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Egg white hydrolysate prevents reproductive impairments induced by cadmium in rats

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ABSTRACT

Here, we investigated the ability of an egg white hydrolysate (EWH) to counteract the reproductive toxic effects induced by Cd in rats. 3-month-old male Wistar rats were treated for 14 days: (a) Untreated group (distilled water i.p.); (b) Cd group ($CdCl_2$ at 1 mg/kg body weight i.p.); (c) EWH group (1 g/kg body weight/day per gavage); (d) CdEWH group ($CdCl_2$ with EWH). Cd exposure was able to be accumulated in testis and epididymis, increasing oxidative stress and compromising sperm quality and daily sperm production. Co-treatment with EWH prevented increased deposition of Cd in testis (Untreated: 0.04 ± 0.02; Cd: 4.68 ± 1.03*; EWH: 0.01 ± 0.00; CdEWH: 1.85 ± 0.25$^{\#}$ μg Cd/g dry tissue $p < 0.05$; *vs Untreated; $^{\#}$vs Cd), prevented reduction of sperm motility and the increased oxidative stress. Therefore, EWH could represent a powerful natural alternative to protect the male reproductive system against Cd-induced toxicity.

1. Introduction

Humans are constantly exposed to numerous harmful substances, such as pesticides (Gasnier et al., 2009), metals (Tchounwou, Yedjou, Patlolla, & Sutton, 2012), organic solvents and propellants (Huijbregts, Rombouts, Ragas, & Van De Meent, 2005) through different routes of exposure (Mehlman, 1991). Cadmium (Cd) is a contaminant strongly present in human daily life mainly by exposure to cigarette smoke (Scherer & Barkemeyer, 1983; Wu et al., 2016). However, this metal represents one of the most harmful environmental contaminants including for non-smokers due to the increasing human exposure related to contaminated food (Wu et al., 2016), and water (Fakhri et al., 2015), and environmental accidents, as occurred in Brazil in 2015 (Fernandes et al., 2016). Therefore, Cd has been associated with several human health disorders (Kumar & Sharma, 2019), such as increased incidence of cancer, related to non-occupational exposure (Cho, Kim, Woo, & Kang, 2013; Julin, Wolk, Bergkvist, Bottai, & Åkesson, 2012) or air pollution (White, O'Brien, Niehoff, Carroll, & Sandler, 2019), neurodegenerative (Branca, Morucci, & Pacini, 2018), kidney and cardiovascular diseases (Eum, Lee, & Paek, 2008). Particularly, Cd has high a affinity to the reproductive system (Siu, Mruk, Porto, & Cheng, 2009), and is related to infertility (Benoff, Jacob, & Hurley, 2000), embryonic developmental impairments (Zhao et al., 2017), reduced spermatogenesis (Yari et al., 2010), sperm quality (Adamkovicova et al., 2016), promotion of hormonal imbalance (Boujelben, Abdennabi, Guermazi, & Elfeki, 2018), testicular dysfunction (Siu et al., 2009), blood-testis barrier disruption (de Angelis et al., 2017), impaired oocyte maturation (Cheng et al., 2019) and, increased incidence of prostate cancer (Waalkes & Rehm, 1994). The toxic effects of Cd in the reproductive system seem to be related to increased oxidative stress, DNA damage,

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inflammation and apoptosis (Badisa et al., 2006; de Angelis et al., 2017).

Given the increased human exposure to toxic agents, the study of therapeutic substances with antioxidant or chelating properties is required (Amadi, Offor, Frazzoli, & Orisakwe, 2019). In this context, functional food seems to be a promising natural strategy against current human toxicity concerns. Among food ingredients, egg white is considered a useful and low-cost source of protein (egg white composition: 88–90% water and 10–12% protein) and bioactive peptides which can be released after enzymatic hydrolysis (Miguel & Aleixandre, 2006). The hydrolysis of proteins may result in the production of different biologically-active peptide sequences, including peptides with either or both antioxidant and anti-inflammatory properties, and these peptides could improve the toxic effects produced by metals in the organism. Although the literature describes bioactive peptides from different structures of the egg, the hydrolysis process has commonly been performed using egg white (Davalos, Miguel, Bartolomé, & Lopez-Fadiño, 2004; Garcés-Rimón et al., 2016; Manso et al., 2008).

Our research group obtained egg-white hydrolysate (EWH) by enzymatic hydrolysis with pepsin for 8 h. Thus hydrolysate seems to have several biological activities that could be used to improve human cardiometabolic diseases (De Campos Zani, Wu, & Chan, 2018) and, to prevent or treat adverse effects induced by human exposure to contaminants (Mine & D'Silva, 2008). *In vitro* and *in vivo* studies suggest that EWH has potent antioxidant (Davalos et al., 2004), antihypertensive (Miguel, López-Fandiño, Ramos, Aleixandre, 2005, 2006; Miguel et al., 2007), hypocholesterolaemic (Garcés-Rimón et al., 2016; Moreno-Fernández et al., 2018) and anti-inflammatory (Garcés-Rimón et al., 2016) properties. The dietary ingestion of EWH seems to improve the cardiometabolic profile, reducing blood pressure and adjusting lipid metabolism in experimental models of hypertension and obesity (Garcés-Rimón et al., 2016; Moreno-Fernández et al., 2018). Recently, EWH was shown to counteract metal-induced systemic toxic effects by controlling the increased oxidative stress and inflammation caused by long-term exposure to contaminants (Martinez et al., 2019; Rizzetti et al., 2016; Rizzetti et al., 2017). The EWH was previously characterised by RP-HPLC (degree of hydrolysis = 63% based on hydrolysis of ovalbumin) and HPLC-MS/MS. Several peptide sequences were identified (FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, FIV, YQIGL, SALAM) of which some demonstrated different biological properties *in vitro* and *in vivo* (Miguel et al., 2005, 2006). Herein, we have evaluated the efficacy of EWH to protect against the reproductive impairments induced by Cd exposure in rats.

2. Methods

Three-month-old male Wistar rats (375.8 ± 6.3 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at constant room temperature, humidity and light cycle (12:12 h light–dark), with *ad libitum* access to water and standard chow. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology. Approval for the study was granted by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 013/2019).

