EGG WHITE-DERIVED PEPTIDES PREVENT MALE REPRODUCTIVE DYSFUNCTION INDUCED BY MERCURY IN RATS

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ABSTRACT

Mercury (Hg) is a trace metal implicated in the oxidative damage of male and female reproductive organs and infertility. In this sense, peptides from enzymatic hydrolysis of egg white have *in vitro* antioxidant activity. Thus, our study aimed to investigate whether the egg white hydrolysate (EWH) is able to prevent the effects of prolonged mercury exposure at low levels on male reproductive system of rats. For this, rats were treated for 60 days with: a) Untreated - saline solution (*i.m.*); b) Hydrolysate - EWH (1 g/kg/day, gavage); c) Mercury - $HgCl_2$ (1^{st} dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day, *i.m.*); d) Hydrolysate-Mercury. At the end of the treatment, sperm motility, count and morphological studies were performed; Reactive Oxygen Species (ROS) levels, lipid peroxidation, antioxidant capacity, histological studies and immunohistochemical assay on testis and epididymis were also carried out. As results, the treatment with $HgCl_2$ for 60 days decreased testicular and epididymal sperm number, increased sperm transit time in epididymis and impaired sperm morphology. However, these harmful effects were prevented by EWH. $HgCl_2$-treatment also increased ROS levels, lipid peroxidation and antioxidant capacity in testis and epididymis as well as promoted testicular inflammation and histological changes in testis and epididymis. EWH improved histological and immunohistochemical alterations, probably due to its antioxidant and anti-inflammatory properties. In conclusion, the EWH could represent a powerful natural alternative to protect the male reproductive system against Hg-induced sperm toxicity.

Keywords: Mercury; Male reproductive dysfunction; Sperm quality; Oxidative stress; Functional food; antioxidant and anti-inflammatory properties.

1. INTRODUCTION

Male reproductive dysfunction can be induced by several conditions such as genetic abnormality or external agents which promote damage on reproductive organs mainly through oxidative stress and inflammation factors (Aitken 1997; Saalu 2010). Heavy metals have become one of this toxic agents found in the environment and, in recent years, they have received greater concern as a putative cause for decline in semen quality and infertility (Mathur and D'Cruz 2011). Many of these metals, including lead, cobalt, cadmium, chromium and mercury are known to exert toxic effects on testicular function, by acting as a endocrine disruptor or inducing the pro-oxidant/antioxidant imbalance in testicular cells, thereby activating associated downstream pathways such as apoptosis (Saxena et al. 1989; Anderson et al. 1993; Orisakwe et al. 2001; Cheng et al. 2011; Kalender et al. 2013; Manfo et al. 2014).

Mercury (Hg) is a trace metal released into the environment from several sources, such as seafood diet, use or handling of dental amalgams and occupational activities in industries or mining areas. This metal has been implicated in the etiology of human and rodents infertility (Choy and Ellsworth 2012; Tchounwou 2014; Kim et al. 2016). Epidemiological data findings demonstrated menstrual cycle abnormalities and anovulation among women dentists and gold miners occupationally exposed to Hg (Davis et al. 2001; Pollack et al. 2011; Rodriguez-Villamizar et al. 2015). This metal has been also reported as an endocrine disruptor, exerting deleterious effect on the ovarian response to gonadotrophin therapy in patients submitted to *in vitro* fertilization (Dickerson et al. 2011). All chemical forms of Hg administered in animals, even

at low levels, result in female reproductive problems, including spontaneous abortion, stillbirths, congenital malformations, inhibition of the ovulation and infertility (Rzymski et al. 2015).

Regarding male disorders, Hg toxicity on reproductive system was associated with reduced libido, problems with sperm production and function, mutations to germ cells and low fertility in men occupationally exposed to this metal (Ernst and Lauritsen 1991; Mendiola et al. 2011). Previous reports have also demonstrated a positive correlation between blood Hg concentration and infertility parameters and abnormalities in semen quality in exposed men (Choy et al. 2002). In animals, acute and chronic Hg exposure are related to hypothalamic-pituitary axis disruption, deficit in the testicular spermatogenic and steroidogenic functions (Martinez et al. 2014b), decreased secretion of sperm maturation components by the epididymis (Rao and Sharma 2001) and reduction in the sperm count, motility and morphology (Martinez et al. 2014a).

Several reports have suggested that Hg exposure enhances the production of ROS (Clarkson et al. 2003; Boujbiha et al. 2009; Rizzetti et al. 2013) and it alters the antioxidant enzyme activities in different tissues of rats (Jadhav et al. 2007; Rao and Chhunchha 2010; Amara et al. 2013). Corroborating with these studies, previously, we have shown that both, 30 (Martinez *et al.* 2014a) and 60 days (Martinez *et al.* 2014b) of exposition to $HgCl_2$ at low concentrations, similar to human occupational contact to this metal, induce male reproductive dysfunction associated with hormonal imbalance and increased oxidative stress.

