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Multi-functional egg white hydrolysate prevent hypertension and vascular dysfunction induced by cadmium in rats

Paola Zambelli Moraes [a], José Eudes Gomes Pinheiro Júnior [a,2], Caroline Silveira Martinez [a,1], Camila Rodrigues Moro [a], Grazielly Caroline da Silva [a], Marina Diaz Rodriguez [a], Maylla Ronacher Simões [c], Fernando Barbosa Junior [b], Franck Maciel Peçanha [a], Dalton Valentim Vassallo [c], Marta Miguel [d], Giulia Alessandra Wiggers [a,*]

[a] Cardiovascular Physiology Laboratory, Universidade Federal do Pampa, BR 472, Km 592, Uruguaiana, Rio Grande do Sul, Brazil
[b] Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, 14049-903 Ribeirão Preto, São Paulo, Brazil
[c] Cardiac Electromechanical and Vascular Reactivity Laboratory, Universidade Federal do Espírito Santo, Marechal Campos, 1468 Vitória, Espírito Santo, Brazil
[d] Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM), Nicolás Cabrera, 9, Campus Universitario de Cantoblanco, Madrid, Spain

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ABSTRACT

We have investigated if EWH could counteract or prevent cardiovascular damage induced by high level of Cd exposure in rats. Male *Wistar* rats were treated for 14 days with: (A) Untreated - intraperitoneal (i.p.) injections of distilled water and tap water by gavage; (B) Cd – 1 mg/kg of bw/day of CdCl2 (i.p.) and tap water by gavage; (C) EWH – distilled water (i.p.) and 1 mg/kg/day of EWH by gavage; (D) CdEWH – both treatments. EWH prevented the increase on systolic blood pressure, vascular dysfunction, and inflammation after Cd exposure; prevent the activation of cyclooxygenase (COX)-2 and its derived contractile protanoids, inhibits angiotensin II by the reduction of ACE activity and prevents the increased oxidative stress mainly mediated by NADPH oxidase. Multifunctional EWH could be considered as a natural alternative therapy to counteract the deleterious effects caused by high level of Cd exposure.

1. Introduction

Cadmium (Cd) is a non-essential and toxic heavy metal whose main sources of human exposure are cigarettes, contaminated soil, air, drinking water, food and occupational activities (Bhattacharyya et al., 2021). Human environmental or occupational exposure is related to harmful health effects, such as hepatic, renal, cardiovascular, musculoskeletal, gastrointestinal, pulmonary, and reproductive disorders (Bernard, 2008; Balali-Mood et al., 2021). Specifically, in the cardiovascular system, Cd is related to the development of arterial hypertension (Tellez-Plaza et al., 2008; Eum et al., 2008; Martins et al., 2021), atherosclerosis (Knoflach et al., 2011; Tinkov et al., 2018; Diaz et al., 2021), peripheral arterial disease (Navas-Acien et al., 2004), stroke and heart failure (Tellez-Plaza et al., 2012; Tellez-Plaza et al., 2013).

In vitro and *in vivo* studies also point to this metal as a toxic environmental factor linked to endothelial and vascular smooth muscle damage (Taniyama and Griendling, 2003; Touyz and Schiffrin, 2008; Gökalp et al., 2009; Almenara et al., 2013) associated with a reduction in the bioavailability of nitric oxide and increased on reactive oxygen species (Cuypers et al., 2010; Broseghini-Filho et al., 2015; Vassallo et al., 2018), mainly derived from NOX-1. The deleterious Cd-vascular effects have been associated with the of renin-angiotensin system (RAS) and COX-2 system activation in resistance and conductance arteries (Pinheiro Júnior et al., 2020).

Besides the high environmental presence of Cd and its effects on human health, this metal has a high capacity to bioaccumulate in the human body (half-life 25–30 years) (Genchi et al., 2020). Therefore, alternative chelating and antioxidant strategies are needed and could be considered a good and valuable strategy to the protection, prevention, and/or reduction of harmful effects caused by Cd exposure (Rahimzadeh

* Corresponding author at: Cardiovascular Physiology Laboratory, Universidade Federal do Pampa, BR 472, Km 592, Uruguaiana, Rio Grande do Sul, Brazil.
E-mail address: giuliapecanha@unipampa.edu.br (G.A. Wiggers).
[1] Present address: William Harvey Research Institute, Heart Centre, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.
[2] Present address: Department of Physiotherapy, Universidade Federal de Sergipe - UFS, Rosa Elze, São Cristovão, Sergipe, Brazil.

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et al., 2017). Therapeutic strategies based on the biological action of food compounds with antioxidant properties (Lobo et al., 2010), such as tocopherols (vitamin E) (Jahan et al., 2014), and phytochemical from grape juice (Lamas et al., 2017) have been reported as potential non-pharmacological allies of natural origin in the management of toxic effects produced in the body by exposure to heavy metals (Abeyrathne et al., 2013).

In previous studies multi-functional properties of an egg white hydrolysate treated by pepsin (EWH) seem to work as a cardiovascular protective agent. In previous works, EWH has demonstrated antioxidant, vascular relaxing, anti-inflammatory properties (Manso et al., 2008; Garcia-Redondo et al., 2010; Garcés-Rimón et al., 2016) blood pressure lowering effects in spontaneously hypertensive rats (Miguel et al., 2006) and cardiovascular protection against different models of exposure to environmental contaminants such as mercury and aluminum (Rizzetti et al., 2017; Martinez et al., 2019; Escobar et al., 2020). More recently, Cd exposure for 14 days at high dose induced important reproductive impairments in rodents, with reduced sperm quality and increased oxidative damage, confirming the high toxicity of this metal on reproductive system. Co-treatment with EWH partially prevented the reproductive dysfunctions induced by Cd, and these effects were associated with an inhibition on Cd deposition and preventing the increased oxidative stress (Pinheiro Júnior et al., 2020).

The purpose of this study was to investigate if multi-functional EWH properties may counteract or prevent cardiovascular damage induced by high level of Cd exposure in rats and attempt to elucidate the mechanism of action involved.

2. Materials and methods

2.1. EWH preparation

The hydrolysate (EWH) was obtained by enzymatic hydrolysis of egg white with pepsin for 8 h according to Garcés-Rimón et al. (Garcés-Rimón et al., 2016). After enzymatic inactivation, the product was centrifuged 2500*g* for 15 min and the supernatant was frozen and lyophilized for later use.

2.2. Animals

Three-month-old male Wistar rats (375.8 ± 8.9 g) from the Central Animal Laboratory of the Federal University of Santa Maria, Brazil, were housed in an environment with controlled temperature and humidity, light and dark cycle (12:12 h), with free access to water and food. All experiments were conducted following the guidelines of the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Experimentation of the Federal University of Pampa, Brazil (Protocol 017/2018).

2.3. Experimental model

The animals were randomly divided into 4 groups and treated for 14 days with (1) Untreated group - intraperitoneal (i.p.) injections of distilled water and tap water by gavage; (2) Group Cd - i.p. 1 mg/kg of cadmium chloride ($CdCl_2$) (Balaraman et al., 1989) and tap water by gavage; (3) EWH group – i.p. of distilled water and EWH 1 g/kg/day by gavage (Miguel et al., 2005); (4) CdEWH group - both treatments ($CdCl_2$ + EWH). General health, body weight, diet and water intake were evaluated and recorded once a week. The volumes of EWH and Cd were adjusted weekly for each animal's weight to ensure correct administration of the dose stipulated by the study.

2.4. Hemodynamic and vascular reactivity parameters

Systolic blood pressure (SBP) was measured, non-invasively by caudal plethysmography (AD Instruments Pty Ltd, Bella Vista, NSW, Australia) according to Buñag (Buñag, 1973), before the start and at the end of treatment. Rat's faeces were collected in a metabolic cage for 24 h before euthanasia.

On the fifteenth day, the animals were anesthetized with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, i.p.) and sacrificed by decapitation. Blood was collected, a sample was stored at −80 °C to measured Cd content, and another sample was centrifuged at 500g for 20 min at 4 °C to obtain plasma which was kept frozen at −80 °C until biochemical analysis. Liver, kidney, aorta and third branchial mesenteric arteries (MRA) were immediately excised and stored at −80 °C for biochemicals assays. Liver and kidney were also used to measured Cd content.