2.1. EWH preparation

EWH was prepared by pepsin hydrolysis of crude egg white as previously described (Garcés-Rimón et al., 2016). EWH was prepared by pepsin hydrolysis of crude egg white, as previously described. The commercial pasteurised egg white (Guillén, Spain) was hydrolysed with pork stomach 1:3000 (E.C. 3.4.23.1; from pork stomach, enzyme:substrate ratio of 2:100, w/w), purchased from Biocatalysts (Cardiff, UK). Egg white was acidified to pH 2.0 with HCl 37% (Panreac Quimica S.L.U., Spain). Hydrolysis was performed in a thermostatic water bath for 8 h, with constant agitation. Enzyme inactivation was achieved by increasing the pH to 7.0 5 N NaOH. The hydrolysate was centrifuged at 4500*g* for 15 min, and the supernatants were frozen and lyophilised. For treatment, EWH was resuspended in water and administered by gavage. The EWH resulting from pepsin hydrolysis has HPLC-MS/MS identified bioactive peptide sequences (FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, FIV, YQIGL, SALAM) (Miguel et al., 2005, 2006).

2.2. Experimental design

Rats were divided into four groups and treated for 14 days (N = 10 per group): (a) Untreated group - intraperitoneal (i.p.) injections of distilled water; (b) Cd group - $CdCl_2$.2.5 H_2O at 1 mg/kg body weight i.p., according to Balaraman, Gulati, Bhatt, Rathod, and Hemavathi (Balaraman, Gulati, Bhatt, Rathod, & Hemavathi, 1989); (c) EWH group − 1 g EWH/kg body weight/day administered by gavage, according to Rizzetti et al. (2017); (d) CdEWH group - both treatments ($CdCl_2$ and EWH). The animals received the same type of feed (Presence, Brazil) that has the following composition: 10.2% protein; 62.6% carbohydrate; 7.3% lipid; 1% vitamins; 3.6% mineral salts; 5% fibre, energy content of 3976 kcal/kg feed). The Cd dose represents 1/7 to 1/11 of the LD_{50} for 6–18-week-old male rats (Kostial, Kello, & Blanusa, 1979) and 14 days of treatment of a rat corresponds to 3.9 months of treatment in the life of a human (Sengupta, 2013). Body weight, water and feed consumption were monitored weekly.

At the end of the treatments, animals were anaesthetised and then euthanised by decapitation. The weights of the testis, epididymis, ventral prostate, vas deferens and seminal vesicle (empty, without coagulation gland) were registered. The right testis, epididymis and left vas deferens were used for sperm analysis, while the left testis and epididymis were used for histological and biochemical studies. For biochemical determination, tissues were quickly homogenised in 50 mM Tris–HCl, pH 7.4 (5/10, w/v). Afterwards, samples were centrifuged at 2400 *g*, 4 °C for 10 min and the resulting supernatant fraction was frozen at −80 °C for further assay.

2.3. Cadmium content in tissue

Cd was determined in testis and epididymis according to Batista, Grotto, Rodrigues, de Oliveira Souza, and Barbosa (Batista, Grotto, Rodrigues, de Oliveira Souza, & Barbosa, 2009) (Batista et al., 2009). Briefly, samples were accurately weighted into a 15 mL propylene Falcon® tubes (Becton Dickinson). Then, 1 mL of 50% (v/v) tetramethylammonium hydroxide solution was added to the samples, incubated at room temperature for 12 h in a rotational homogenization. After solubilization the volume was made up to 10 mL with a dilute solution containing 0.5% (v/v) HNO_3, 0.01% (v/v) Triton® X-100 and 10 µg / L Rh. Analytical calibration standards were prepared daily over the range of 0–100 µg / L Cd in a diluent containing 5% (v/v) TMAH, 0.5% (v/v) HNO_3, 0.01% (v/v) Triton® X-100 and 10 µg / L Rh. Analyses were carried out with an inductively coupled plasma mass spectrometer (ICP-MS) (NexION 2000, PerkinElmer, Norwalk, CT, USA) operating with high-purity argon (99.99%, Praxair, Brazil). Sample introduction system was composed by cyclonic spray chamber and a Meinhard® nebulizer connected by Tygon® tubes to the ICP-MS's peristaltic pump. In order to verify the accuracy of data, the certified reference materials, bovine liver SRM 1577b, bovine muscle powder SRM 8414 and whole egg powder SRM 8415, provided by National Institute of Standards and Technology (NIST) were analysed before and after 5 ordinary samples. Results were in good agreement with the certified values *(t test, 95%)*.

2.4. Sperm analysis

2.4.1. Sperm motility

Sperm motility was analysed according to Martinez et al. (2014). The sperm was removed from vas deferens and mixed with 1 mL of Human Tubular Fluid (DMPBS, Nutricell, São Paulo, Brazil) pre-heated to 34 °C heated plate (37 °C), An aliquot of 10 μL was transferred to a slide and was analysed under optical microscopy, with an objetive of 400× (Binocular, Olympus CX31, Tokyo, Japan). Sperm samples were analysed and classified according to their motility: motile with progressive movement, motile without progressive movement and immotile, as described by Perobelli et al. (2010).

2.4.2. Sperm morphology

Sperm were obtained by vas deferens using 1 mL of human tubular fluid (DMPBS-Nutricell-SP-Brazil). 100 μL of the sample were added to 900 μL of 10% formal-saline and, for analysis, histological slides were prepared and, 200 sperm per animal were evaluated by optical microscope under 400× magnification (Binocular, Olympus CX31). Morphological abnormalities were classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail), according to Filler (2013)).

2.4.3. Membrane integrity test

Sperm were removed from the right vas deferens by injection of 1 mL of human tubular fluid (Dulbecco Modified DMPBS Flush; Nutricell) preheated to 34 °C. The membrane functional integrity was determined by the hypo-osmotic test (HOS), as described by Lomeo and Giambersio (1991). This assay was performed by mixing sperm with hypo-osmotic solution (1: 5) and incubating the mixture at 37 °C for 45 min. 100 cells were analysed by animal by optical microscope under 400× magnification. Tails without changes or straight tails were considered as positive for membrane damages.

