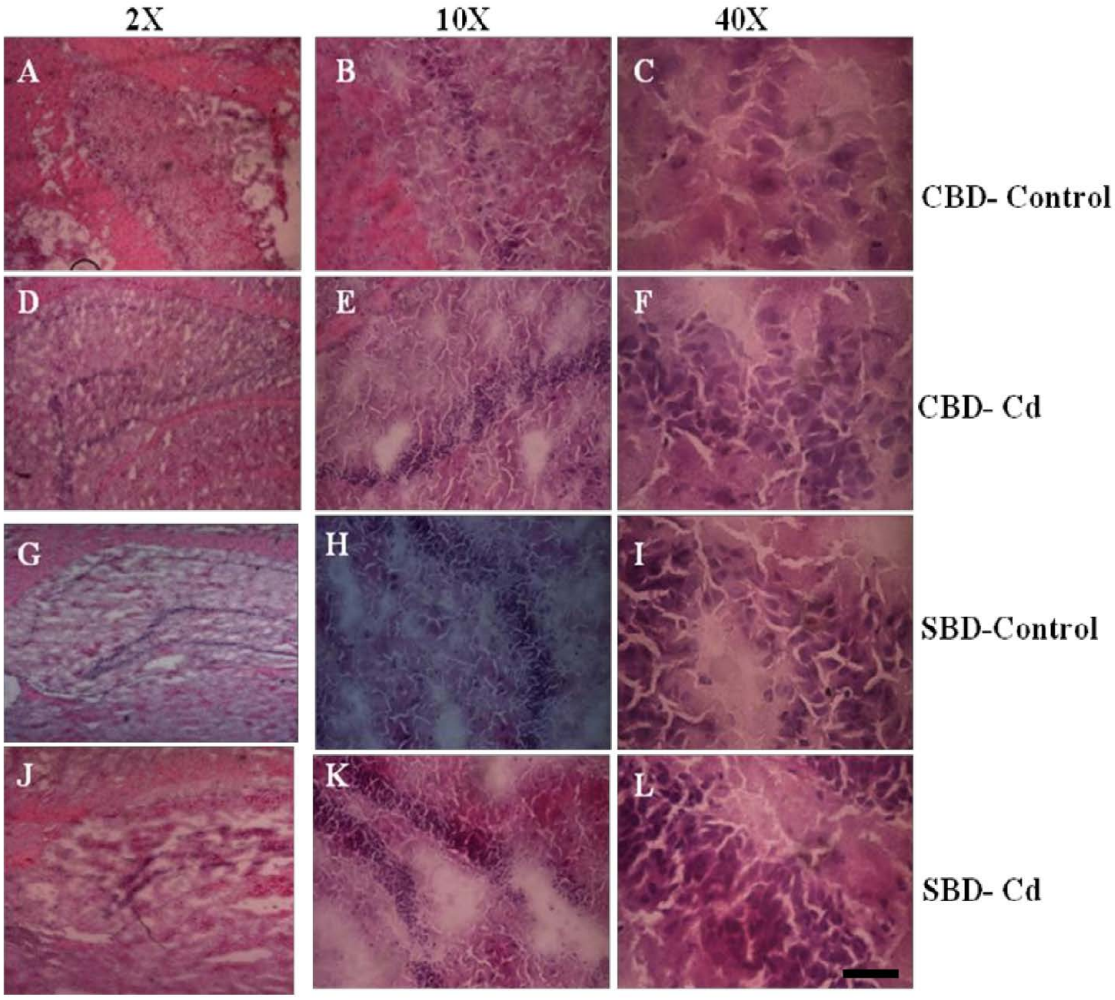

Figure 3: Histomorphology of the hippocampus. Cuts of the hippocampus were stained with H&E. Cuts were made at -4.16 mm respect to the Bregma in an adult animal brain according to the Paxinos & Watson rat brain atlas. Figures show a representative image at magnifications of 2x, 10x and 40x. Measurement bar is 50 µm.

