AMELIORATIVE EFFECTS OF EGG WHITE HYDROLYSATE ON RECOGNITION MEMORY IMPAIRMENTS ASSOCIATED WITH CHRONIC EXPOSITION TO LOW MERCURY CONCENTRATION

Danize Aparecida Rizzetti[a]*; Caroline Dalla Colletta Altermann[b]*; Caroline Silveira Martinez[a]; Franck Maciel Peçanha[a]; Dalton Valentim Vassallo[c]; José Antonio Uranga-Ocio[d]; Marta Miguel Castro[e]; Giulia Alessandra Wiggers[a]; Pâmela Billig Mello-Carpes[b]

Affiliations:

[a]Cardiovascular Physiology Laboratory, Universidade Federal do Pampa, BR 472, Km 592, Uruguaiana, Rio Grande do Sul, Brazil.

[b]Stress, Memory and Behavior Laboratory, Universidade Federal do Pampa, BR 472, Km 592, Uruguaiana, Rio Grande do Sul, Brazil.

[c]Cardiac Electromechanical and Vascular Reactivity Laboratory, Universidade Federal do Espírito Santo, Marechal Campos, 1468, Vitória, Espírito Santo, Brazil.

[d]Histology Laboratory, Universidad Rey Juan Carlos, Atenas s/n, Alcorcón, Spain.

[e]Bioactivity and Food Analysis Laboratory, Instituto de Investigación en Ciencias de la Alimentación, Nicolás Cabrera, 9, Campus Universitario de Cantoblanco, Madrid, Spain.

*authors contributed equally to this work.

Corresponding Author:

Danize Aparecida Rizzetti

Address: Cardiovascular Physiology Laboratory, Universidade Federal do Pampa, BR 472, Km 592, Uruguaiana, Rio Grande do Sul, Brazil.

Phone: (5555) 81064776 / E-mail: danize.rizzetti@gmail.com

ABSTRACT

The study aimed to investigate if the EWH is able to prevent the recognition memory disorders associated with long-term Hg exposure in rats. For this, male Wistar 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. Object recognition memory test was performed to verify Short (STM) and Long-Term Memory (LTM) and Open Field, Plus Maze and Tail Flick tests were performed as control for behavioral experiments. Reactive Oxygen Species (ROS) in hippocampus were determined by dichlorofluorescein diacetate (DCFH-DA) method, malondialdehyde (MDA) levels by TBARS, antioxidant power by FRAP assay and total Hg concentration by atomic fluorescence spectrometry. We confirm that the STM and LTM were impaired in adult rats exposed to Hg at low concentrations, which may be related to the increased metal deposition, ROS production and subsequently the oxidative damage in hippocampus. In addition, we demonstrated for the first time that EWH treatment is able to prevent memory impairment induced by Hg exposure, reducing Hg content and ROS production in hippocampus. In conclusion, EWH ameliorates memory impairments induced by chronic exposure to low doses of Hg. These findings may represent a good public health strategy since they indicate that EWH is a promising candidate as a new natural therapy for heavy metals intoxication.

Keywords: Mercury; Memory Impairments; Oxidative Stress; Egg White Hydrolysate; Antioxidant Activity.

1. INTRODUCTION

Memory formation is an important function of the hippocampus (Morris *et al*. 1982; Eichenbaum *et al*. 2007; Sokolowski *et al*. 2013), which plays a role in the consolidation of information from short-term (STM) to long-term memory (LTM) (Aggleton and Pearce 2001; Abo El-Khair *et al*. 2014). However, this cortical area is often a target of environmental contaminants that promotes neurological impairments and neurodegenerative diseases in early and later in life (Onishchenko *et al*. 2007; Grandjean and Landrigan 2014; Tolins *et al*. 2014).

Heavy metals are hazardous environmental contaminants related to various human health disorders, including neuropsychological dysfunctions (Sharma *et al*. 2014; Kim *et al*. 2016). Mercury (Hg) is known to be an environmental neurotoxicant potentially causing learning and emotional disturbances in humans and rodents (Nishchenko *et al*. 2007; Grandjean and Landrigan 2014; Tolins *et al*. 2014) and is associated to detrimental effects on memory (Morris *et al*. 1982; Eichenbaum *et al*. 2007; Falluel-Morel *et al*. 2007; Sokolowski *et al*. 2013). Previously, we described recognition memory deficits in adulthood rats after a chronic Hg exposure to low doses, similarly to human occupational exposition (Mello-Carpes *et al*. 2013). These effects were probably associated, at least in part, to oxidative stress evidenced in different tissues and organs in this experimental model (Wiggers *et al*. 2008) and others (Shim and Kim 2013; Cobbina *et al*. 2015; Wu *et al*. 2016b).