2.4.4. Daily sperm production per testis, sperm number and transit time in epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were counted as described by Robb, Amann, and Killian (1978). To calculate daily sperm production, the number of spermatids at stage 19 was divided by 6.1, which is the number of days these spermatids are present in the seminiferous epithelium. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the daily sperm production.

2.4.5. Testis and epididymis histology

After weighing, epididymis tissues were fixed in 10% formaldehyde and testis in Bouin's solution for 1–2 days. Thus, tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin/eosin. Tissues were studied under a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany). The analysis was made in 10 random fields measured in 20× or 40× magnifications per section.

2.5. Biochemical Analyses in testis and epididymis

2.5.1. Lipid peroxidation

Lipid peroxidation was analysed by malondialdehyde (MDA) levels using colorimetric method, as previously described by Ohkawa, Ohishi, and Yagi (1979). The tissues were incubated with thiobarbituric acid (0.8% TBA), phosphoric acid (H_3PO_4) and sodium dodecyl sulfate (0.8% SDS) for 60 min and the results were expressed as nanomoles of MDA per mg of protein. The protein quantification was determined using the Bradford (1976) method (Kruger, 1994).

2.5.2. The total antioxidant capacity

The total antioxidant capacity were measured by FRAP assay (iron-reducing antioxidant power) described by Benzie and Strain (1996), with modifications (Martinez et al., 2017). Absorbance was read at 532 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50–1000 μM - water-soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are expressed in mMol/L.

2.5.3. Reactive oxygen species (ROS) levels

Reactive oxygen species (ROS) levels were determined by the spectrofluorometric method described by Loetchutinat, Kothan, and Dechsupa (2005), with some modifications (Martinez et al., 2017). The ROS levels were expressed as fluorescence units. The sample was diluted (1:10) in 50 mM TrisHCl (pH 7.4) and 2.7-dichlorofluorescin diacetate (DCHF-DA, 1 mM) was added. The emission of the DCF fluorescence intensity was recorded for the detection of intracellular ROS at 520 nm (480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The results are expressed in units of fluorescence.

2.5.4. Reduced glutathione level (GSH) level

Reduced Glutathione Level (GSH) level were determined by the spectrofluorometric method described by Armstrong, Browne, and Armstrong (1998). Briefly, samples were homogenized in perchloric acid (0.1 M) and centrifuged at 3000 rpm. Subsequently, 100 μL o-phthalaldehyde (1 mg/mL in methanol) and 800 μL of phosphate buffer (0.1 M, pH 8.0) were added to the supernatant in the dark and incubated for 15 min. Next, 200 μL of the mixture was placed in an opaque 96-well plate and the fluorescence was read, the emission set at 420 nm and the excitation set at 350 nm (SpectraMax M5 Molecular Devices, CA, USA). The results are expressed in nmol GSH/g tissue.

2.5.5. Catalase activity

Catalase Activity was performed according Aebi (1984). Samples were added to the phosphate buffer (50 mM, pH 7.0) and H_2O_2 (0.3 M), the decrease in absorbance at 240 nm due to the decrease in H_2O_2 extinction was referred to the catalase activity was recorded for 3 min. Results were expressed in units per milligram of protein (U/mg of protein). One unit (U) was defined as 1 pmol of H_2O_2 degraded per minute.

2.5.6. Superoxide dismutase activity

Superoxide Dismutase Activity was measured according to a technique described by Misra and Fridovich (1972). The sample was added to carbonate buffer (0.05 M Na $_2CO_3$, pH 10.2) and then adrenaline (60 mM) in the dark. The unit of SOD inhibits 50% self-oxidation of adrenaline. The increase in absorbance at 480 nm was monitored every 30 s for 120 s at 37 °C and calculated the Δ minute. The results were expressed as units per milligram of protein (U/mg protein).

2.5.7. Glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was determined according to Paglia and Valentine (1967) using reaction medium (phosphate buffer 0.1 M, pH 7.6; NADPH 0.1 mM; sodium azide 4.28 mM and glutathione reductase 2 U/mL). The absorbance was measured every 30 s for 2 min at 340 nm after the addition of 0.72 mM H_2O_2. The GPx activity was expressed as mM/ min/mg protein.

2.6. Data analysis and statistics

Data are expressed as mean ± SEM. Differences were analysed using One-Way ANOVA followed by post hoc Bonferroni multiple comparison test for parametric data and *Kruskal-Wallis* test for non-parametric data. Values of $P < 0.05$ were regarded as being significantly different.

Table 1
EWH effects on body weight (g), feed intake (g), water consumption (mL), absolute (g or mg) and relative (g/100 g or mg/100 g) weights of reproductive organs after Cd-exposure.

Parameters	Experimental groups			
	Untreated	Cd	EWH	CdEWH
Initial body weight (g)	356.4 ± 10.3	374.5 ± 12.4	382.6 ± 9.12	373.8 ± 14.7
Final body weight (g)	409.5 ± 13.5	338.2 ± 16.0*	396.0 ± 14.1	348.0 ± 17.6*
Feed intake (g)	30.1 ± 1.9	8.1 ± 1.8*	24.9 ± 5.9	15.3 ± 2.1*
Water Consumption (mL)	66.1 ± 6.1	29.6 ± 5.9*	78.6 ± 5.2	36.6 ± 3.2*
Testis (g)	1.9 ± 0.0	1.1 ± 0.1*	1.9 ± 0.5	1.2 ± 0.2*
Testis (g/100 g)	0.4 ± 0.0	0.2 ± 0.0*	0.4 ± 0.0	0.3 ± 0.05*
Epididymis (mg)	651.2 ± 25.1	515.3 ± 40.0*	668.1 ± 25.6	549.9 ± 44.6
Epididymis (mg/100 g)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Ventral prostate (mg)	436.1 ± 70.0	350.4 ± 47.2	582.9 ± 80.3	247.4 ± 34.2
Ventral prostate (mg/100 g)	95.9 ± 16.9	87.7 ± 11.8	111.0 ± 17.8	63.6 ± 8.8
Full seminal vesicle (g)	1.4 ± 0.0	1.0 ± 0.1*	1.3 ± 0.0	1.3 ± 0.1
Full seminal vesicle (g/100 g)	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Empty seminal vesicle (g)	0.7 ± 0.0	0.5 ± 0.0*	0.7 ± 0.0	0.7 ± 0.0#
Empty seminal vesicle (g/100 g)	0.1 ± 0.0	0.0 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0#
Vas deferens (mg)	0.1 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Vas deferens (mg/g)	33.9 ± 0.0	76.0 ± 0.0	32.2 ± 0.0	30.9 ± 0.0

Data are expressed as means ± SEM. The relative organ weight is expressed in organ weight/body weight × 100. Units: g: gram, mg: milligram; One-way ANOVA, *P < 0.05 vs Untreated, # vs Cd (N = 10 per group).