Based on the histology of Hp that suggests stress response, we studied the redox profile in this tissue after 60 days of dieting and Cd intoxication (Table 3). Compared to CBD, Cd intoxication reduced TAC in Hp (CBD vs CBD-Cd). Changing casein for soybean in the diet did not affect TAC in Hp (CDB vs SBD). When CBD-Cd and SBD-Cd were compared we found no effect on TAC. TBARS in Hp was higher in SBD than in CBD fed animals. Cd intoxication changed TBARS in Hp in neither CBD nor SBD. Protein carbonyls were similar in the CBD and the SBD Hp, however, Cd intoxication increased protein oxidation in SBD fed animals (compare SBD vs SBD-Cd). Cd intoxication increased CAT activity in Hp in CBD fed rats. The SBD increased specific CAT activity in Hp in relation to a CBD. SOD specific activity did not change in Hp when CBD and CBD-Cd groups where compared. However, the Hp of SBD showed reduced SOD activity when compared to CBD fed animals. Cd intoxication increased SOD activity in the Hp of rats fed an SBD (SBD vs SBD-Cd) but did not in CBD (CBD vs CBD-Cd). Glutathione peroxidase activity is responsible for the reduction of lipid peroxides. GPX activity increased almost twice in the Hp SBD fed rats in relation to CBD fed rats. In animals fed a CBD, Cd intoxication did not change GPX activity. Cd intoxication reduced GPX activity in Hp of rats fed an SBD (SBD vs SBD-Cd). Interestingly, GPX activity was higher in SBD

Table 3. Redox profile in the hippocampus tissue. [1]TAC, total antioxidant capacity, TBARS, thiobarbituric acid reactive substances, CAT, catalase, SOD, superoxide dismutase and GPX, glutathione peroxidase. [2]Values are shown as mean values ± SEM for n = 6/group. The following letters indicate p<0.05 when comparing groups: a: CBD vs. CBD-Cd, b: CBD vs. SBD, c: CBD-Cd vs. SBD-Cd and d: SBD vs. SBD-Cd.

	CBD	CBD-Cd	SBD	SBD-Cd
TAC[1] (% de ABTS$^{\bullet+}$ quenching)	12,74 ± 1,31[2]	6,88 ± 0,33 a	11,34 ± 1,85	9,12 ± 0,86
TBARs (nmol /g protein)	4,454 ± 0,58	6,69 ± 1,4	8,943 ±1,35 b	8,415 ± 1,19
Carbonyls (nmol /mg protein)	2,26 ± 0,29	2,44 ± 0,16	2,23 ± 0,2 c	3,86 ± 0,51 d
CAT (IU/mg protein)	9,77 ± 1,37	10,61 ± 1,14 b	15,33 ± 1,52 c	16,22 ± 1,31
SOD (IU/mg protein)	0,81 ± 0,08	0,69 ± 0,07	0,33 ± 0,10 b c	1,08 ± 0,09 d
GPX (IU/mg protein)	2,22 ± 0,13	2,036 ± 0,16	6,01 ± 0,7 b c	4,06 ± 0,51 d

fed rats, either with or without Cd intoxication, than in CBD fed rats (Table 3).

Next, we measured parameters of inflammation in homogenates of Hp tissue to explain those histology and redox changes as we observed in our experimental model. TNFα concentration was measured by ELISA because we had the available resources to perform this determination. Cd intoxication did not affect TNFα concentration in Hp of rats fed a CBD (Figure 4A). Changing casein for soybean protein did not affect TNFα concentration in Hp (CBD vs SBD). However, TNFα concentration was reduced in animals intoxicated with Cd and fed an SBD in relation to SBD (SBD vs SB-Cd). The expression of iNOS did not change in the Hp of CDB in