For many situations of metal induced-oxidative damage, strong chelating agents can be used to remove heavy metals or synthetic antioxidants can help to eliminate free radicals generated (Haber and Gross 2015). However, the toxicity of these chemical compounds limits its therapeutic application (You and Wu 2011). In this context,

exogenous dietary antioxidants may represent a safe and natural therapeutic alternative (Yu and Paetau-Robinson 2006).

Egg white proteins such as ovalbumin have demonstrated to possess antioxidant action and beneficial functions to human health (Sun *et al*. 2014). Previously we described that egg white hydrolysate protein (EWH) possess bioactive peptides with several biological properties such as antihypertensive and antioxidant activities (Davalos *et al*. 2004; Miguel *et al*. 2004). Additionally, peptides, released from ovalbumin by pepsin, have been shown to have the peroxyl radical-scavenging activity *in vitro* and *in vivo* and act in cardiovascular diseases (Miguel *et al*. 2006; Pokora *et al*. 2014).

Despite well-established evidence of oxidative damage to the Central Nervous System (CNS) and memory consolidation promoted by Hg, there are no studies reporting the effects of bioactive peptides from egg proteins as antioxidant therapeutic alternative for this metal exposure. Thus, the aim of our study was to investigate if the dietetic supplementation with EWH is able to prevent the recognition memory disorders associated with long-term Hg exposure in rats.

2. MATERIAL AND METHODS

2.1. Preparation of EWH

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 *Wistar* rats were purchased from Central Vivarium of Federal University of Santa Maria (RS/Brazil) and maintained in cages (5 animals each cage) in controlled environmental conditions (temperature 23 °C, humidity 60%) with 12 h light/darkness cycle with free access to tap water and fed with standard chow *ad libitum*. Rats were divided into four groups (n = 12/group), 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) Hydrolysate: received intramuscular injections of saline solution 0.9% and EWH diluted in tap water in a doses of 1 g/kg/day by gavage, according to prior work (Miguel *et al*. 2007); c) Mercury: received intramuscular 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 a model previously described (Wiggers *et al*. 2008) and tap water by gavage; d) Hydrolysate plus Mercury: received both treatments, $HgCl_2$ by intramuscular injections and EWH by gavage.

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. At the last week of treatment the animals were submitted to control behavioral experiments (open field, plus maze and tail flick; day 01), followed by memory tasks (object recognition test; 02–06 days). At the end of the treatment period, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *i.p.*), and euthanized by decapitation. Subsequently, the

hippocampus of some animals were excised from surrounding tissues and processed for biochemical analysis and metal determination.

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, Uruguaiana, Rio Grande do Sul, Brazil (institutional review board 0052014) 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. Short and Long-Term Memory Evaluation: Object recognition memory test (OR)

To verify the effects of Hg exposition and analyze the possible neuroprotection promoted by EWH on the short (STM) and long-term recognition memory (LTM) it was performed an OR task involving exposure to two different stimuli objects. For this, an open-field arena (50 cm × 50 cm × 50 cm) built in polyvinyl chloride plastic, plywood and transparent acrylic was used. All the OR procedures were performed in the light period in the absence of any specific behavioral stimulus, according to previously described (Myskiw *et al*. 2008). Animals firstly were habituated to the open-field apparatus for 20 min per day during 4 days before the training. After the habituation, in the training day, two different objects (named X and Y) made of metal, glass, or glazed ceramic were placed in the apparatus and the animals were allowed to explore them freely for 5 min. The testing was performed 3 h later to evaluate STM and 24 h later to evaluate LTM, in each case the rats were reintroduced into the apparatus for a 5 min period freely to explore; one of the objects was randomly changed for a novel object (W

or Z). The positions of the objects (familiar or novel) were randomly chosen for each experimental animal. Exploration was defined as sniffing or touching the objects with the nose and/or forepaws (sitting on or turning around the objects was not considered exploratory behavior). The object and the arena were cleaned with 70% ethanol after testing each animal to avoid confounds by lingering olfactory stimuli and preferences. The experiments were performed by an observer blind to the treatment condition of the animals. To statistical analyzes the data from OR task were converted in percentage of total exploration time (Mello-Carpes and Izquierdo 2013).