The effectiveness of different agents and nutrients to prevent or reverse Hg toxicity has been investigated. Selenium, vitamin E, zinc and some bioactive

components derived from plants have been postulated to exert protection on the reproductive system against heavy metal toxicity (Rao and Sharma 2001; Beyrouty and Chan 2006; El-Desoky et al. 2013; Frenedoso da et al. 2014; Abarikwu et al. 2016). Moreover, the World Health Organization (WHO) recommended that nutrients that alter toxicity associated with environmental contaminants, such as Hg, must be more investigated.

In this respect, eggs are economically and nutritionally important because they can form a significant component of the diet and they are also an excellent source of bioactive substances (Mine 2007; Garces-Rimon et al. 2016). In previous work, we demonstrated that the enzymatic hydrolysis of egg white with pepsin resulted in the production of peptides with *in vitro* peroxyl radical-scavenging activity (574 μmol Trolox/g protein), reducing the intracellular ROS levels in t-BOOH challenged RAW 264.7 macrophages, without any effect on cell viability, which suggests that this egg white hydrolysate (EWH) could be useful to improve oxidative stress related pathologies, including reproductive dysfunction (Davalos et al. 2004; Miguel et al. 2004). These bioactive peptides from EWH, which sequences were previously identified (Miguel *et al.* 2004), showed *in vivo* to reduce hypertension, oxidative stress and hyperlipidemia in spontaneously hypertensive (SHR) and obese rats (Miguel et al. 2005; Miguel et al. 2006; Moreno et al. 2015; Garces-Rimon et al. 2016).

Taking into account the involvement of oxidative mechanisms in the toxic manifestation of Hg on the male reproductive system and the proven antioxidant functions of the EWH; this study aimed to investigate whether the EWH is able to prevent or mitigate the effects of prolonged Hg exposure at low levels on

sperm quality, biomarkers of oxidative stress and inflammation and, histological aspects on male reproductive system of rats.

2. MATERIALS AND METHODS

2.1. EWH obtaining

EWH was prepared by pepsin hydrolysis of crude egg white as previously described (Garces-Rimon *et al.* 2016). Briefly, commercial pasteurized egg white was hydrolysed with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S: 2:100 w:w, pH 2.0, 38 ºC), purchased from Biocatalysts (Cardiff, United Kingdom), for 8 h. Enzyme inactivation was achieved by increasing the pH to 7.0 with 5N NaOH. The hydrolysate was centrifuged at 2500 x g for 15 min and the supernatants were frozen and lyophilised.

2.2. Animals and experimental design

Male young adult *Wistar* rats (Charles River, Barcelona) of 8-week-old (200-250 g) and in the reproductive phase were maintained in cages (5 animals each cage) and in controlled environmental conditions (temperature 23 °C, humidity 60 %) with 12 h light/darkness cycles with free access to tap water and fed with standard chow *ad libitum*. Rats were divided into four groups, which were treated for 60 days with: a) Untreated – received intramuscular injections (*i.m.*) of saline solution 0.9 % and tap water by gavage; b) Mercury – received *i.m.* injections of mercury chloride ($HgCl_2$) diluted in saline solution, the 1st dose of 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day, to cover daily loss, using model previously described (Wiggers *et al.* 2008) and tap water by gavage; c)

Hydrolysate – received *i.m.* injections of saline solution 0.9 % and egg white hydrolysate (EWH) diluted in tap water in a concentration of 1 g/kg/day by gavage, according to dose described in prior work (Miguel *et al.* 2006); d) Hydrolysate plus Mercury – received both treatments, $HgCl_2$ by *i.m.* injections and EWH by gavage. This experimental model of controlled chronic exposure to low concentration of Hg reproduces the human exposure to this metal during approximately six years (Quinn 2005; Andreollo et al. 2012). This is the most similar model of human occupational exposure known so far. Doses administered took into account the half-life of inorganic Hg as well as the daily loss that can occur by the metal removal via urine and feces. These were administered in a controlled manner, by intramuscular injections, to ensure full access to the bloodstream without loss during the gastrointestinal absorption. The blood concentration of Hg in this model is about 3 ng/ml (Rizzetti et al., submitted), which is within the safety limit established by US Environmental Protection Agency's (5.8 ng/ml) and similar to the blood Hg concentration of people exposed to the metal by the workplace or through diet (Rice 2004). Moreover, to make the model more similar to human exposure condition, we prefer to carry out a simultaneous treatment of Hg and EWH, considering that humans are rarely completely free of any level of exposure to this metal. So our concern was to investigate the benefits of EWH during a continuous and concurrent exposure to Hg.