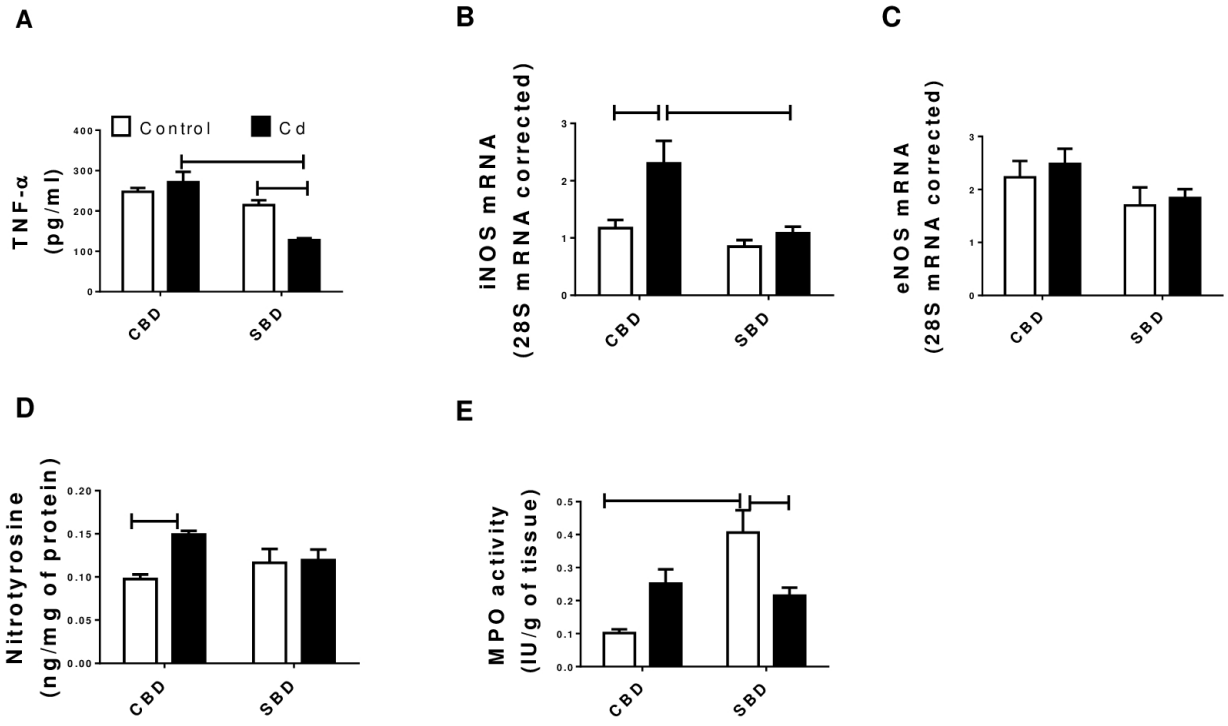

Figure 4: Pro-inflammatory markers in the hippocampus. A) tumor necrosis factor-α, TNF-α concentration was measured by ELISA; whereas **B**) inducible nitric oxide synthase (iNOS) and **C**) endothelial nitric oxide synthase (eNOS) expression were measured by reverse transcriptase (RT)-PCR. Transcript levels were normalized against 28S mRNA intensity. **D)** nitrotyrosine concentration in tissue was measured by ELISA and **E)** myeloperoxidase (MPO) specific activity was measured spectrophotometrically using O-dianisidine as substrate. Values are shown as mean values ± SEM for n = 6/group. Segments connecting bars indicate p<0.05.

relation to SBD fed rats (Figure 4B). However, iNOS expression increased in CBD intoxicated with Cd in relation to CDB. This effect was blocked in SBD (CBD vs SB-Cd). eNOS expression did not change in our experiment groups (Figure 4C). We also observed that Cd intoxication increased protein nitration assessed as nitrotyrosine-a marker of protein nitration in the Hp of CBD fed rats (Figure 4D). An SBD did no change nitrotyrosine content in Hp proteins. MPO is a marker if inflammation due to increased infiltration of neutrophils or a restored synthesis of MPO in myeloid cells [40]. We found an increased activity of MPO in the Hp of SBD fed rats in relation to the Hp of rats fed a CBD. Cd intoxication increased MPO activity in the Hp of CBD (Figure 4E). However, Cd intoxication in SBD fed animals caused a reduced MPO activity almost to the activity of MPO found in the Hp of CBD fed rats.