For the vascular reactivity experiments, the aorta and MRA were carefully dissected and cleaned of the adipose and connective tissues, divided into 2 mm long segments, and placed in Krebs-Henseleit solution (in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 23; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O_2 and 5% CO_2 (pH 7.4). The aorta segments were mounted in an isolated tissue chamber and maintained at a resting tension of 1.5 g at 37 °C. Isometric tension was recorded using an isometric force transducer (TSD125BX8, Biopac Systems, Inc, Santa Barbara, CA, USA) connected to an acquisition system (MP150WSW-SYS, Biopac Systems). The MRA segments were mounted on a small vessel chamber myograph (Multi-Wire Myograph System, DMT620, ADInstruments, Australia) to measure the isometric tension (Wiggers et al., 2008). The segments were stretched to the ideal lumen diameter for the development of active tension, determined based on the internal circumference ratio of the wall stress of the segments, setting the internal circumference to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg. After a 45-minute equilibrium period, the aortic and MRA segments were exposed twice to 75 mM and 120 mM KCl respectively, first to verify their functional integrity and again to assess the maximum developed tension. Subsequently, endothelial integrity was tested with acetylcholine (ACh, 10 μM) in segments of the aorta or MRA previously contracted with phenylephrine (Phe) or norepinephrine (NE) respectively, in a concentration that produced tension close to 50% of the induced contraction by KCl. After 60 min of washing, a single concentration–response curve for Phe or NE (0.01 nM − 300 μM) was performed. The role of the endothelium in vasoconstrictor responses to Phe and NE was analyzed removing the endothelium mechanically, and its absence was confirmed by the inability of ACh to induce relaxation greater than 10% of the previous contraction due to Phe or NE. Their administration evaluated the effects of the following drugs 30 min before the Phe or NE concentration–response curve: nonspecific nitric oxide synthase (NOS) inhibitor, N (ω) -nitro-L-arginine methyl ester (L- NAME, 100 μM); the NADPH oxidase inhibitor, apocynin (0.3 μM); the selective COX-2 inhibitor, NS398 (1 μM); the AT-1 receptor blocker, losartan (10 mM), and the NOX1 inhibitor, ML171 (0.5 μM). Concentration-response curves to ACh (0.1 nM − 300 μM) and sodium nitroprusside (SNP, 0.1 nM − 300 μM) were performed, respectively, in previously segments contracted with Phe or NE to evaluate endothelium-dependent (ACh) in intact vessels or incubated with Apocynin or NS398 in MRA and independent relaxation (NPS) in both vessels.

2.5. Cadmium content in the blood, liver, kidney and feces

Cd content in blood, liver, kidney, and feces was determined using mass spectrometry according to Batista et al. (Batista et al., 2009) (Batista et al., 2009) as previously described (Pinheiro Júnior et al., 2020).

2.6. Biochemical analyses

Biochemical studies of oxidative stress biomarkers have been performed in the plasma, aorta, MRA, liver and kidney. The tissues were

homogenized in 50 mM Tris-HCl, pH 7.4, centrifuged at 2400g for 10 min at 4 °C.

The lipid peroxidation was measured as malondialdehyde (MDA) levels using a colorimetric method, as previously described by Ohkawa, Ohishi and Yagi (Ohkawa et al., 1979) (Ohkawa et al., 1979), with modifications (Martinez et al., 2017). The results were expressed in nanomoles of MDA per mL of plasma or per mg of tissue.

The levels of reactive oxygen species (ROS) were determined by the spectrofluorometric method described by Loetchutinat et al. (Loetchutinat et al., 2005) (Loetchutinat et al., 2005) with modifications (Martinez et al., 2017). The results were expressed in fluorescent units (FU).

The total antioxidant capacity by the Ferric Reducing Antioxidant Power (FRAP) test described by Benzie and Strain (Benzie and Strain, 1996) (Benzie and Strain, 1996), with modifications (Martinez et al., 2017). A standard Trolox dose–response curve (50–1000 μM - water-soluble analog of vitamin E) was prepared, and the FRAP assay was compared to reference to the Trolox equivalents and expressed in mM/ mL of Trolox.

The SOD activity was spectrophotometrically assayed in liver and kidney as described by Misra and Fridovich (Misra and Fridovich, 1972) (Misra and Fridovich, 1972). The capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome is measured by color reaction at 480 nm and expressed as Units (U) per mL of plasma or per mg of protein.

The catalase (CAT) activity was spectrophotometrically assayed as described by Aebi (Aebi, 1984) (Aebi, 1984). The enzymatic activity was expressed in Units (1 U decomposes 1 μM H_2O_2/min at pH 7.0 at 25 °C) per mg of protein.

The level of Glutathione Reductase was assessed using the spectrofluorometric method by Armstrong et al. (Armstrong et al., 1998) (Armstrong et al., 1998). Sample fluorescence was read at 420 nm emission and 350 nm excitation and expressed in nmol GSH / g tissue.

Glutathione peroxidase (GPx) activity was spectrophotometrically assayed according Wendel (Wendel, 1981) (Wendel, 1981). The enzymatic activity was expressed as nmol NADPH/ min/ mg of protein.

We perform protein quantification in all samples using the Bradford method (Kruger, 1994).

2.7. In situ detection of vascular superoxide radical anion production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* superoxide radical anion ($O_2^{\cdot-}$) production in aortic and MRA segments, as previously described (Miller et al., 1998). Fluorescence was detected with a 568 nm long-pass filter. For quantification, five rings per animal were sampled for each experimental condition and averaged. The mean fluorescence densities in the artery were calculated using NIH Image J software version 1.46r (https://rsbweb.nih.gov/ij/), using the same imaging settings in each case. The results are expressed as arbitrary units (AU) of fluorescence intensity.

2.8. Determination of angiotensin-converting enzyme (ACE) activity

The ACE activity was fluorometrically measured in plasm (Friedland and Silverstein, 1977; Miguel et al., 2007). Briefly, aliquots of plasma (3 μl) were incubated for 15 min at 37 °C with assay buffer (40 μl) containing the ACE substrate 5 mM Hip-His-Leu (Sigma). The reaction was stopped by the addition of 0.35 N HCl (190 μl). The generated product, His-Leu, was measured fluorometrically after 10 min incubation with 2% o-phathal dialdehyde in methanol (100 μl). Fluorescence measurements were carried out at 37 °C in a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with 350 nm excitation and 520 nm emission filters. The results are expressed as mU ACE / mL plasma.

2.9. Immunofluorescence for detection of NOX-1, AT-1 receptor, and COX-2

Artery segments were prepared and analyzed according to Jimenez-Altayó (2006) (Jiménez-Altayó et al., 2006). The primary antibodies used were against the NOX-1 receptor (1: 200), COX-2 (1: 200), and AT1 (1: 400) (Sigma Aldrich, St. Louis, MO, USA), and secondary antibody (anti-goat conjugated with Alexa 488). -M IgG) diluted (1: 400). For the staining of the nucleus, we used 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI, 1: 10,000; MBD0015, Sigma). In the preparation of the negative control sections, we omitted the primary antibody. The EVOS® Floid® Cell Image Station (Life Technologies, Carlsbad, CA) was used to acquire the images. For quantifications, sections of five different animals per group were analyzed with the same capture parameters. The readings were taken from the mean fluorescence densities (histogram) and were calculated using NIH Image J software version 1.46r (HTTP: // rsbweb. Nih.gov/ij/); the data were expressed concerning the control group.

2.10. Western blot analysis

To analyze COX-2 expression in aortic samples, proteins were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes before being incubated with mouse polyclonal antibody to COX-2 (1: 1000, Cayman Chemical, Ann Arbor, MI, USA). After washing, the membranes were incubated with an anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (1: 5000, Sigma Aldrich, St. Louis, MO, USA). After careful washing, the immunocomplexes were detected using an improved horseradish peroxidase / luminal chemiluminescence system (ECL Plus, GE Healthcare, GE Healthcare, Buckinghamshire, UK). Image acquisition was performed using ChemiDoc (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the signals in the immunoblot were quantified using the computer program ImageJ. The same membrane was used to determine the expression of the ponceau staining protein. The data are expressed as the ratio between the signals in the immunoblot corresponding to the studied protein and Ponceau.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as mean ± SEM. In vascular reactivity experiments, vasoconstrictor responses of the aorta and MRA were expressed as a percentage of KCl-induced contraction. Vasodilator responses were expressed as a percentage of contraction before Phe or NE. To compare the effect of the drugs in response to Phe in the segments of each group, some results were expressed as differences in area under the concentration–response curves (dAUC) in control and experimental situations. The dAUCs were calculated from the graphs of the response curve to individual concentration; the differences were expressed as the percentage of the dAUC of the corresponding control situation. The results were analyzed using the two-way ANOVA for comparison between groups. When ANOVA showed a significant treatment effect, the Bonferroni post hoc test was used to compare individual averages. Values of $p < 0.05$ were consider as significantly different.