During the treatment, the manipulation of the animals was performed following the appropriate safety measures and general health, body weight, food and water intakes were recorded once a week. All experiments were conducted in compliance with the guidelines for biomedical research stated by

the Brazilian Societies of Experimental Biology and the European and Spanish legislation on care and use of experimental animals (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013) and approved by the Ethics Committees on Animal Use at both Universidade Federal do Pampa (CEUA/UNIPAMPA), Uruguaiana, Rio Grande do Sul, Brazil (Protocol Number: 005/2014) and Universidad Rey Juan Carlos, Madrid, Spain. The experiments also were designed to minimize the number of animals used and their suffering during the execution of the protocols.

2.3. Reproductive organs collection

At the end of the treatment period, rats were euthanized by decapitation and subsequently, testis, epididymis, vas deferens, prostate glands and seminal vesicles were excised from surrounding tissues and placed into tube. Thus, organs were dried between two sheets of filter paper and their wet weight was determined. Next, the relative organ weight was calculated by use of the formula: organ weight/body weight x 100. Left epididymis was divided in two segments, one of this was processed for histological study and the other one for biochemical determination as well as left testis was processed for biochemical, histological and immunohistochemical studies. Right epididymis and testis were used for sperm count.

2.4. Mercury Quantification

Total Hg concentration was determined in testis and epididymis samples by a Hg analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Atomic Spectrometry Service at the Universidad de Málaga, Spain, using the principles of thermal decomposition, amalgamation and atomic absorption described in EPA Method 7473 (Boylan *et al.* 2003). This protocol uses a decomposition

furnace to release Hg vapor instead of the chemical reduction step used in traditional liquid-based analyzers. Samples were weighed directly into a Ni capsule using an analytical balance. For determination of total Hg, a calibration line was performed with a range of 8 to 10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as total Hg (ng/g of tissue).

2.5. Sperm analysis

2.5.1. Sperm motility

Sperm motility was assessed according to previous study (Martinez *et al.* 2014a). The sperm was removed from vas deferens and mixed with 1 ml of Human Tubular Fluid (DMPBS, Nutricell, São Paulo, Brazil) pre-warmed to 34°C. After this, an aliquot of 10 ml was transferred to a histological slide. Using a light microscope (20X magnification, Binocular, Olympus CX31, Tokyo, Japan), 100 spermatozoa were analyzed and classified as type A: motile with progressive movement, type B: motile without progressive movement and type C: immotile. Sperm motility was expressed as % of total sperm.

2.5.2. Sperm morphology

Morphological studies were performed as previously described (Martinez *et al.* 2014b). The sperm removed from vas deferens was stored with 1 ml of formol saline and kept at room temperature until the analysis. For the analysis, smears were prepared on histological slides and 200 spermatozoa per animal were evaluated (40X magnification, Binocular, Olympus CX31, Tokyo, Japan). Morphological abnormalities were classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail).

2.5.3. 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 (Robb *et al.* 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.

2.6. Biochemical studies

2.6.1. Tissue preparation

Testis and epididymis were homogenized in 50 mM Tris–HCl at pH 7.4 (1/5, weight/volume [w/v]). The homogenate was centrifuged for 10 min at 2500 rpm, 4°C and the pellet was discarded, while the low speed supernatant (S1) for each tissue was kept for subsequent biochemical measures.

2.6.2. Reactive Oxygen Species (ROS) measure

ROS levels were assessed spectrofluorometrically using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe as previously described (Ali *et al.* 1992). The sample (S1) was diluted (1:5) in 50 mM Tris-HCl (pH 7.4) and the DCHF-DA (1 mM) was added to the medium. The DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2´,7´-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of reactive species formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 488 nm excitation)

(Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence unit (FU).

2.6.3. Lipid peroxidation determination

Lipid peroxidation was evaluated in testis and epididymis by the Thiobarbituric Acid Reactive Substance (TBARS) assay (Ohkawa *et al.* 1979). In this procedure, an aliquot of S1 was incubated with a 0.8 % thiobarbituric acid solution, acetic acid buffer (pH 3.2) and sodium dodecyl sulfate solution (8 %) at 95 °C for 1 h, and the color reaction was measured at 532 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Results were expressed as nmol of malondialdehyde (MDA) per mg of protein.