The transcription factor p53 controls the expression of a number of proteins involved in stopping cell cycle progression when genomic DNA damage occurs [41, 42]. The expression of p53 expression was increased in the Hp of SBD fed rats in relation to CBD (Figure 5A). Cd intoxication increased p53 expression in CBD fed rats. However, Cd intoxication did not change the expression of p53 in the Hp of SBD fed rats. To assess a potential effect of genomic damage in the Hp in the cell death by apoptosis, we measured the molecular marker of this process such as Bax and Bcl-2 [43]. We found that none of the treatments affect the expression of

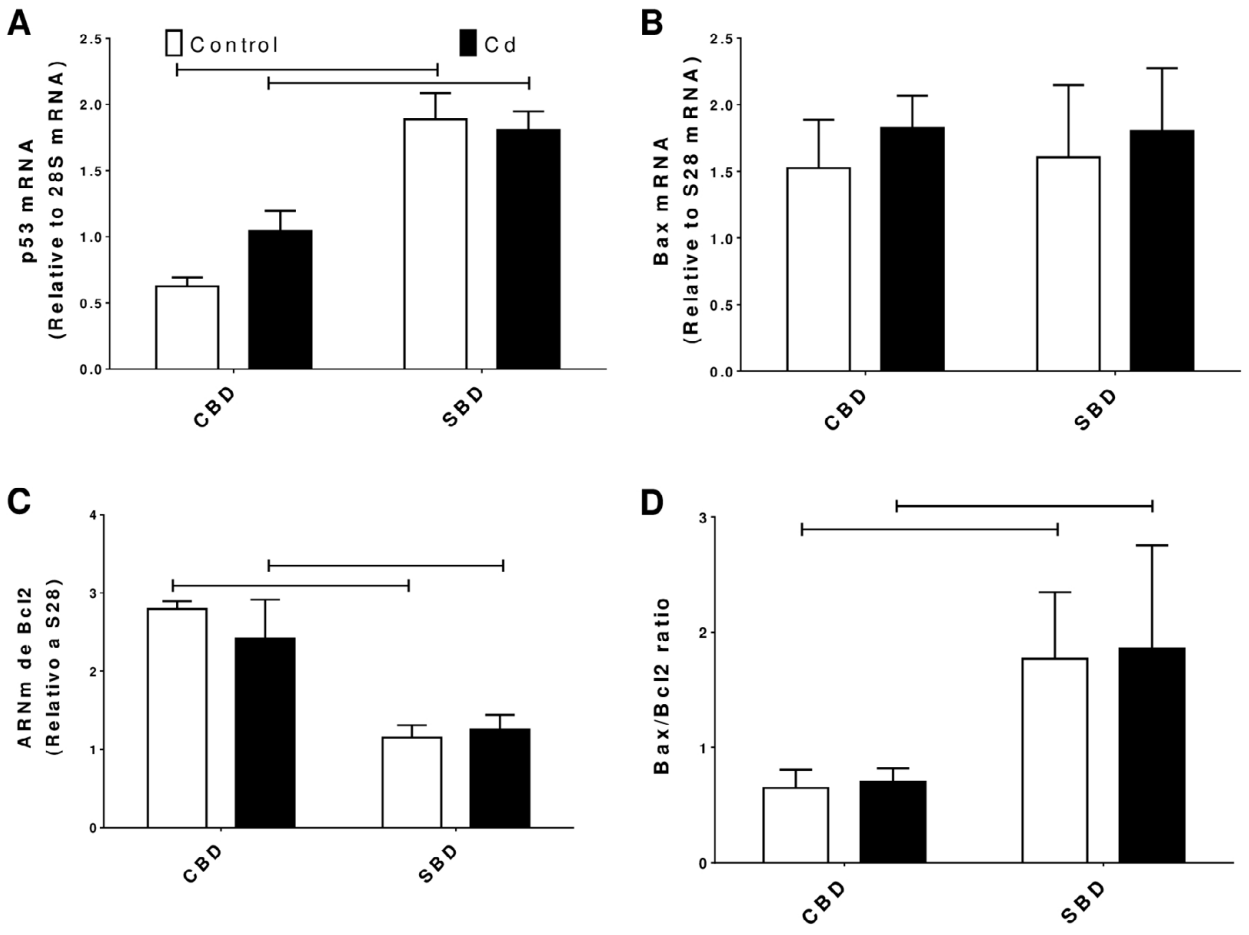

Figure 5: Molecular markers of genomic damage and apoptosis in the hippocampus. Relative expression of **A**) p53, **B**) Bax and **C**) Bcl-2 was measured in hippocampus tissue by RT-PCR. The 28S mRNA was used as a housekeeping gene. **D**) Bax/Bcl-2 intensity ratio. Data are shown as mean values ± SEM in three experiments by duplicate. Segments connecting bars indicate $p<0.05$.