3. Results

The initial weight of the animals was similar in all groups evaluated. However, we observed that the body weight of both groups receiving Cd was lower in relation to the groups that did not receive the metal at the end of treatment, but that difference was only significant when Cd group was compared to control group (Table 1). In the same way, water and feed intake were also lower in the Cd and CdEWH groups, in this case the differences were significant in both groups when they were compared to control (Table 1).

Table 1
EWH effects on weight, feed and water consumption and Cd content in blood, kidney, liver and feces.

Parameters	Untreated	Cd	EWH	CdEWH
Initial Weight (g)	356.4 ± 10.3	374.5 ± 12.4	382.6 ± 9.1	373.8 ± 14.7
Final Weight (g)	409.5 ± 13.5	338.2 ± 16.0*	396.0 ± 14.1	348.0 ± 17.6
Feed (g/day)	30.1 ± 1.9	8.1 ± 1.8*	24.9 ± 2.1	15.3 ± 2.1*
Water (ml/day)	66.7 ± 6.1	29.6 ± 5.9*	78.6 ± 5.2	36.6 ± 3.2*
Blood Cd content (μg/L)	1.02 ± 0.27	496.7 ± 15.4*	1.00 ± 0.38	397.6 ± 21.8*#
Kidney Cd content (μg/g)	130.8 ± 79.4	98342.1 ± 13101.1*	193.0 ± 54.9	49827.5 ± 3478.5*#
Liver Cd content (μg/g)	8.4 ± 1.7	173453.7 ± 19251.8*	155.6 ± 47.7	212519.9 ± 20386.5*
Feces Cd content (μg/g)	54.7 ± 2.2	978.9 ± 97.7*	48.7 ± 1.5	5852.2 ± 2123.2*#

*P < 0.05 *vs* Untreated, # *vs* Cd; n = 8 per group; Two-way ANOVA.

After 14 days of exposure, the Cd content was extremely higher in the blood, liver, and kidney of rats that received only Cd treatment. However, the group Cd-EWH significantly reduced the Cd deposition in blood and kidney but not in liver (Table 1). The co-treatment with EWH significantly enhanced the Cd elimination by the feces (Table 1).

When analyzing the blood pressure data, hypertension produced by Cd exposure was completely prevented by EWH co-treatment, which restored the values of blood pressure to control (in mmHg - Untreated: 119 ± 2.2, Cd: 149 ± 5.0*, EWH: 124 ± 2.6, CdEWH: 123 ± 3.2#).

Regarding vascular reactivity experiments, the response to KCl was similar for all groups (Aorta in g, Untreated: 1.60 ± 0.04, Cd: 1.54 ± 0.06, EWH: 1.57 ± 0.06, CdEWH: 1.61 ± 0.06; MRA in mN/mm, Untreated: 5.64 ± 0.13, Cd: 5.37 ± 0.12, EWH: 5.24 ± 0.15, CdEWH: 5.42 ± 0.15). EWH co-treatment prevented the increase in the contractile response promoted by Cd, which can be observed after the contractile response to Phe and NE (Table 2, Table 3 and Fig. 1 A, a). The endothelium dependent and independent vasodilator response mediated by ACh and NPS, respectively, was not affected by exposure to Cd or EWH in the aorta (Fig. 1 - Aorta B and C) but in MRA, exposure to Cd showed an endothelial dysfunction that was totally prevented by EWH co-treatment (Fig. 1b). The endothelium independent relaxation response mediated by NPS in MRA was not affected (Fig. 1c).

To investigate whether co-treatment with EWH modifies NO modulation in the vasoconstrictor responses of rats exposed to Cd, the endothelium was removed, and rings incubated with a NOS inhibitor (L-NAME). Endothelial removal and NOS inhibition shift to the left the concentration response curve to Phe in all groups in aorta (Fig. 2 A – D, Table 2). However, the magnitude of the response was smaller in the Cd group and recovered in preparations from rats co-treated with EWH, suggesting a protective effect of EWH against the endothelial modulation reduction and NO pathway damage promoted by Cd as demonstrated in dAUC representation (Fig. 2 E, E′). Fig. 2 (a-d) shows that the co-treatment with EWH completely prevented the reduction of endothelial modulation caused by exposure to Cd in MRA (Fig. 2 a-d; a′-d′).

The effects of EWH on vascular dysfunction related to oxidative stress induced by Cd were investigated by the role of the superoxide anion using apocynin, a non-selective inhibitor of NADPH oxidase. Co-treatment with EWH prevented the increased participation of ROS in the vasoconstrictor response of the aorta and MRA (Table 2, Fig. 3A-E, A′-D′, a-d) and of the vasodilator response to ACh in MRA (Fig. 3a″-d″); Corroborating these functional data, the increased ROS levels in both vessels induced by Cd were restored by EWH co-treatment, and lipid peroxidation normalized in MRA and reduced in the aorta (Fig. 7). The antioxidant capacity was maintained elevated in groups that receive Cd cotreated with EWH or not (Fig. 7). Confirming the vascular protective role of EWH, the increased *in situ* production of superoxide anion was also restored by EWH co-treatment in both vessels (Fig. 4).

Additionally, the dietary supplementation with EWH seems to be protecting metabolism organs such as kidney and liver from the high toxicity induced by Cd. Our results demonstrated that EWH reduced ROS and improved non-enzymatic antioxidant defense in both organs. Specifically, EWH supplementation reduced GSH levels and decreased SOD activity in kidney and GPx activity in liver to values similar to those found in control animals (Fig. 7).

The inhibition of NOX1 subunit of NADPH oxidase with ML171 showed that EWH prevented the decrease of the contractile response to Phe and NE caused by Cd exposure in both vessels (Table 2, Fig. 3 A′-D′, a′-d′). Reinforcing these findings, we observed that EWH reduced the detection of the NOX-1 subunit in the aorta and MRA Cd-exposed segments (Fig. 8).

Increased vascular contractile prostanoids from COX seems to be an important mechanism by which Cd increases vascular reactivity. We have used a specific COX inhibitor, NS398, to investigate whether the administration of EWH can reduce the activation of the COX pathway. The dietary treatment with EWH prevented the reduction of vasoconstrictor responses caused by Cd in the presence of NS398 (Table 2, Fig. 5A-E, a-e) and the increase in the vasodilator response to ACh in MRA (Fig. 5 a′-d′). Moreover, Cd exposure increased *in situ* expression of COX-2 in aorta (Fig. 5F) which was prevented by EWH (immunofluorescence analysis of aorta and MRA - Fig. 8).

Considering the importance of the RAS in the control of the blood pressure and vascular reactivity, and the possible protective role of the EWH against ACE effects, aorta and MRA were incubated with losartan, an AT-1 receptor blocker. As expected, losartan decreased the vasoconstrictor response to Phe and NE in Cd-treated rats, but the magnitude of this reduction was smaller in CdEWH arteries (Table 2, Fig. 6A-D, a-

Table 2
Effect of EWH on sensitivity (pD2) and maximal effect (Emax) to Phe in aortic rings of rats exposed to of $CdCl_2$ for 14 days.

	pD_2				E_{max} (%)			
	Untreated	Cd	EWH	CdEWH	Untreated	Cd	EWH	CdEWH
Ct	6.6 ± 0.0	6.5 ± 0.2	6.2 ± 0.1	6.4 ± 0.1	84.9 ± 1.5	101.6 ± 2.0#	90.6 ± 1.5	91.7 ± 3.6
E-	7.7 ± 0.2	7.0 ± 0.2#	7.5 ± 0.1	7.0 ± 0.1	129.0 ± 2.5*	119.6 ± 4.4*#	127.8 ± 4.7*	108.0 ± 1.3*
L-NAME	6.7 ± 0.3	6.7 ± 0.1	6.9 ± 0.2	6.9 ± 0.1	130.3 ± 3.7*	126.7 ± 2.2*#	121.8 ± 4.2*	119.5 ± 5.0*
APO	6.4 ± 0.3	6.8 ± 0.2	6.4 ± 0.2	6.1 ± 0.1	54.0 ± 8.1*	41.7 ± 5.7*#	46.2 ± 7.6*	69.1 ± 1,4*&
INDO	6.4 ± 0.2	6.4 ± 0.3	6.0 ± 0.1	6.4 ± 0.2	64.9 ± 4.5*	46.2 ± 6.2*#	77.7 ± 4.7	41.0 ± 4.3 *#
NS398	6.3 ± 0.2	6.5 ± 0.1	6.3 ± 0.1	6.7 ± 0.3	57.8 ± 3.0*	37.1 ± 3.7*#	56.1 ± 4.5*	53.0 ± 3.9*&
LOS	6.4 ± 0.1	6.5 ± 0.1	6.1 ± 0.2	6.6 ± 0.2	69.9 ± 4,5*	40.9 ± 3,7*#	70.6 ± 3,2*	61,9 ± 6.8*&
ML171	6.7 ± 0.1	6.5 ± 0.1	6.7 ± 0.1	5.9 ± 0.1	79.3 ± 3.3	51.8 ± 8.6*#	85.5 ± 8.6*	79.8 ± 1.7*

Parameters of sensitivity (pD_2) and maximal effect (Emax) of the concentration–response curves to Phe in aortas from rats Untreated, treated with Cadmium, Hydrolysate and Hydrolysate plus Cadmium in intact (Ct) and endothelium removal (E-) segments and in the presence of L-NAME (100 μM), Apocynin (APO – 0.3 μM), Indomethacin (INDO – 1 μM), NS398 (1 μM), Losartan (LOS – 10 mM) and ML171 (0.5 μM) incubation. Results are expressed as mean ± SEM. Emax: maximal effect (expressed as a percentage of maximal response induced by 75 mM KCl and pD_2 expressed as a -log one-half Emax. n = 8, *p < 0.05 *vs* corresponding control in each group, # *vs* Untreated group, & *vs* Cd group.