2.6.4. Ferric Reducing Antioxidant Power (FRAP) assay

FRAP was performed according to a colorimetric method (Benzie and Strain 1996). To prepare working FRAP reagent, acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) were mixed in a 10:1:1 ratio (v:v:v). After this, the reagent was mixed with S1. The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the samples was monitored after incubation of the samples for 15 min at 37 °C, by measuring the absorbance at 593 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Antioxidant potential of the samples was determined against standards of Trolox, which were processed in the same manner as the samples. Results are presented with particular reference to Trolox equivalents.

2.6.5. Protein quantification

Protein concentration was measured by the Bradford method (Bradford 1976), using bovine serum albumin as a standard.

2.7. Testis and epididymis histology

Histological studies on testis and epididymis were carried out. After weighing, epididymis tissues were fixed in 10 % formaldehide 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) equipped with the image analysis software package AxioVision 4.6 to evaluate the morphometric parameters. The analysis was made in 10 random fields measured in 40X magnification per section.

2.8. Testis immunohistochemistry

Testis immunohistochemistry was performed on paraffin-embedded sections of 5 μm thickness. Deparaffined slides were washed with phosphate buffered saline (PBS) with 0.05 % Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3 % (vol/vol) in hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal bovine serum for 30 minutes to minimize nonspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with a monoclonal antibody against macrophage-associated antigen (CD163, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to quantify the number of activated macrophages, which is consistent with the presence of inflammation. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with

Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining the preparations were incubated without the primary antibody.

2.9. Data analysis and statistics

Data are presented as the mean values±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.

3. RESULTS

3.1. Body and organ weight measures

There was no change in body weight of rats after Hg exposure for 60 days neither in those groups that received the EWH co-treatment. The absolute and relative organ weights were also similar between the experimental groups (Table 1).

3.2. Mercury quantification

Hg levels in testis and epididymis exhibited a significant increase after 60 days of $HgCl_2$ treatment. Testis in Hydrolysate-Mercury group had lower concentrations of Hg compared to those of $HgCl_2$-treated rats. However, epididymis remained high levels of Hg in the rats that received the co-treatment with EWH (ng/g of tissue: Testis – Untreated: 0.57±0.13; Mercury: 1.33±0.09*; Hydrolysate: 0.48±0.07; Hydrolysate-Mercury: 0.99±0.23; Epididymis – Untreated: 0.63±0.11; Mercury: 1.30±0.12*; Hydrolysate: 0.52±0.03; Hydrolysate-Mercury: 1.18±0.06*; n=4; One-Way ANOVA *$P<0.05$ vs Untreated).

3.3. Sperm analysis

The treatment with $HgCl_2$ for 60 days decreased the testicular and epididymal (cauda) sperm number compared to untreated group as well as increased sperm transit time in epididymis (caput/corpus). These harmful effects were prevented by the EWH intake, as shown by the results of the group Hydrolysate-Mercury (Table 2). Hg exposition also resulted in a significant reduction in daily sperm production per testis, which was totally prevented by the administration of EWH (Table 2).

Analysis of sperm morphology showed that chronic Hg exposure diminished the percentage of morphologically normal spermatozoa compared with the untreated group, whereas the predominant abnormalities were in the sperm head, mainly amorphous and banana head morphology. Concerning tail morphology, there was predominantly bent tail abnormality. Interestingly there were not morphological changes in those groups that received the EWH alone or associated with the Hg treatment, and they presented similar to untreated group (Table 3).

Regarding the sperm motility, we demonstrated in $HgCl_2$-treated rats a decrease on type A sperm (motile with progressive movement) (Figure 1A) accompanied by an increase on type C sperm (immotile) (Figure 1C). However, there were not motility changes in type B sperm (motile without progressive movement) (Figure 1B). The EWH co-treatment was able to prevent these alterations and promoted a return to normal sperm motility like as untreated group.

3.4. Biochemical assays

Hg intoxication increased ROS production in testis and epididymis of $HgCl_2$-treated rats compared to untreated rats. The levels of MDA were also significantly elevated in these tissues. However, the co-treatment with EWH caused a significant reduction in these effects, preventing testis and epididymis against the oxidative stress and lipid peroxidation caused by the long-term Hg exposure (Figures 2 and 3).

Respect to testicular and epididymal antioxidant capacity, the results showed that antioxidant capacity was increased in both testicular and epididymal tissues of Hg-treated group when compared to untreated group. EWH intake avoided the increase in the antioxidant capacity in testis, whereas in epididymis this parameter remained elevated (Figures 2 and 3).

3.5. Histological and Immunohistochemical analysis of testis and epididymis

Histological analysis of testis did not reveal tissue damage in Hg-treated rats, and all the groups were morphologically similar respect to untreated group (Figure 4). However, immunohistochemical analysis showed an increase in the number of activated macrophages in Hg-treated rats, while the groups that received EWH alone or associated with Hg were similar to untreated group and no immunohistochemical changes were observed (Figure 5).