3. Results

3.1. Body weight, water and feed consumption and Cd content

Body weight, feed intake and water consumption of all animals receiving Cd were markedly reduced at the end of treatment (Table 1).

Rats exposed to Cd showed a high presence of the metal in the testis and epididymis. However, the co-treatment with EWH was able to reduce Cd deposition in testis (Untreated: 0.04 ± 0.02; Cd: 4.68 ± 1.03*; EWH: 0.01 ± 0.00; CdEWH: 1.85 ± 0.25$^{\#}$ μg Cd/g/ dry tissue, where *vs Untreated, $^{\#}$vs Cd) but not in the epididymis (Untreated: 0.00 ± 0.00; Cd: 6.99 ± 1.29*; EWH: 0.00 ± 0.00; CdEWH: 3.61 ± 1.29* μg Cd/g dry tissue).

Cd exposure reduced the weight of the seminal vesicle (empty and full), testis and epididymis. EWH co-administration prevented the weight reduction of the empty seminal vesicle without preventing other weight losses (Table 1).

3.2. Sperm quality and histopathology

Cd exposure for 14 days induced a marked reduction in daily sperm production and sperm count in the testis and epididymis, as well as increased the sperm transit time in the epididymis (caput/body and tail). EWH co-treatment prevented the increased sperm transit time but did not prevent the reduction in sperm count and daily sperm production (Table 2). Cd strongly impaired sperm motility, decreasing the number of sperm with progressive movement and increasing the number of immotile sperm (Fig. 1). In contrast, the administration of EWH prevented the motility impairments found after Cd exposure (Fig. 1). Cd exposure also impaired sperm morphology, inducing head and tail abnormalities Figure Supplementary figure 1, while the co-treatment with EWH prevented the reduced number of sperm with normal morphology (Table 3). Cd impaired the sperm membrane integrity, whereas the co-treatment with EWH prevented the membrane impairment (Fig. 2).

Histopathological analysis showed normal histoarchitecture of the testis and epididymis of rats in the Untreated and EWH groups (Fig. 3A, C; Fig. 4A, C). However, Cd induced a marked degeneration, with reduction of germ cell layers, presence of multinucleated cells and sperm reduction in the testis (Fig. 3B) EWH co-treatment partially prevented the histopathological alterations in the testis, maintaining the structure of the seminiferous tubules and the germinal epithelium (Fig. 3D). In the epididymis, Cd exposure induced severe reduction of sperm stock, and impairment of both the basement membrane and the layer of surrounding smooth muscle (Fig. 4B). EWH partially prevented the epididymal histopathological damages induced by Cd (Fig. 4D).

3.3. Oxidative stress

Cd exposure raised the oxidative stress in the testis and epididymis, increasing reactive species in both organs, as well as lipid peroxidation in the epididymis. Cd also impaired the antioxidant defences, with decreased testicular glutathione (GSH) level and catalase activity in the epididymis and increased superoxide dismutase (SOD) activity and glutathione peroxidase (GPx) in the epididymis (Fig. 5). EWH prevented the increased oxidative stress in the testis and epididymis, reducing reactive species and lipid peroxidation levels, besides increasing the total antioxidant capacity and GSH levels in the testis and normalised the GPx activity in the epididymis.

4. Discussion

The toxic effects of exposure to Cd in the male reproductive system are widely described (Adamkovicova et al., 2016; Benoff et al., 2000; do Carmo Cupertino et al., 2017; Djuric et al., 2015). In the current study, Cd exposure for 14 days led to Cd accumulation in the reproductive organs, consequently impairing sperm quality and inducing oxidative damage in the testis and epididymis. We have tested the ability of EWH to counteract the toxic effects induced by Cd, and despite the high reproductive toxicity of Cd, the co-treatment with EWH was able to prevent, partially, the reproductive dysfunction found after Cd exposure.

In the present study, rats exposed to Cd showed a high content of the metal in their reproductive organs, but the co-treatment with EWH prevented the deposition of Cd in the testis. The reduction in metal deposition may be related to a possible chelating effect of EWH because cysteine residues present in the hydrolysate have a high electrochemical affinity for Cd. Cd is easily linked to thiol groups (Fothergill & Fothergill, 1970) contained abundantly in egg proteins and peptides (Garcés-Rimón, Sandoval, Molina, López-Fandiño, & Miguel, 2016), forming chelates and interfering with cell metabolism (Flora, Mittal, & Mehta, 2008). Thus, this interaction could prevent absorption and thereby the toxic effects of Cd.

Acute and chronic Cd toxicity typically decreases the body weight

Table 2
EWH effects on sperm counts in testis and epididymis of reproductive organs after Cd-exposure.