Table 3
Effect of EWH on sensitivity (pD2) and maximal effect (Emax) to NE in MRA of rats exposed to of $CdCl_2$ for 14 days.

	pD_2				E_{max} (%)			
	Untreated	**Cd**	**EWH**	**CdEWH**	**Untreated**	**Cd**	**EWH**	**CdEWH**
Ct	4.60 ± 0.03	4.47 ± 0.11	4.39 ± 0.09	4.40 ± 0.13	96.87 ± 2.17	116.13 ± 4.14$^{\#}$	101.97 ± 2.38	98.21 ± 4.53$^{\&}$
E-	4.80 ± 0.0	4.64 ± 0.10	4.53 ± 0.14	4.61 ± 0.14	112.27 ± 2.11*	113.98 ± 2.32	112.17 ± 2.97*	108.42 ± 3.24*
L-NAME	4.72 ± 0.11	4.43 ± 0.09	4.48 ± 0.07	4.51 ± 0.11	116.12 ± 4.08 *	109.99 ± 4.08	105.65 ± 2.40*	112.35 ± 2.05*
APO	4.55 ± 0.11	4.50 ± 0.13	4.35 ± 0.13	4.50 ± 0.11	92.83 ± 1.85	66.33 ± 5.71$^{*\#}$	92.43 ± 3.30*	84.64 ± 5.33$^{*\&}$
INDO	4.40 ± 0.07	4.41 ± 0.12	4.33 ± 0.08	4.55 ± 0.14	88.79 ± 3.40	55.34 ± 6.78$^{*\#}$	93.80 ± 0.92	64.18 ± 7.80$^{*\&}$
NS398	4.41 ± 0.08	4.63 ± 0.09	4.41 ± 0.13	4.60 ± 0.07	90.91 ± 3.34	55.90 ± 7.51$^{*\#}$	97.80 ± 1.56	76.72 ± 5.21$^{*\&}$
LOS	4.59 ± 0.07	4.58 ± 0.10	4.32 ± 0.09	4.52 ± 0.08	95.16 ± 1.51	82.44 ± 2.73$^{*\#}$	97.24 ± 4.28	80.06 ± 3.44$^{*\&}$
ML 171	4.59 ± 0.07	4.58 ± 0.10	4.11 ± 0.18	4.26 ± 0.07	95.16 ± 1.51	82.44 ± 2.73$^{*\#}$	98.97 ± 4.26	89.03 ± 3.21$^{*\&}$
Ach	7.64 ± 0.13	6.85 ± 0.28$^{\#}$	7.04 ± 0.11	7.11 ± 0.15	102.59 ± 0.45	107.04 ± 1.92$^{\#}$	103.53 ± 0.44	102.62 ± 0.26$^{\&}$
AChAPO	7.37 ± 0.09	7.47 ± 0.29*	7.46 ± 0.10	7.36 ± 0.09	96.05 ± 0.77*	96.10 ± 0.45*	96.54 ± 0.53*	98.53 ± 0.53$^{*\&}$
AChNS398	7.95 ± 0.09	7.80 ± 0.05*	7.55 ± 0.12$^{*\#}$	7.47 ± 0.12$^{\#}$	101.84 ± 0.50	101.92 ± 0.30*	102.45 ± 0.56	102.06 ± 0.54

Parameters of sensitivity (pD_2) and maximal effect (Emax) of the concentration–response curves to NE in MRA from rats Untreated, treated with Cadmium, Hydrolysate and Hydrolysate plus Cadmium in intact (Ct) and endothelium removal (E-) segments and in the presence of L-NAME (100 μM), Apocynin (APO – 0.3 μM), Indomethacin (INDO – 1 μM), NS398 (1 μM), Losartan (LOS – 10 mM) and ML171 (0.5 μM) incubation and concentration–response curve to ACh in presence of Apocynin (APO – 0.3 μM) and NS398 (1 μM). Results are expressed as mean ± SEM. Emax: maximal effect (expressed as a percentage of maximal response induced by 120 mM KCl and pD_2 expressed as a -log one-half Emax. n = 8, *p < 0.05 *vs* corresponding control in each group, $^{\#}$ *vs* Untreated group, $^{\&}$*vs* Cd group.

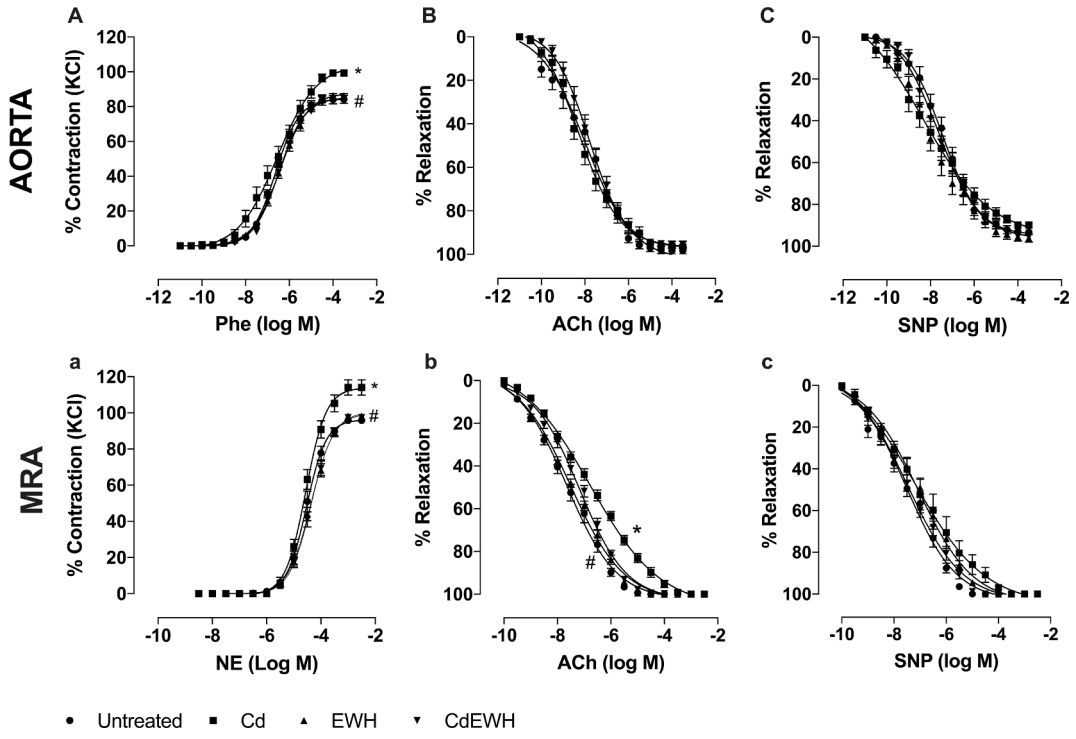

Fig. 1. Effect of EWH in cadmium exposure on vascular reactivity. Concentration response curves to (A) phenylephrine (Phe), (B) acetylcholine (ACh) and (C) sodium nitroprusside (SNP) in aorta segments and to (a) norepinephrine (NE), (b) ACh and (c) SNP in MRA segments. Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l of KCl in aorta or 120 mmol/l in MRA. n = 8, *p < 0.05 *vs* Untreated group and $^{\#}$ *vs* Cd group (Two-Way ANOVA followed by Bonferroni).

d), as show in dAUC graphs (6 E, e). These results suggesting an involvement of RAS in the altered vascular function after Cd exposure and prevented by EWH. Confirming our functional data, the biochemical analysis of plasma ACE activity showed that EWH prevented the increase in ACE activity triggered by exposure to Cd (Fig. 6 F) and the increased vascular expression of AT-1 receptors after Cd exposure (Fig. 8).