Histopathological evaluation of epididymis showed a significant reduction in the sperm amount in the lumen of efferent ducts in $HgCl_2$-treated group relative to the Untreated and Hydrolysate groups. There were a large number of empties efferent ducts in Hg-treated rats. The co-treatment with EWH improved histological changes, and the Hydrolysate-Mercury group presented in efferent ducts sperm amount similar to untreated group (Figure 6).

4. DISCUSSION

To our knowledge, this is the first study to verify the effects of a dietetic supplementation with EWH on male reproductive dysfunction induced by chronic exposure to Hg at low doses. We confirmed that 60-day $HgCl_2$ exposure affects sperm quality through the increase in the oxidative and inflammatory factors and proved that EWH is able to prevent the decreased motility, daily sperm production, sperm quantity in testis and epididymis and the increased sperm abnormalities in exposed-animals. These findings suggest that the EWH intake is effective against Hg-induced male reproductive toxicity in rats and this potential beneficial is related to its antioxidant and possible anti-inflammatory properties.

Hg is a known spermatoxic agent that impairs male fertility through the inhibition of spermatogenesis and disorders in sperm morphology and motion (Choy *et al.* 2002; Heath *et al.* 2012). *In vitro* and *in vivo* studies have showed sperm production and motility impairments in addition to morphological abnormalities in rodents submitted at both, acute and chronic $HgCl_2$ exposure (Boujbiha et al. 2009; Mendiola et al. 2011; Heath et al. 2012; Abarikwu et al. 2016). Chronic administration of $HgCl_2$ promoted a decrease in testis, epididymis and accessory sex organs weight, associated with a reduction in sperm count in the testis, vas deferens, and cauda epididymis (Heath *et al.* 2012) and a decrease in testosterone level in adult male rats (Abarikwu *et al.* 2016). However, the metal exposition enhanced the relative and absolute testis weight after 90 days of treatment in another study, which was related to the presence of edema in this organ (Boujbiha *et al.* 2009).

In previous studies our research group reported a moderate alteration of the sperm parameters in 30-days $HgCl_2$ treated rats and a severe disorder when the metal exposure was prolonged for 60 days, demonstrating that Hg cumulatively affects the male reproductive system, despite any changes in body and reproductive organs weight were observed (Martinez *et al.* 2014b). The current study corroborates with previous and also shows marked alterations in 60-days $HgCl_2$ treated rats, characterized by impairs on sperm production and count following by motility and morphological abnormalities mainly banana head and bent tail. These results show that Hg can induce severe damage in the male reproductive system, even if apparent physical changes are not evident.

It has been demonstrated that consumption of natural antioxidants present in certain foods can prevent toxic effects caused by exposure to trace metals, once it protects the cell from DNA damage and changes its redox state induced by Hg (Rao and Sharma 2001; Beyrouty and Chan 2006; El-Desoky et al. 2013; Frenedoso da et al. 2014; Abarikwu et al. 2016). In this work we showed that the EWH intake was able to prevent the damage on sperm quality promoted by Hg at low concentrations. The EWH had previously demonstrated effectiveness on cardiovascular (Miguel et al. 2007a; Miguel et al. 2007b; Garcia-Redondo et al. 2010), metabolic (Manso et al. 2008; Moreno et al. 2015; Garces-Rimon et al. 2016) and neurologic (Rizzetti *et al.* 2016) diseases. In this study, for the first time, we showed that its functional properties make it a good therapy on male reproductive dysfunction.

In this sense, natural bioactive compounds, such as minerals, vitamins and phytochemicals have been described to possess functional activities on sperm disorders induced by heavy metals. Treatment with selenium and vitamin

E ameliorated the adverse effects of $HgCl_2$ on testicular parameters (Rao and Sharma 2001; Beyrouty and Chan 2006; El-Desoky et al. 2013; Frenedoso da et al. 2014; Abarikwu et al. 2016). Some medicinal plants were reported to protect testis against $HgCl_2$-induced testicular damage, and its effects were associated with the effective restoration of oxidative stress markers, activities of enzymatic antioxidant biomarkers and histopathological alterations (Boujbiha et al. 2009; Siouda and Abdennour 2015; Abarikwu et al. 2016).

In fact, it has been postulated that the Hg induces male infertility mainly through oxidative damage. This metal is able to bind with sulfhydryl groups in the membrane, head, midpiece, and tail of the sperm and, subsequently, affects the sperm membrane permeability, mitochondrial functional integrity and DNA synthesis in mitotic spindles (Clarkson et al. 1985; Choy et al. 2002). Our results show an increase on ROS production and MDA levels in testis and epididymis, consistent with previous reports (Boujbiha et al. 2009; Boujbiha et al. 2011).