Parameters *Sperm count*	Untreated	Cd	EWH	CdEWH
Testis				
Sperm number ($\times 10^6$)	218.0 ± 15.9	26.5 ± 3.5*	194.7 ± 9.2	43.6 ± 9.6*
Sperm number ($\times 10^6$/g)	128.5 ± 6.8	31.2 ± 3.6*	122.4 ± 6.2	54.7 ± 6.3*
DSP ($\times 10^6$/testis/day)	35.7 ± 2.6	5.1 ± 1.1*	31.7 ± 1.8	6.2 ± 2.1*
DSP ($\times 10^6$/testis/day/g)	21.5 ± 1.4	5.8 ± 0.2*	19.9 ± 1.2	8.9 ± 1.0*
Epididymis Caput/ Corpus				
Sperm number ($\times 10^6$)	150.7 ± 7.4	4.6 ± 1.9*	163.9 ± 12.5	39.9 ± 5.0*
Sperm number ($\times 10^6$ /g)	464.8 ± 17.6	33.2 ± 14.5*	512.6 ± 52.9	144.9 ± 9.7*
Sperm transit time (days)	6.2 ± 0.3	11.5 ± 0.7*	6.2 ± 0.4	8.6 ± 0.8#
Epididymis/cauda				
Sperm number ($\times 10^6$)	131.1 ± 7.8	8.8 ± 4.4*	163.1 ± 14.0	31.0 ± 6.0*
Sperm number ($\times 10^6$/g)	420.6 ± 11.7	34.1 ± 15.2*	518.0 ± 46.3	111.8.7 ± 9.4*
Sperm transit time (days)	6.2 ± 0.3	11.0 ± 0.4*	6.3 ± 0.2	7.3 ± 1.0#

DSP: daily sperm production; DSPr: daily sperm production relative to testis weight. Data are expressed as mean ± SEM. Units: g: gram. *P < 0.05 vs Untreated, # vs Cd. (N = 10 per group) (One-way ANOVA).

(Miller, Lampp, Powell, Salotti, & Blackmon, 1967) as observed in our Cd-treated rats, although the underlying mechanisms are unclear. Administration of antioxidants, such as vitamin E, sulforaphane, *Ficus religiosa* extract (Jahan, Khan, Ahmed, & Ullah, 2014) and lycopene (Rencuzogullari & Erdogan, 2007) show abilities to restore body weight reduction after Cd exposure at different doses. In the present study, although it prevented Cd deposition and toxicity in the reproductive organs, the co-treatment with EWH was not able to prevent body weight loss after Cd exposure. This unlikely effect could be due to the marked toxicity of this experimental model, considering the high i.p. absorption of Cd (~91%) when compared with oral administrations (~1%) (Moore, Stara, Crocker, Malanchuk, & Iltis, 1973).

Cd toxicity is strongly related to male infertility (Kumar & Sharma, 2019; Siu et al., 2009). In the present study, Cd deposition in the testis and epididymis strongly impaired sperm quality, reducing sperm motility and daily sperm production per testis. Similarly, short- or long-term exposure to Cd seems to impair sperm quality, with reduced sperm motility, viability and sperm number, as well as increased morphological abnormalities (Adamkovicova et al., 2016; Yari et al., 2010). In our study, the high amount of immotile sperm and morphological abnormalities, such as broken or bent tails, was prevented by the concomitant treatment with EWH. Moreover, EWH prevented the membrane impairment, maintaining the sperm viability in Cd-exposed rats.

Histopathological analysis of the testis and epididymis supported the reproductive toxicity of Cd. In the current study, exposure to Cd for 14 days led to marked disorganisation throughout the epididymis and in the seminiferous tubules, including reduced sperm. The histopathological damage induced by Cd was already seen after high levels of metal exposure, with increased interstitial oedema and loss of spermatogenic cells (Alkhedaide et al., 2016). Here, we co-treated rats with EWH in an attempt to counteract the reproductive toxic effects of Cd exposure. EWH co-treatment partially prevented the histopathological damages induced by Cd in the testis and epididymis, maintaining the structure of the seminiferous tubules and the basement membrane of the epididymis. Similarly, other food-derived ingredients, such as curcumin (Oguzturk et al., 2012), saffron (*Crocus sativus*) (Asadi et al., 2014) and grape juice (Lamas et al., 2017) have shown beneficial

Table 3
EWH effects on sperm morphology of reproductive organs after Cd-exposure.

Parameters *Sperm morphology*	Untreated	Cd	EWH	CdEWH
Normal	92 (90–95)	53 (48–60)*	92 (90–94)	90.9 (88–93)#
Head Abnormalities				
Amorphous	2 (2–4)	15 (12–18)*	2 (1–4)	3.5 (1–5) #
Banana Head	1 (0–1)	16 (12–18)*	0 (0–1)	3.5 (1–4)
Detached Head	1 (1–3)	4 (3–5)*	1 (0–2)	3 (2–4)
Total of Head Abnormalities	5 (3–5)	36 (33–44)*	3 (2–4)	14 (7–13)
Tail Abnormalities				
Bent Tail	0 (0–1)	4 (2–5)*	1 (0–2)	2 (0–3)
Broken Tail	0 (0–1)	5 (3–7)*	0 (0–1)	2 (1–4)
Total of Tail Abnormalities	1 (0–1)	9 (7–10)*	1 (0–2)	3 (2–6)

Data are expressed as median (Minimum–Maximum). *P < 0.05 vs Untreated, # vs Cd. (n = 10 per group), Kruskal-Wallis test.

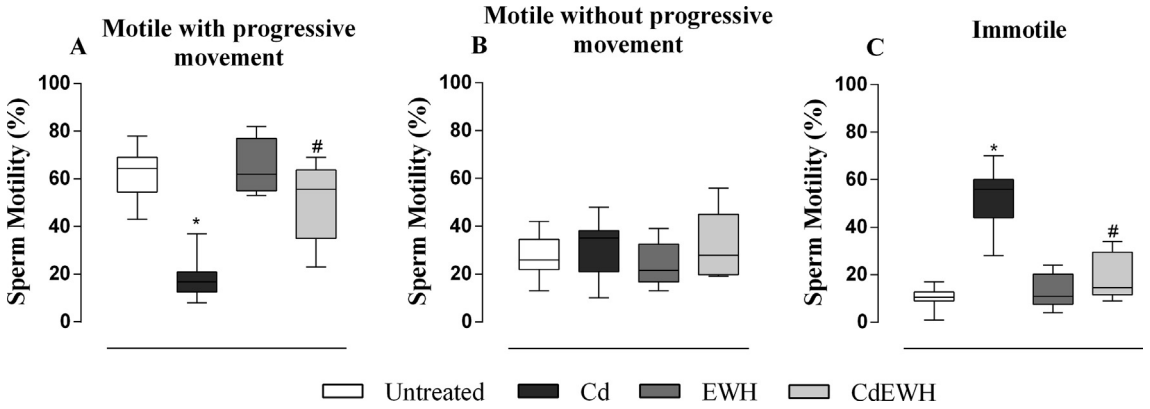

Fig. 1. EWH effects on sperm motility after Cd exposure. A: Motile with progressive movement, B: Motile without progressive movement, C: Immotile. Data are expressed as median (Q1 – Q3). * P < 0.05 vs Untreated; # vs Cd, Kruskal-Wallis test.