4. Discussion

In this study it has been demonstrated that EWH evidenced a clear improvement on these cardiovascular alterations and prevented the increase of the systolic blood pressure and the vascular reactivity dysfunction in conductance and resistance arteries caused by Cd exposure at high level. This effect seems to be related to the capacity of EWH to reduce oxidative stress, inhibit the COX-2 vascular pathway, and reduce AT1 activation at vascular level induced by this metal.

Regarding to the effect of Cd on body weight, we observed that Cd and CdEWH groups showed a weight loss. This effect produced by Cd was previously reported by our group ([bPinheiro et al., 2020]) and in different models of Cd exposure (Liu et al., 2021). Although this effect is still poorly understood, it has been associated with increased levels of lipid peroxidation and dysbiosis in gut microbiota induced by metal toxicity (Gökalp et al., 2009; Sompamit et al., 2010; Kim et al., 2017). In our study, the weight loss observed in groups treated with Cd could be associated to the less food and drink intake and also with a worsening of

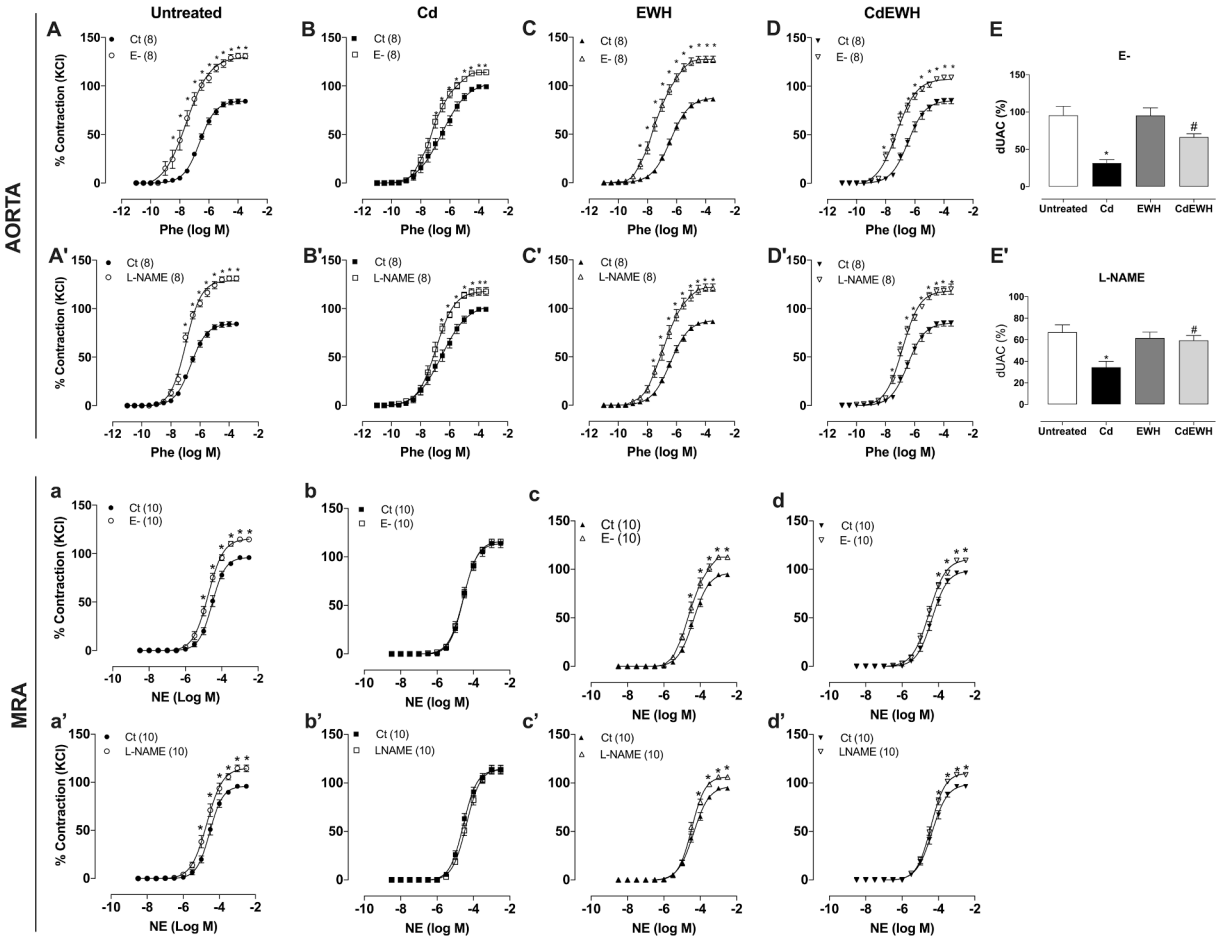

Fig. 2. Effect of EWH in cadmium exposure on endothelium and NO-mediated vascular response. Effect of endothelium removal (E−) (A, B, C and D in aorta and a, b, c and d in MRA) and L-NAME (100 μM) (A′, B′, C′, and D′ in aorta and a′, b′, c′, and d′ in MRA) on the concentration response curve to Phe and NE compared to control curves. The differences in the area under the concentration–response curves (dAUC) in (E) endothelium denuded and LNAME incubate aorta (E′) in all experimental groups. Data are expressed as mean ± SEM as a percentage of the response to 75 mmol/l of KCl in aorta or 120 mmol/l in MRA. Number of animals are expressed in parenthesis in each graph. *p < 0.05 *vs* control curve (Two-Way ANOVA followed by Bonferroni) *p < 0.05 *vs* Untreated, # *vs* Cd in dAUC graphs.

the healths conditions of the animals related to Cd exposure. This situation seems to be less serious in the case of group Cd-EWH, suggesting that the intake of EWH could led an improvement in general health conditions associated to Cd toxicity.

Cd exposure at high and low concentrations are related to the development of hypertension (Almenara et al., 2013; Subramanyam et al., 1992; Lall et al., 1997; Choudhary and Bodakhe, 2016), and the continuous human exposure its associated to changes in cardiovascular health markers playing a crucial role in cardiac pathologies (Obeng-Gyasi, 2020). In fact, it has been known that hypertensive patients have higher concentration of Cd in urine and the population of contaminated Cd areas shows higher incidence of atherosclerosis (Houtman, 1993; Madrigal et al., 2019). In this study an increase in systolic blood pressure was observed after Cd exposure, and that induced hypertension was completely prevented by EWH co-treatment. The antihypertensive properties of EWH and some of peptides included in it have been already reported in other experimental models such as in spontaneously hypertensive rats (Miguel et al., 2006; Miguel et al., 2005; Miguel et al., 2007), and in animals exposed to other metals such as mercury (Rizzetti et al., 2017; Escobar et al., 2020) and aluminum (Martinez et al., 2019). All this information allows us to consider EWH as antihypertensive natural agent.

Several mechanisms have been suggested for Cd-induced hypertension, especially increased oxidative stress by superoxide anion, mediated by NADPH oxidase and RAS activation. Furthermore, the increase in ROS may favor endothelial dysfunction by increasing vascular resistance with reduced NO bioavailability and vasodilatory capacity, seems to play an important role in the development of hypertension. Similar findings have been reported in different models of exposure to the Cd (Martins et al., 2021; Pinheiro Júnior et al., 2020; Cannon, 1998; Triggle et al., 2003; Kolluru et al., 2006; Kolluru et al., 2010).

The antihypertensive effect exerted by EWH has been explained by different mechanisms. Among them the angiotensin converting enzyme (ACE) inhibitory activity seems to be closely related to the antihypertensive action produced by EWH (Miguel et al., 2007; Miguel et al., 2004). The antioxidant and vasoprotective effect attributed to this food ingredient and their peptides have been also demonstrated in hypertensive rats (Manso et al., 2008; Miguel et al., 2007). In rats exposed to Hg or Al exposure the EWH administration was strongly related to their antioxidant and chelating power (Rizzetti et al., 2017; Martinez et al., 2019).

Reactive species such as O_2^-, $ONOO^-$, OH^- are the most unstable and reactive (Taniyama and Griendling, 2003). Their formation in vascular tissues is related to the activity of some enzymes, especially NADPH oxidase, xanthine oxidase and eNOS. In this study we observed that Cd increased the functional and biochemical activity of NADPH oxidase, specifically from the NOX1 subunit in the arteries of animals in the Cd group. However, in the CdEWH group we observed a reduction of ROS derived from that subunit preventing the increment of the contractile response in MRA and aorta and the reduction of the vasodilator response in MRA.