Sperm cells are exceedingly susceptible to oxidative stress because the spermatozoa membranes are rich in polyunsaturated fatty acids, so they represent a fragile target of ROS attack and lipid peroxidation as a result of exposure to Hg (Orisakwe et al. 2001; Kalender et al. 2013). Lipid peroxidation reaction causes membrane damage which leads to a decrease in sperm motility, presumably by a rapid loss of intracellular ATP, and an increase in sperm morphology defects (Clarkson et al. 1985; Choy et al. 2002), corroborating with our study that found increased frequency of spermatozoa with abnormal head and tail.

The defense enzymes are also an important indicator of the oxidative imbalance promoted by the $HgCl_2$ chronic exposure. The testis, epididymis, sperm and seminal plasma contain high activities of antioxidant enzymes that may be dramatically affected by the metal (Orisakwe et al. 2001; Kalender et al. 2013). In the present study, $HgCl_2$ exposure was correlated with increased levels of antioxidant capacity in the male reproductive organs of rats, represented by the testicular and epididymal FRAP values. The rise in the antioxidant capacity represents a compensatory mechanism to scavenge ROS levels produced as a result of $HgCl_2$ accumulation, consistent with results of our prior work in this experimental animal model (Martinez *et al.* 2014b) and others reports (Boujbiha et al. 2009; Penna et al. 2009; Abarikwu et al. 2016).

The co-treatment with EWH normalized the oxidant and antioxidant status of testis and epididymis in terms of MDA contents and antioxidant power, suggesting that EWH consumption might have a potential role in preventing $HgCl_2$-induced testicular and epididymal injuries due to its antioxidant and free radical scavenger properties. In this sense, the EWH could act on the increment of the endogenous cellular antioxidant defense system and on the neutralization of ROS in the reproductive organs. It has been reported that the presence of Tyr and Phe amino acids in some of protein hydrolysates is related to scavenging free radicals properties (Sun *et al.* 2014). Prior studies of our research group have described the presence of peptides with Tyr, His, Pro, Phe and Leu amino acids in the EWH (Miguel et al. 2004), thus we can suggest that its effect on the oxidative damage observed in testis and epididymis in this study is probably due to the antioxidant properties provided by EWH.

Regarding the Hg deposition in male reproductive organs of rats, it has been proposed that the Hg is able to cross the blood-testis barrier to induce testicular damage (Rao and Sharma 2001; Penna et al. 2009). In addition, Hg ions also can enter in the epididymis through the blood-epididymal barrier (Sharma *et al.* 1996). Once into the testicular and epididymal tissues, this metal induces disruption of the physiology function in these organs probably due to alterations in ATPase enzymes and in sialic acid levels (Rao and Sharma 2001; Rao and Gangadharan 2008; Penna et al. 2009). Despite several studies have reported some effects on the male reproductive system after chronic administration of $HgCl_2$ (Vachhrajani and Chowdhury 1990; Ernst and Lauritsen 1991), only a few of them have quantified the levels of metals in the organs of treated animals. In the current study, animals that received $HgCl_2$ accumulated approximately 2 ηg/g of Hg in the testis and epididymis. This concentration is lower than the others studies that reported after an $HgCl_2$ exposure a metal accumulation ranged from 60–80 ηg/g of tissue (Penna *et al.* 2009). However, the low levels of Hg founded in testis and epididymis were sufficient to cause serious damage to sperm quality, suggesting that during chronic exposure to inorganic Hg, there is not a direct relationship between the amount of Hg deposited and the damage to the male reproductive system.

The co-treatment of rats with EWH attenuated the Hg accumulation on the testis. However, the Hg deposition in the epididymis was not modified by EWH consumption. This result can suggest that, despite others protein hydrolysates have been found chelating activity (Gallegos-Tintore *et al.* 2011), in this condition, the potential effects of the EWH against Hg toxicity on the male reproductive system of rats were due to its powerful antioxidant and free radical

scavenger activity. Co-treatments with zinc and some herbs were reported to play a crucial rule in the absorption of Hg (El-Desoky *et al.* 2013). In addition, the chelating property on Hg ions demonstrated by some foods was related to the presence of selenium compounds, which exerts protection, due to its capability to alter the distribution of Hg in tissues and induce binding of the Hg-Se complexes to proteins (Joshi *et al.* 2010).