Bax (Figure 5B), however, Bcl-2 expression was reduced in the Hp of SBD in relation to CBD rats (Figure 5C). Cd intoxication did not change Bcl-2 expression in the Hp of CBD or SBD rats. To assess the global impact of dieting and Cd intoxication in the apoptotic process we calculated the Bax/Bcl-2 ratio [43] and found that compared to a CBD, an SBD increased the ratio and it was not affected in the SBD when animals were also intoxicated with Cd (Figure 5D). These data suggest that the observed changes in the ration Bax/Bcl-2 are due to a reduction in the expression of Bcl-2, but not to any changes of Bax expression.

DISCUSSION

Because the diet is a modifiable factor to reduce the impact of environmental exposure to neurotoxic compounds, a diet based in soybean proteins may prove to be effective to reduce the impact of cadmium intoxication [44,45]. In our study, a sub chronic model of intoxication was used to assess whether an SBD can ameliorate the neurotoxicity of Cd intoxication in the Hp and determine potential effects on cell death. Casein, a protein from animal origin was used to compare the effect of Cd intoxication in a diet based in soy flour [46].

A soybean-based diet caused increased TAC but reduced the PON-1 activity at a systemic level. Interestingly, carbonyl content in serum increased in SBD fed animals. Cadmium did not modify SBD effects suggesting that at a systemic level, both dieting and intoxication may cause similar effects. Histology of the Hp showed that both Cd and SBD increased eosinophilia. This effect has been observed in other models of cell damage due to stress [47]. Cd picnosis is a remarkable feature of apoptosis at the tissue level [48], and in our model picnosis and fusion of the nuclei was increased with Cd intoxication and was also enhanced in SBD fed rats. This observation indicates that apoptosis may be occurring in the Hp and oxidative stress may play a role. Indeed, most of the effects of Cd in tissues are due to changes in the redox balance towards oxidative stress [49].

To assess the role of redox changes in the Hp in the morphology observed in the Hp, we measured the redox profile in Hp tissue. In a diet based in casein subchronic Cd intoxication reduced TAC in Hp, but increased TBARS suggesting that reduced small and enzyme responsible to scavenge, decompose ROS to protect the tissue against deleterious effects of its overproduction [50]. Neither carbonyls, CAT, SOD or GPX were changed in CBD fed animals when intoxicated by Cd. Replacement in the diet of casein for soybean protein caused increased lipid peroxidation marker (TBARS), did not change TAC, but increased CAT and GPX activity, suggesting an organic response to ROS and or inflammation due to the diet. Superoxide radical anion ($^{\bullet}O_2^-$) and nitric oxide ($^{\bullet}NO$) are two of the major reactive species from where most of the known reactive species are produced, among other ONOO-, HOCl, and $^{\bullet}OH$ [51-53]. Excess of ROS can cause oxidative damage in tissues resulting in oxidative stress, i.e., oxidative damage to lipid, proteins, carbohydrates, and nucleic acids [54]. This excess ROS can result from increased synthesis or reduced scavenging [53]. SOD-1 activity decreased in SBD suggesting increased superoxide anion content and derived reactive species [55]. These data suggest that superoxide radical anion can be involved in further oxidative damage to the Hp in animals fed an SBD. Although we did not observe Nrf2 expression in the Hp of SBD fed rats (data not shown), Nrf2 signaling pathway and genes under the antioxidant response element control may be induced. Indeed, we observe an antioxidant response in the Hp, which may account for the induction of antioxidant enzyme expression (i.e., CAT and GPX) [56].