It has been also reported that the increased local activity of the RAS

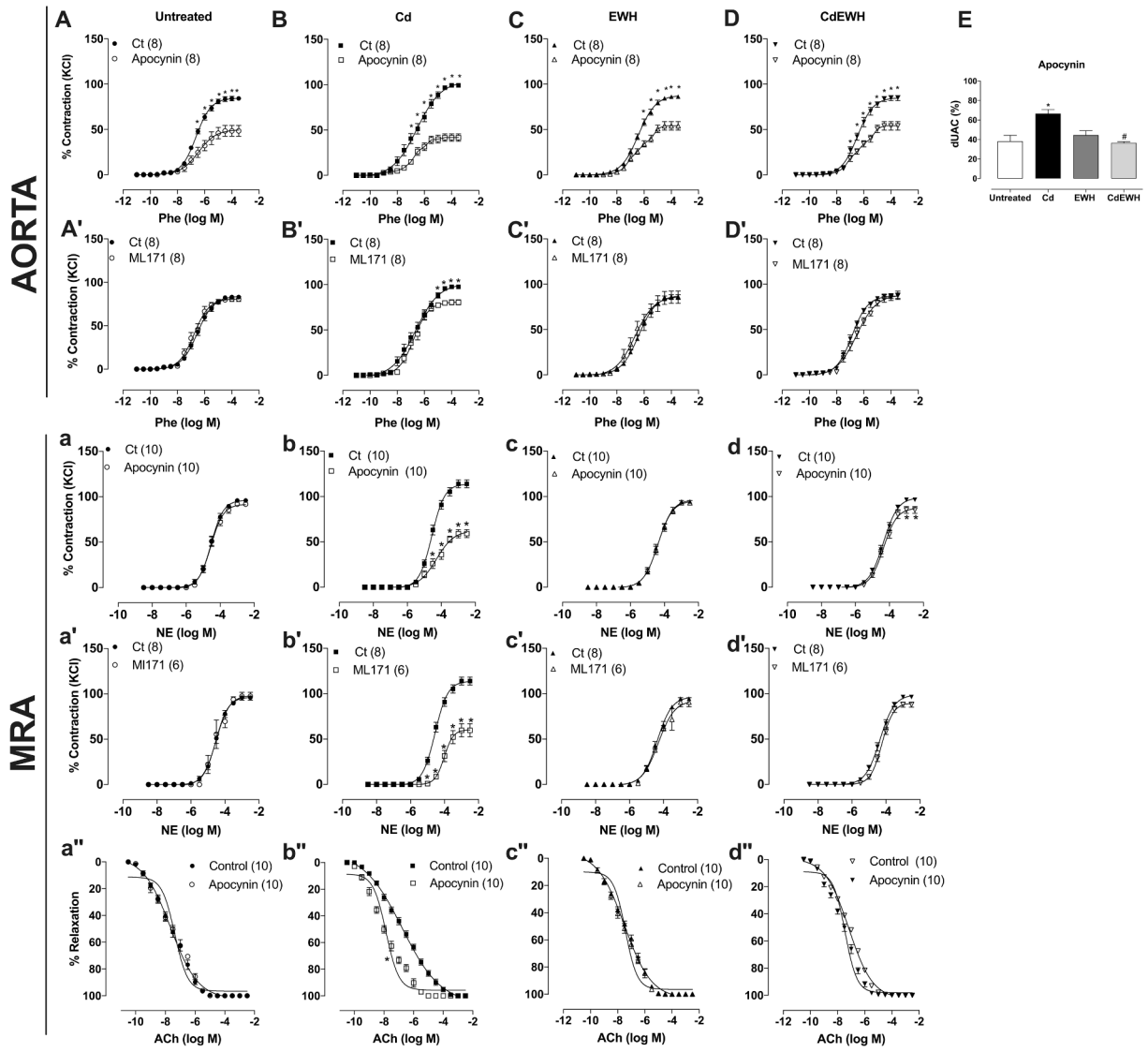

Fig. 3. Effect of EWH in cadmium exposure on NAD(P)H oxidase inhibitor, Apocynin (0.3 μM) (A in aorta and a in MRA) and NOX 1 inhibitors, ML171 (0.5 μM) (A′ in aorta and a′ in MRA) on the concentration response curves to Phe and NE compared to control curves. The differences in the area under the concentration–response curves (dAUC) in (E) the presence of apocynin in aorta. Data are expressed as mean ± SEM as a percentage of the response to KCl 75 mmol/l in aorta or 120 mmol/l in MRA. The relaxation response on the concentration response curves to ACh was verified in the presence of apocynin in MRA (a″- d″). Number of animals are expressed in parenthesis in each graph. *p < 0.05 *vs* control curve (Two-Way ANOVA followed by Bonferroni) *p < 0.05 *vs* Untreated, # *vs* Cd in dAUC graph.

and the production of vasoconstrictor prostanoids promote an increase on ROS levels in arteries of normotensive and SHR rats (Alvarez et al., 2007). Our functional and biochemical findings also showed that the disturbances presented by Cd exposure were associated with the RAS by increased ACE activity in the resistance and conductance vessels exposed to Cd. Therefore, it could generate more angiotensin II and increase the vascular reactivity to Phe. It is also known that angiotensin II regulates the production of prostanoids from COX-2 and the expression of this protein, as well as the activation of NADPH oxidase (Griendling et al., 2000; Ohnaka et al., 2000; Weseler and Bast, 2010). In our study Cd exposure also increased participation of vasoconstrictor prostanoids and COX-2 expression. Using a low-exposure Cd model Angeli et al. (Angeli et al., 2013) (Angeli et al., 2013) observed that Cd induces an increase of local release of angiotensin II and COX-2 and also an increase of the activity of NADPH oxidase and postulated that this may be the mechanism for ROS generation and consequently the deleterious effects of the metal.

The biomarkers analyzed in plasma, kidney and liver further showed that Cd increased ROS level and reduced antioxidant defenses and bioaccumulated in target-organ as previously demonstrated in other models of high exposure in rodents (El-Maraghy et al., 2001; Lovásová et al., 2013; Almeer et al., 2019) and low metal (Jurczuk et al., 2004; Alghasham et al., 2013). Some nutritional options such as apple juice, herbal medicines and plant extracts have already demonstrated the ability to reduce oxidative stress and increase antioxidant defenses, especially in the kidney (Yan and Allen, 2021).

Cd exerts time and dose dependent nephro and hepatotoxic effects (Bhattacharyya et al., 2021; Kara et al., 2005) and both tissues, are target organ where Cd bioaccumulates, and this situation was also observed in our study, showing high level of Cd in blood, kidney and liver of Cd treated group. Due to its long biological half-life of 15 to 30 years, its toxicity is long-lasting, leading to an increase in ROS (Ishizaki et al., 2015) and reduced bioavailability of calcium, iron, and zinc by impairing the competitive action by homeostasis (Nair et al., 2013; Nemmiche, 2016). Although we have not investigated in this study, it is known that Cd damages the nephron structure, impairing glomerular filtration, increasing the excretion of proteins, amino acids, and electrolytes (Prozialeck et al., 2007) promoting vascular dysfunction and

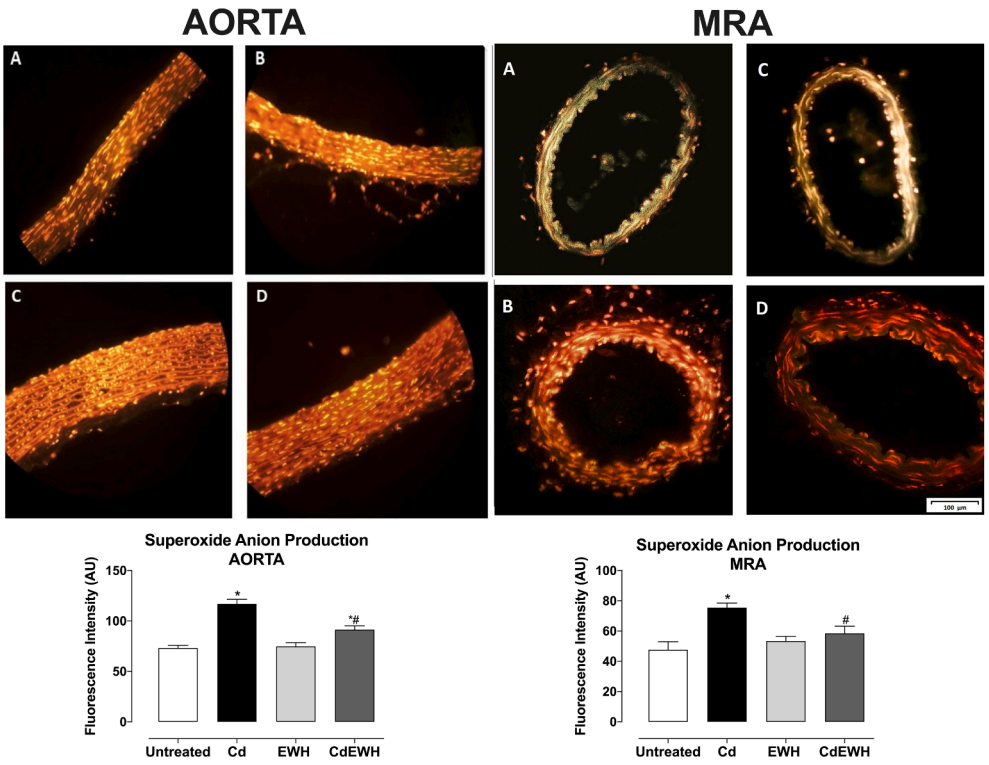

Fig. 4. Effects of EWH on local anion superoxide production in aorta and MRA, in both vessels we show untreated (A), Cd (B), EWH (C) and Cd plus EWH (D) groups. Representative images from aorta and MRA exposure to DHE x20 objective, zoom 4x, n = 8, * $p < 0.05$ *vs* untreated; # *vs* Cd.