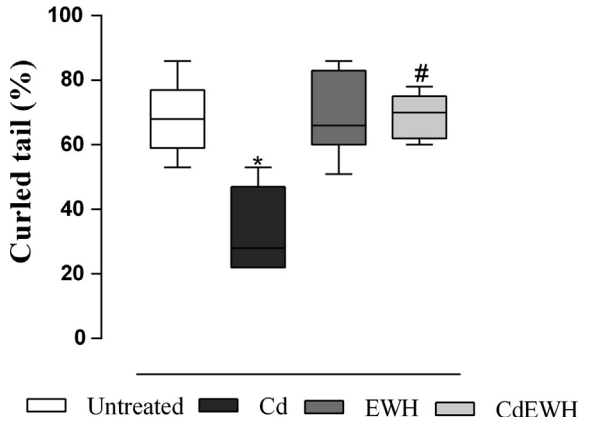

Fig. 2. EWH effects on sperm membrane integrity after Cd-exposure. The results are expressed the percentage of spermatozoa curled tail. Data are expressed as median (Q1 – Q3). * P < 0.05vs Untreated; #vs Cd, Kruskal-Wallis test.

effects on infertility, improving sperm motility and quality after Cd exposure in rats.

The reproductive toxicity induced by Cd seems to be linked to oxidative damage, either by increasing the production of reactive oxygen species (ROS) or reducing antioxidant defences or both (do Carmo Cupertino et al., 2017; de Angelis et al., 2017; Kara, Karataş, & Canatan, 2005). The raised oxidative stress, evidenced by the increased levels of the superoxide anion radical ($O_2^{\cdot -}$) was found in different experimental models of Cd exposure (Djuric et al., 2015; Wang, Fang, Leonard, & Rao, 2004). In the present study, Cd-exposure for 14 days increased the ROS and lipid peroxidation levels and impaired the enzymatic and non-enzymatic antioxidant defences in the reproductive organs. Therefore, the reproductive impairments found could be related to the increased level of oxidative stress after metal exposure, which was also described with a five-fold smaller dose of Cd (Jahan, Zahra, Irum, Iftikhar, & Ullah, 2014). Here, we investigated whether or not EWH could be acting as an antioxidant to prevent the deleterious effects of Cd. Rats exposed to Cd and co-treated with EWH show pro-oxidant levels similar to the Untreated group, suggesting that the beneficial effects of EWH could be linked to its antioxidant power.

This antioxidant action of EWH has been demonstrated metabolic syndrome (Moreno-Fernández et al., 2018), obesity (Garcés-Rimón et al., 2016), hypertension (Miguel et al., 2005, 2006, 2007, 2004) and metals exposure models *in vitro* and *in vivo* (Martinez et al., 2019; Rizzetti et al., 2016; Rizzetti et al., 2017; Rizzetti, Martinez, et al., 2017). EWH-derived peptides and amino acids have antioxidant effects, which could be acting *per se* or synergistically (Davalos et al., 2004). EWH seems to reduce the oxidant–antioxidant imbalance, mainly by acting as an $O_2^{\cdot -}$ scavenger and controlling the lipid peroxidation levels (Garcés-Rimón et al., 2016; Martinez et al., 2019; Rizzetti et al., 2016; Rizzetti et al., 2017). EWH antioxidant effects could be attributed to several identified peptide sequences (Tyr-Ala-Gly-Gly-Arg-Tyr-Pro-Ile-Leu, Tyr-Ala-Glu-Arg-Tyr-Pro-Ile-Leu, Ser-Ala-Leu -Ala-Met, Tyr-Gln-Ile-Gly-Leu, Tyr-Pro-Ile, Tyr-Arg-Gly-Gly-Leu-Glu-Pro-Ile-Asn-Phe, Tyr-Gln- Ile-Gly-Leu) and the amino acids (Tyr and Met) that present significant free radical scavenging activities (Davalos et al., 2004; Garcia-Redondo, Roque, Miguel, López-Fandiño, & Salaices, 2010).

A previous report described the *in vitro* (Yang et al., 2019;20.) and *in vivo* (Yang et al., 2016;17.) damage caused by Cd, including Nrf2-mediated activation and transcription of genes responsible for the