Increased superoxide radical anion besides being involved n generating dangerous reactive biochemical species can also enhance inflammation by activation of tissue phagocytes [55,57]. Interestingly in our model subchronic Cd intoxication reduced TN-α content in Hp of rats fed an SBD. As observed by other authors Cd intoxication enhances iNOS expression in other tissues [58-60]. Here in we observed that Cd intoxication enhanced iNOS expression in CBD fed rats. Changing casein for soybean proteins in the diet did not change iNOS expression. Interestingly, Cd intoxication in SBD fed rats did not affect iNOS expression, suggesting that somehow soybean protein may be protecting against inflammation in the Hp [61-63]. As expected, nitrotyrosine-a marker

of nitrosative stress also increased in the Hp of CBD fed rats when they were intoxicated with Cd [64]. This marker of nitrosative stress results from the reaction of peroxynitrite with tyrosine residues in proteins [65].

Another marker of oxidative stress and source of oxidative damage is myeloperoxidase [66,67]. This is the only enzyme that under physiological conditions produces HOCl, a random oxidant that can damage at a high rate any macromolecule in biological systems [68]. Cd intoxication increased MPO activity in the Hp of CBD fed rats. Intriguingly, an SBD increased MPO activity in the Hp in relation to CBD animals. Cd intoxication reduced MPO activity in the Hp of SBD fed rats. It is possible that soybean proteins increase inflammation and oxidative stress in Hp [69]. Indeed, it was demonstrated that soybean protein induces a

pro-inflammatory profile in the gut of zebrafish [70]. Moreover, when dieting with soybean proteins inflammation is reduced by Cd intoxication as a compensatory mechanism [71].

The morphological examination of the Hp tissue indicates that an SBD and Cd intoxication cause picnosis and nucleus fusions as well as eosinophilia in the cytoplasm. These structural changes in Hp are consistent with changes in the cell function that may indicate the mechanism of cell damage in the Hp [72]. As stated above both an SBD and Cd intoxication may, in some cases, have an enhancing effect if oxidative and inflammation damage or they may trigger compensatory mechanisms [70]. P53 expression-a marker of genotoxic damage in the Hp was increased by an SBD, but Cd intoxication did not change this effect, whereas in CBD, Cd intoxication increased p53 expression [73]. These data are consistent with a tissue response to the genotoxic damage triggered by an enhanced ROS production due to feeding the animals with an SBD. In order to investigate the consequence of this induction of p53 in the cell death process in Hp we measured Bax and Bcl-2 expression [74], these are molecular markers of pro-apoptosis and anti-apoptosis, respectively. These data indicate that the picnosis observed in the histology of Hp may be a consequence of an ongoing cell apoptosis process.

In a recent study aimed at investigating the underlying mechanism of neurotoxicity of cadmium, the effects of intraperitoneal injection of cadmium on messenger RNA (mRNA) expression of Bcl-2 (B-cell lymphoma 2) and Bax (Bcl2-associated x) genes and caspase-3/7 activation in rat Hp and frontal cortex were determined [19]. In agreement with our study this study showed that the mRNA level of Bax increased compared to the control group at the doses of 1, 2 and 4 mg/kg in rat Hp. Cadmium increased caspase-3/7 activity at doses of 1, 2 and 4 mg/kg in rat Hp. In relation to our study, they found that the decreased Bcl- 2/Bax mRNA is due to increased Bax expression and results in cell apoptosis in the hippocampus. These authors suggested that the apoptotic effect of cadmium may be through the mitochondrial pathway by the activation of caspase-3/7 [19].

Finally, although a number of studies have shown beneficial effects of soybean in the diet, caution should be taken when using a diet supplemented with soybean proteins to reduce oxidative stress, inflammation, and genotoxicity in the Hp. Herein we show that an SBD feeding for 60 days caused increased oxidative stress, inflammation, and apoptosis in the Hp of rat intoxicated with cadmium in the drinking water. An SBD may not be the right diet to reduce the neurotoxic effect of a Cd sub chronic intoxication in the Hp. More mechanistic studies are needed to clarify the toxicity or protective effects of a diet supplemented with soybean proteins, especially in subjects exposed to environmental toxicants, such as heavy metals.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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