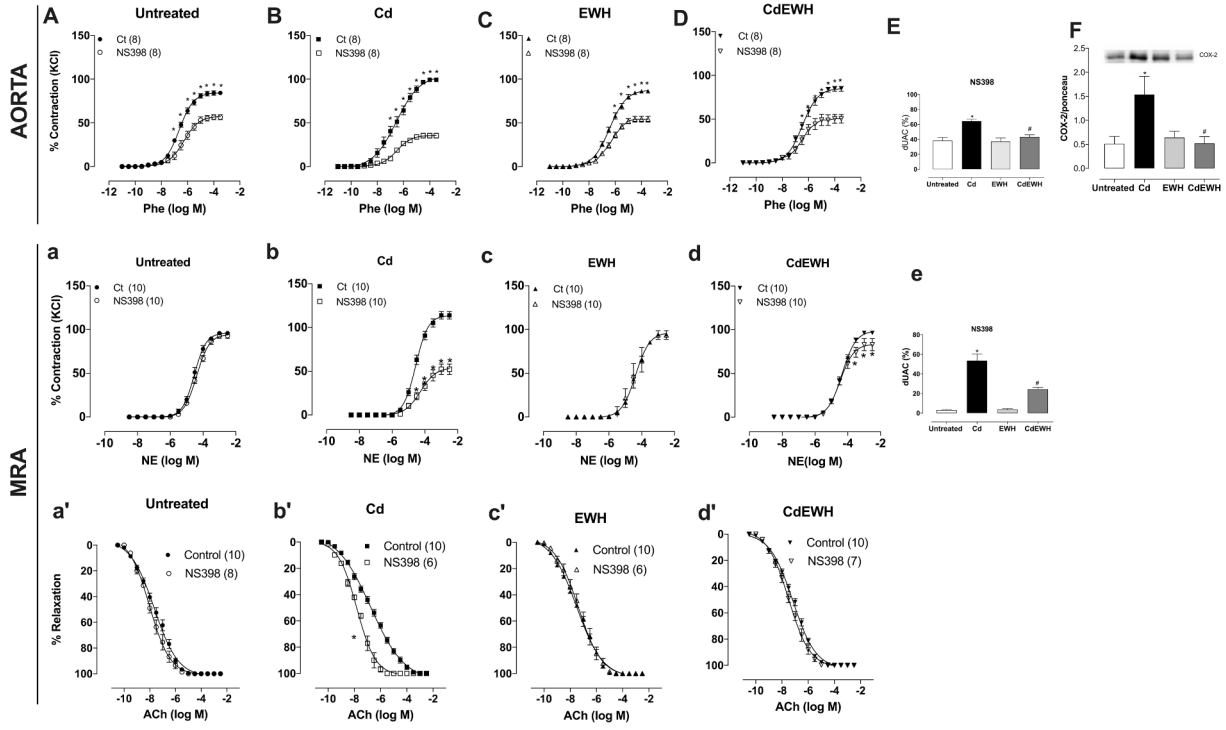

Fig. 5. Effect of the selective COX-2 inhibitor NS398 (1 μM) on the concentration response curves to Phe and NE compared to control curves (A - E aorta and a - e MRA). The differences in the area under the concentration–response curves (dAUC) in (E and e) the presence of NS398 in aorta and MRA respectively. Data are expressed as mean ± SEM as a percentage of the response to KCl 75 mmol/l in aorta or 120 mmol/l in MRA. In a-d′ are represented the relaxation response on the concentration response curves to ACh in the presence of NS398 in MRA (a′- d′). Number of animals are expressed in parenthesis in each graph. *$p < 0.05$ *vs* control curve (Two-Way ANOVA followed by Bonferroni) *$p < 0.05$ *vs* Untreated, # *vs* Cd in dAUC graph.

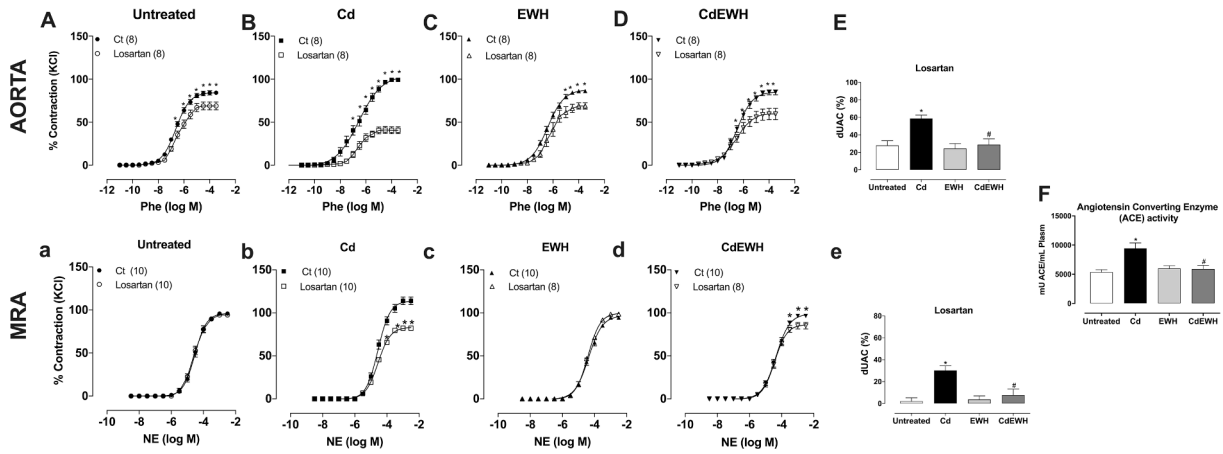

Fig. 6. Effect of EWH on participation of local renin-angiotensin system in vasoconstrictor responses to Phe in aorta and MRA of rats exposed to cadmium exposure. Concentration-responses curve to Phe in the absence (Ct) and the presence of the AT-1 receptors blocker (Losartan 10 mM) in aorta (A-E) and MRA (a-e) segments of all groups. The differences in the area under the concentration–response curves (dAUC) in the presence and absence of Losartan (E in aorta and e in MRA). Activity the angiotensin-converting enzyme in plasma also was demonstrated in (F). Data are expressed as mean ± SEM as a percentage of the response to KCl 75 mmol/l in aorta or 120 mmol/l in MRA. Number of animals are expressed in parenthesis in each graph. *p < 0.05 *vs* control curve (Two-Way ANOVA followed by Bonferroni) **vs* Untreated, # *vs* Cd in dAUC graph.