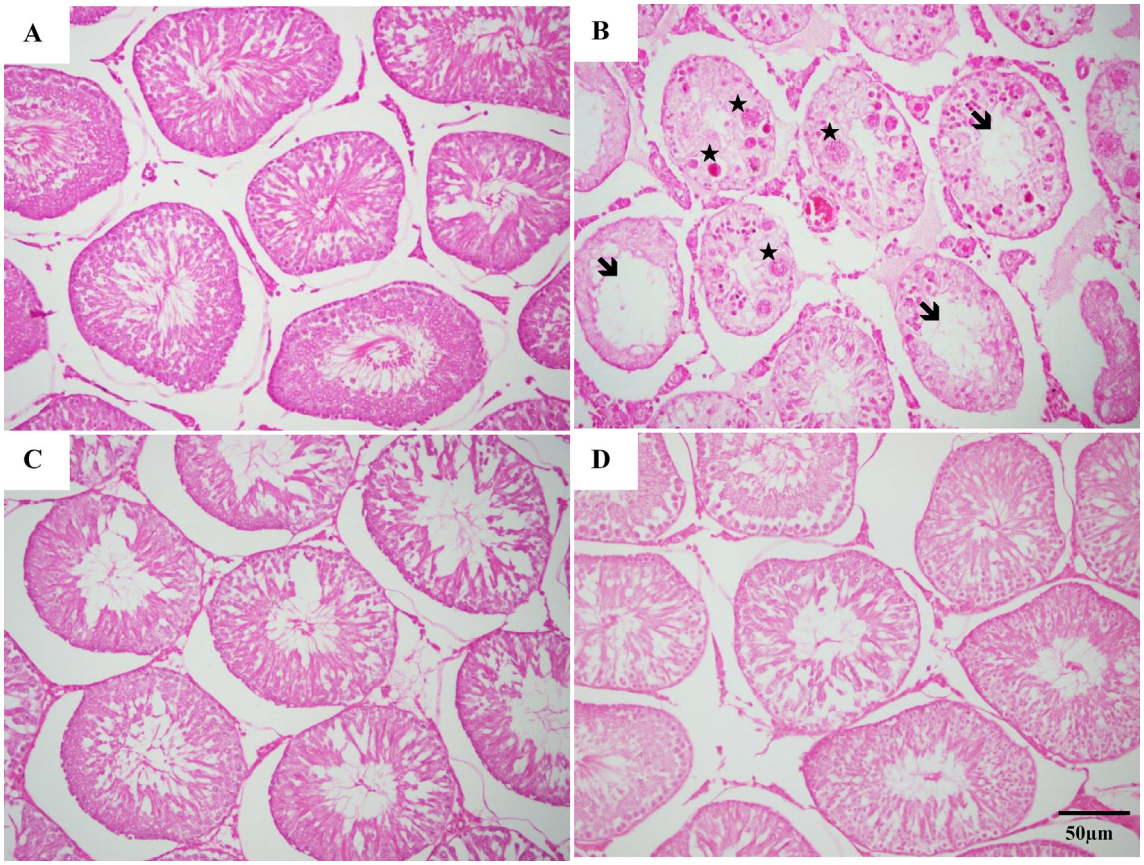

Fig. 3. EWH effects on testis histology after Cd exposure. Untreated (A): Seminiferous tubules without damage; Cd (B): reduction of germ cell layers, presence of multinucleated cells (stars) and sperm reduction in the seminiferous tubules (arrows) EWH (C): Seminiferous tubules without damage; CdEWH (D): Sperm reduction in the seminiferous tubules. 20x objective, scale bars: 50 µm.

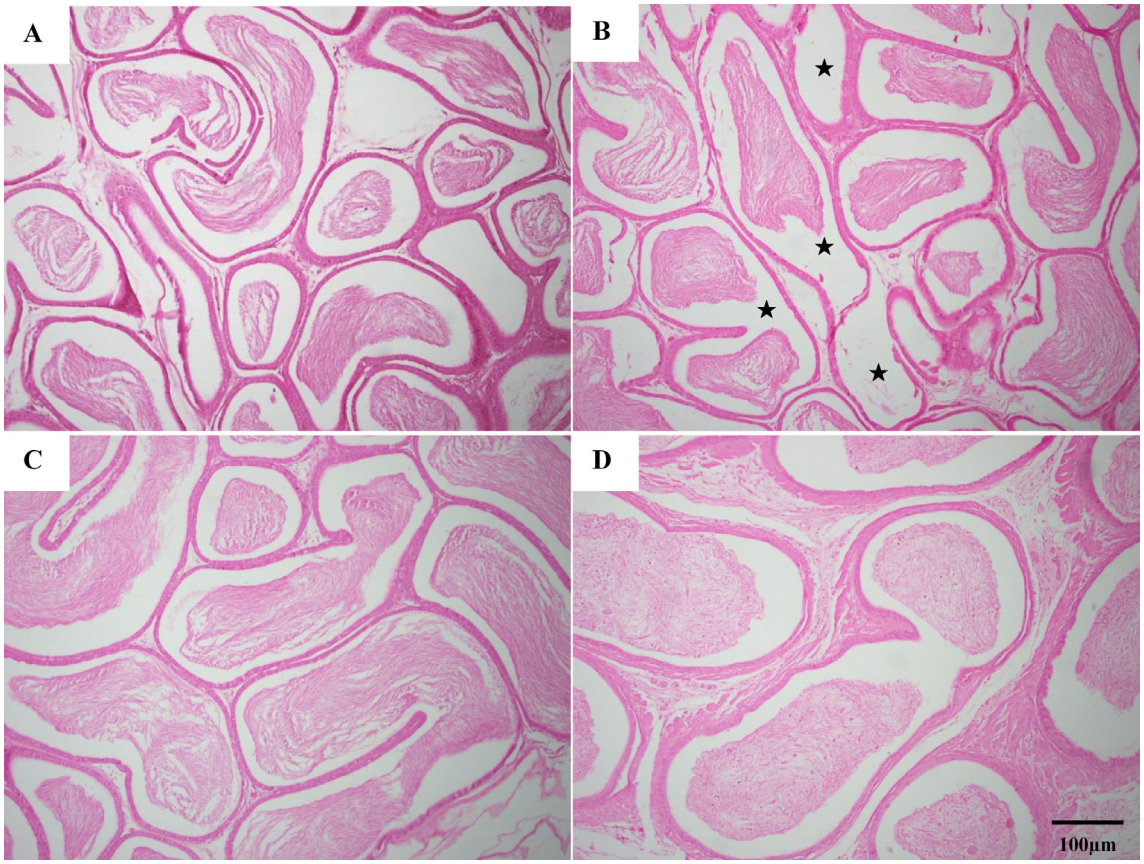

Fig. 4. EWH effects on epididymis histology after Cd exposure. Untreated (A): Epididymal ducts without damage; Cd (B): severe reduction of sperm stockage (stars); impairment of the basement membrane and of the layer of smooth muscle surrounding EWH (C): Epididymal ducts without damage; CdEWH (D): low impairment of the basement membrane and of layer of smooth muscle surrounding, 40x objective, scale bars: 100 µm.

antioxidant enzymes, such as catalase and glutathione transferase Although we did not evaluate the transcription factors, our findings demonstrated that Cd could act on the GSH level, and the co-treatment with EWH exhibited a protective effect. These effects were similar to that found in the above study, in which the authors used sulforaphane as an antioxidant to restore the catalase activity and GSH levels in the testis via Nrf2 activation (Yang et al., 2016, 2019). In addition, Cd induces autophagy and ROS-dependent DNA damage through the activation of AMPK signalling in spermatozoa, affecting an important mechanism of survival and programmed death (Li et al., 2017).