Respect to histological and immunohistochemical parameters, the decrease in the epididymal sperm concentration is consistent with the reduced sperm amount in the lumen of efferent ducts in $HgCl_2$-treated animals. In accordance with our study, others authors reported increased number of empty efferent ducts in the epididymis after Hg exposure, with was related to hypospermatogenesis in testis of $HgCl_2$ treated rats (Penna *et al.* 2009).

Despite some authors reports histological changes in testis, including decrease in the diameter of seminiferous tubules, disorganization of the basal membrane and aspermatogenesis after chronic exposition to $HgCl_2$ (Penna et al. 2009; Boujbiha et al. 2011; Frenedoso da et al. 2014), our work did not show any histological changes in testis of Hg-treated rats even that serious functional dysfunctions were observed. However, the immunohistochemical analysis showed infiltrated macrophages around the seminiferous tubules of rats treated with $HgCl_2$, suggesting the development of an inflammatory process, which was not detected by the conventional microscopy. In fact, it has been postulated that metal-induced imbalance in immune regulation can lead to inadequate or excessive production of either inflammatory or anti-inflammatory cytokines resulting in chronic inflammatory processes or autoimmune diseases (Boujbiha et al. 2009; Boujbiha et al. 2011). Oxidative damage promoted by Hg is also

related to loss of enzymatic activity and structural integrity of enzymes and activation of inflammatory processes (Ansar 2016). In the current study, the lack of histological alterations in testis could be explained by our model of exposure. We found increased oxidative stress and inflammatory process in testis after 60 days of exposure at extremely low levels of Hg, at these experimental condition we were not able to find differences in the testicular histoarchitecture between groups. If another experimental condition was adopted, such as longer or higher Hg exposure, this scenario should be different.

Interestingly, rats that received the EWH dietetic supplementation showed a homogeneous and normal testicular and epididymal tissue structure as demonstrated by the histological findings. Furthermore, the EWH inhibited the inflammatory cells release in testis observed in the immunohistochemistry assay. These finding suggest that the impairment observed in tissue structure is due to oxidative and inflammatory damage on the testis and epididymis induced by the Hg deposition in these organs. Therefore, the EWH, acting as antioxidant and anti-inflammatory compound, was able to prevent the Hg-induced impair on the male reproductive system and sperm quality.

In summary, a dietetic supplementation with EWH promotes protective effects on spermatic parameters against Hg-induced sperm toxicity in rats, preventing the oxidative and inflammatory injuries on male reproductive organs and could represent a good public health strategy against environmental contaminants.

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DUALITY OF INTEREST

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this manuscript. The authors declare that there is no duality of interest associated with this manuscript.

CONFLICT OF INTEREST

The authors have nothing to disclose and no conflicts of interest to report.

DISCLOSURE STATEMENT

The authors are unaware of any affiliation, funding, or financial holdings that might be perceived as affecting the objectivity of this article.

CONTRIBUTION STATEMENT

Conceived and designed the experiments: DAR, CSM, JAUO, FMP, DVV, MMC, GAW; performed the experiments: DAR, CSM, LGE, TMS; analyzed the data: DAR, CSM, JAUO, FMP, DVV, MMC, GAW; contributed reagents/materials/analysis tools: JAUO, DVV, MMC, GAW; wrote the paper: DAR, DVV, MMC, GAW. All authors have approved the final manuscript.

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TABLES

Table 1. Effect of treatment with EWH on body weight (g), absolute (g or mg) and relative (g/100g or mg/100g) weights of reproductive organs of male rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters	Experimental groups			
	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Initial body weight (g)	318.25 ± 16.75	318.50 ± 15.66	305.38 ± 9.22	311.75 ± 13.72
Final body weight (g)	432.38 ± 18.15	435.75 ± 11.65	423.13 ± 6.90	433.75 ± 7.87
Testis (g)	1.58 ± 0.10	1.82 ± 0.04	1.66 ± 0.04	1.78 ± 0.04
Testis (g/100g)	0.37 ± 0.03	0.34 ± 0.06	0.40 ± 0.02	0.41 ± 0.01
Epididymis (mg)	614.17 ± 32.59	666.80 ± 8.36	646.83 ± 21.08	661.14 ± 24.64
Epididymis (mg/100g)	142.78 ± 9.14	151.48 ± 4.03	153.88 ± 5.53	153.20 ± 6.18
Ventral prostate (mg)	545.71 ± 19.97	526.57 ± 59.29	576.00 ± 60.08	578.88 ± 45.72
Ventral prostate (mg/100g)	130.44 ± 7.24	105.59 ± 19.29	135.72 ± 13.40	134.50 ± 12.07
Full seminal vesicle (g)	1.63 ± 0.15	1.53 ± 0.09	1.73 ± 0.13	1.66 ± 0.12
Full seminal vesicle (g/100g)	0.37 ± 0.03	0.35 ± 0.02	0.41 ± 0.03	0.39 ± 0.03
Empty seminal vesicle (g)	0.85 ± 0.19	0.56 ± 0.11	0.61 ± 0.07	0.76 ± 0.11
Empty seminal vesicle (g/100g)	0.19 ± 0.04	0.13 ± 0.02	0.15 ± 0.02	0.18 ± 0.03
Vesicular secretion (g)	0.78 ± 0.08	0.98 ± 0.08	1.05 ± 0.07	0.90 ± 0.04
Vas deferens (mg)	97.50 ± 6.06	112.43 ± 6.64	109.25 ± 11.70	98.88 ± 5.11
Vas deferens (mg/100g)	22.55 ± 1.04	25.77 ± 1.30	25.79 ± 2.64	22.84 ± 1.25