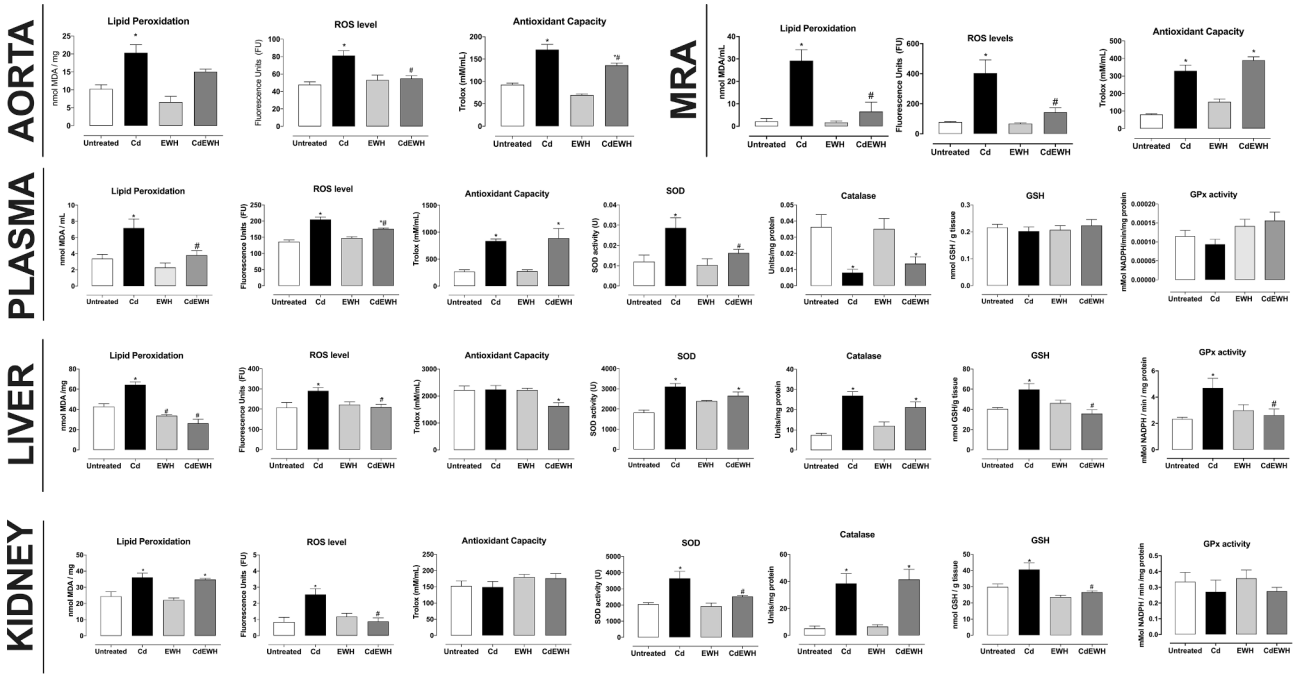

Fig. 7. Effects of EWH treatment on lipid peroxidation, reactive species, and total antioxidant capacity in aorta, MRA, plasma, liver and kidney of rats of all groups. SOD, Catalase, GSH and GPx activity in plasma, liver and kidney. Data are expressed as mean ± SEM, n = 8, *p < 0.05 *vs* Untreated group, # *vs* Cd group.

hypertension.

However, in our study co-administration of EWH showed a reduction in Cd levels in blood and kidney and this could be related to an improvement in oxidative stress biomarkers. The increased Cd levels found in liver may be a consequence of high detoxifying action to remove this heavy metal of the body. In this context, it is important to highlight the chelating properties of EWH since a greater elimination in the feces of this metal was observed and this could be related to the reduced levels of Cd in the blood and kidney founded in EWH-Cd group. Egg white is a food matrix composed to a large extent by proteins that contain thiol groups (-SH) (Abeyrathne et al., 2013). Cd has high affinity for -SH and this affinity makes possible its association with other molecules such as albumin, cysteine, and glutathione (Klaassen et al., 2009). Therefore, the peptides contained in the EWH could act by competing to Cd reducing the absorption of this metal and improving its natural excretion routes as found in our results. In this sense, our study presents a nutritional option which was able to reduce the bioaccumulation of metal in blood and tissues. This ability to remove Cd was also observed in a previous study of our research group in male reproductive organs (Pinheiro Júnior et al., 2020).

In summary, the results obtained in this study reinforce the contribution of EWH as cardiovascular protective agent, since it was able to prevent the increase in blood pressure and the vascular damage induced by Cd. This effect seems to be related to the capacity of EWH to reduce oxidative stress and increase antioxidant defenses, inhibit the COX-2 vascular pathway, and reduce AT1 activation at vascular level induced by this metal. EWH seems to have important antihypertensive, anti-inflammatory, chelating and antioxidant natural properties to improve

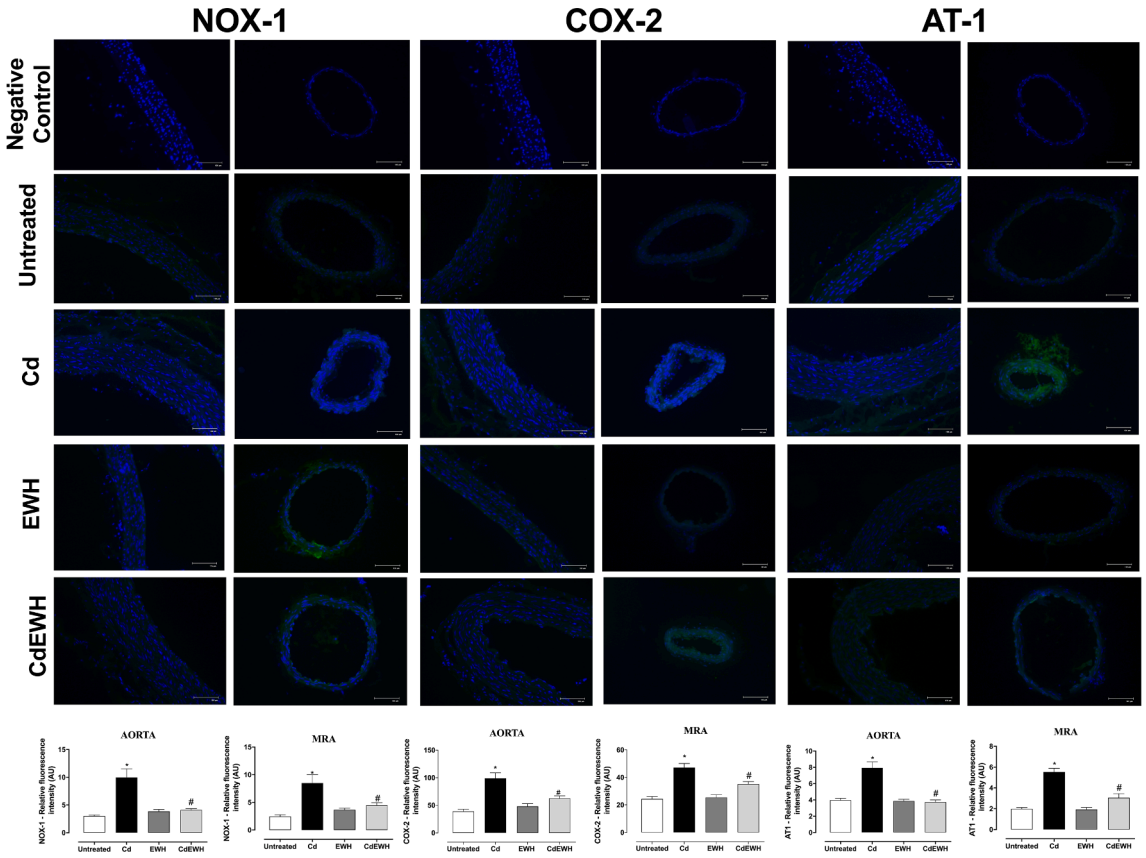

Fig. 8. Representative photomicrographs (×40 magnification) of data of NOX-1, COX-2 and AT-1 immunofluorescence of aortic and MRA sections of rat's exposure to Cd and cotreated to EWH. The results are expressed mean ± SEM; *p < 0.05 *vs* Untreated group, #p < 0.05 *vs* Cd group.

toxicity associated to Cd exposure and cardiovascular related complications. This functional food ingredient could be considered as an alternative therapy to counteract the deleterious effects of Cd exposure at high level of exposure.

Ethics information

We declare that all the procedures followed in this study were in accordance with the ethical standards of the Ethics Committee of the applicant's research institution (approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil - protocol 017/2018) and in accordance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology.

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CRediT authorship contribution statement

Paola Zambelli Moraes: Investigation, Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **José Eudes Gomes Pinheiro Júnior:** Investigation, Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Caroline Silveira Martinez:** Investigation, Conceptualization, Methodology, Formal analysis, Validation, Writing – original draft, Writing – review & editing. **Camila Rodrigues Moro:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Grazielly Caroline da Silva:** Investigation. **Marina Diaz Rodriguez:** Investigation. **Maylla Ronacher Simões:** Investigation, Writing – review & editing. **Fernando Barbosa Junior:** Investigation, Resources, Writing – review & editing. **Franck Maciel Peçanha:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. **Dalton Valentim Vassallo:** Conceptualization, Methodology, Resources, Funding acquisition, Writing – original draft, Writing – review & editing. **Marta Miguel:** Conceptualization, Methodology, Validation, Resources, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. **Giulia Alessandra Wiggers:** Conceptualization, Methodology, Formal analysis, Validation, Visualization, Data curation, Supervision, Project administration, Resources, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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