Few studies have demonstrated the beneficial effects of bioactive peptides (Rizzetti et al., 2017) and amino acids against exposure to heavy metals. Previously, we demonstrated the capacity of EWH to protect against neurological and cardiovascular dysfunctions induced by metals at low doses (Martinez et al., 2019; Rizzetti et al., 2016; Rizzetti et al., 2017; Rizzetti, Martinez, et al., 2017). Specifically, in the reproductive system, the co-administration of EWH protects against reproductive impairments induced by sub-chronic exposure to Hg in rats, attributed to reduced inflammation and oxidative stress in the testis (Rizzetti et al., 2017). In the present study, we show for the first time the efficacy of EWH to protect against a marked reproductive dysfunction induced by Cd exposure at high levels.

In summary, Cd exposure for 14 days induces important reproductive impairments, with reduced sperm quality and increased oxidative damage, confirming the high reproductive toxicity of this metal. Our data show that the co-treatment with EWH, which partially prevented the reproductive dysfunctions induced by Cd, might be associated with inhibiting Cd deposition and preventing the increased oxidative stress. Therefore, the administration of EWH-derived bioactive peptides, as a functional food ingredient, could be an alternative to protect against Cd-induced reproductive toxicity.

Ethics statement

All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology. Approval for the study was granted by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 013/2019).

CRediT authorship contribution statement

J.E.G. Pinheiro Júnior - Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **C.S. Martinez** - Conceptualization, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **P.Z. Moraes** - Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization. **J.E. Stasiaki** - Investigation, Formal analysis, Validation, Visualization. **M.E. Trost** - Investigation, Methodology. **D.V. Vassallo** - Conceptualization, Funding acquisition, Supervision, Writing - original draft. **F. Barbosa (Junior)** - Investigation, Methodology, Validation, Visualization. **F.M. Peçanha** - Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Visualization, Writing - original draft, Writing - review & editing. **F.W. Santos Cibin** - Conceptualization, Formal analysis, Supervision, Visualization, Writing - original draft. **M. Miguel** - Conceptualization, Funding acquisition, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **G.A. Wiggers'** - Conceptualization, Data curation, Formal

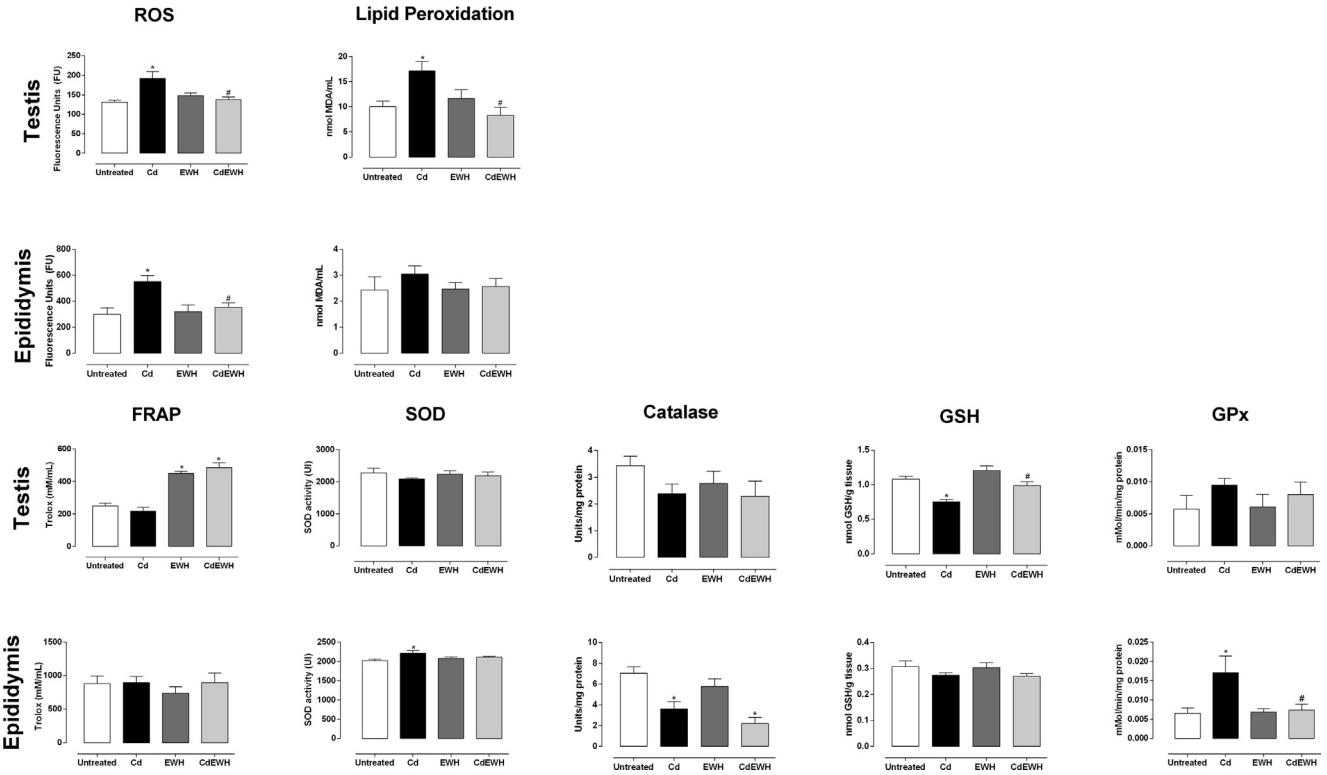

Fig. 5. EWH effects on oxidative stress biomarkers after Cd exposure. Reactive species (UF), lipid peroxidation (nmol MDA/mL), total antioxidant capacity (Trolox nM/mL), SOD activity (UI), Catalase activity (Units/mg protein), GSH level (nmol GSH/tissue), Glutathione Peroxidase Activity (nMol/min/mg protein) in testis and epididymis of Cd exposure rats for 14 days. The results are expressed mean ± SEM; * P < 0.05 vs Untreated; # vs Cd.

analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, 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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103823.

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