Data are expressed as means ± SEM.The relative organ weight was calculated by use of the formula: organ weight/body weight x 100. Units: g: gram, mg: milligram; One-way ANOVA ($P>0.05$).

Table 2. Effect of treatment with EWH on sperm counts in testis and epididymis of rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters			**Experimental groups**	
Sperm count	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Testis				
Sperm number ($x10^6$)	109.4 ± 12.08	66.8 ± 8.40*	109.4 ± 11.69	107.8 ± 8.42#
Sperm number ($x10^6$/g)	82.84 ± 11.97	46.78 ± 8.85*	75.44 ± 6.52	79.50 ± 11.60#
DSP ($x10^6$/testis/day)	17.93 ± 1.98	10.95 ± 1.38*	17.94 ± 1.91	17.67 ± 1.50#
DSPr ($x10^6$/testis/day/g)	13.58 ± 1.96	7.67 ± 1.45*	12.37 ± 1.06	13.03± 1.90#
Epididymis				
Caput/ Corpus				
Sperm number ($x10^6$)	123 ± 20.47	105.7 ± 15.29	119 ± 15.76	120.7 ± 15.47
Sperm number ($x10^6$/g)	385 ± 47.62	343.9 ± 75.59	372.5 ± 41.55	398 ± 40.07
Sperm transit time (days)	6.90 ± 1.26	9.73 ± 1.55*	6.65 ± 0.78	6.91 ± 1.31#
Cauda				
Sperm number ($x10^6$)	186.6 ± 45.03	141.8 ± 11.66*	199 ± 29.21	203.9 ± 36.93#
Sperm number ($x10^6$/g)	856.6 ± 98.81	628.2 ± 69.85*	839.4 ± 67.01	877.5 ± 64#
Sperm transit time (days)	10.55 ± 2.77	13.05 ± 1.35	11.12 ± 1.72	11.58 ± 2.13

Data are expressed as mean ± SEM. Units: g: gram, mg: milligram. *vs Untreated; # vs $HgCl_2$; One-way ANOVA ($P<0.05$).

Table 3. Effect of treatment with EWH on sperm morphology of rats exposed to low doses of $HgCl_2$ for 60 days.

Parameters	Experimental groups			
	Untreated (n=8)	Mercury (n=8)	Hydrolysate (n=8)	Hydrolysate-Mercury (n=8)
Normal	91.75 (90.2 - 92.8)	64.5 (61.5– 76.5)*	86.5 (84.5 – 89)	91.5 (85.5 – 92)#
Head Abnormalities				
Amorphous	2 (1 – 4.2)	12.5(6.5 – 16.5)*	6(3.5 – 7.5)	2 (1 – 6)#
Banana Head	0.5 (0.1 – 0.8)	10.5 (3 – 12.5)*	1 (0 – 4)	3 (1 – 4.5)
Detached Head	3 (1.6 – 6.6)	3 (2 – 5)	1 (1 – 2)	2 (0.5 – 2)
Total of Head Abnormalities	5 (4.1– 8.6)	25.5(8.5 – 32.7)	9.2 (4.5 – 11.6)	5.7 (4.5 – 10.2)
Tail Abnormalities				
Bent Tail	1 (0.5 – 2)	3.2 (1.7 – 4.7)*	1.7 (0.5 – 3.3)	0.7 (0 – 1.5)#
Broken Tail	0 (0.0 – 0.3)	0.5 (0 – 1)	0 (0 – 0.5)	0 (0 – 0.5)
Total of Tail Abnormalities	2 (0.2 – 2.3)	3.2 (2.1 – 5.1)	2 (0.5– 5.2)	1 (0.1 – 2.1)

Data are expressed as median (Q_1 – Q_3). *vs Untreated; # vs $HgCl_2$; Kruskal-Wallis ($P<0.05$).