2.4. Control Behavioral Experiments: Open field (OF), Plus Maze (PM) and Tail Flick (TF)

To confirm that memory experiments did not suffer interference from possible behavioral changes promoted for both treatments, OF, PM and TF tests were performed as control experiments in all groups of rats to evaluate locomotor and exploratory activities, anxiety behavior and pain sensibility. For the OF test, at the end of the treatment rats were placed on the left quadrant of a 50 cm × 50 cm × 39 cm open field made of wooden painted in white, with a frontal glass transparent wall. Black lines were drawn on the floor to divide it into 12 equal quadrants. Crossings and rearing, as measures of locomotor and exploration, respectively, were measured over 5 min as previously described (Barros *et al*. 2006). The PM test was performed to assess the anxiety state after the treatment period as detailed in (Pellow *et al*. 1985). The maze had a central platform (5 cm × 5 cm), two open arms (50 cm long × 10 cm wide, 0.5 cm high borders) and two enclosed arms (50 cm deep × 10 cm wide, with 10 cm-high walls), elevated 50 cm above the ground. The animal was placed in the center of the apparatus facing the open arm and its locomotion was observed for 5 min. Total number

of entries in the open and closed arms and time spent in each one were recorded via infrared sensors over a 5 min session. The pain threshold at the end of the treatment was determined using the TF test, previously described (Tjolsen *et al*. 1989). For the TF test, pain was induced by giving infra-red light on the tail of the mice 5 cm away from the tip of the tail. Reaction time (tail-flick latency) was noted by observing the interval between placing the tail on the infra-red light source and the withdrawal of the tail.

2.5. Biochemical studies

2.5.1. Tissue preparation

Hippocampus was homogenized in 50 mM Tris–HCl at pH 7.4 (1/10, 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) was kept for subsequent biochemical and chemical measures.

2.5.2. Reactive Oxygen Species (ROS) measure

ROS levels were assessed spectrofluorometrically in hippocampus using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe as previously described (Ali *et al*. 1992). The sample (S1) was incubated in the dark with 5 µL of DCFH-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured as a method of detecting intracellular ROS. The formation of the oxidized fluorescent derivative (DCF) was measured by DCF fluorescence intensity recorded at 520 nm (488 nm excitation) (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA) for 60 min at 15 min intervals after the addition of DCFH-DA to the medium. The results were expressed as DCF AFU (arbitrary fluorescence unit of DCF).

2.5.3. Lipid peroxidation determination

Lipid peroxidation was evaluated in hippocampus 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.

2.5.4. Ferric Reducing Antioxidant Power (FRAP) assay

FRAP was performed according to the colorimetric method previously described (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) was mixed in a 10:1:1 ratio (v:v:v). After this, 1000 µL of this reagent was mixed with 10 µL of S1 in a test tube and incubated at 37∘C for 10min. The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex was read against blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). Standard dose-response curve of Trolox (50-1000 µM – water soluble analog of vitamin E) was performed and results are presented with particular reference to Trolox equivalents.

2.5.5. Protein quantification

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

2.6. Brain and Hippocampus Hg Quantification

Total Hg concentration was determined in brain and hippocampus 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 nanograms of Hg per g of tissue.

2.7. Data analysis and statistics

Data are presented as mean ± SEM. The OR task results were converted to a percentage of total exploration time and were analyzed using a one-sample t-test considering a theoretical mean of 50%. The OF, PM and TF tests data were analyzed using ANOVA followed by Duncan post hoc if necessary. Biochemical results were compared by ANOVA followed by Bonferroni post hoc. Values of $P < 0.05$ were considered significant.

3. RESULTS

3.1. Water and food intake and body weight

There was no change in water and food intake of rats after Hg exposure for 60 days neither in those groups that received the EWH co-treatment. The body weight was also similar between the experimental groups ($P > 0.05$, Table 1).

(TABLE 1)

3.2. Short and Long Term memory

During the training session rats from all treatment groups explored for a similar percent of total exploration time the two objects (X and Y). As expected, in the testing sessions the percent of time that untreated rats spent exploring the new object was significantly higher than 50%, indicating a preserved memory (66.96 ± 7.69%; $P < 0.0001$ for STM and 69.31 ± 10.99%; $P < 0.0005$ for LTM). However, Hg-treated rats spent about 50% of total time exploring the familiar and about 50% exploring the new object (W or Z) in both sessions, 3h and 24 h later, suggesting STM and LTM impairments (55.29 ± 22.09%; $P = 0.39$ for STM, and 53.80 ± 16.00%; $P = 0.47$ for LTM). Rats that received both treatments, Hg and EWH, spent more than 50% of total exploration time exploring the new objects (73.55 ± 9.77%; $P < 0.0001$ for STM and 61.47 ± 2.63%; $P < 0.0001$ for LTM), indicating that EWH intake was able to avoid the recognition memory deficits induced by the metal (Figure 1A and 1B).

(FIGURE 1)

3.3. Control behavioral experiments

None of the treatments altered the number of crossings and rearing during the 5 min long free OF exploration session ($P > 0.05$, Table 2). Similarly, there were no alterations in the total number of entries or in time spent at open arms during the PM and in the latency time to reaction in TF ($P > 0.05$, Table 2). These results confirm that

the results observed on OR task are related to $HgCl_2$ chronic exposure effects on memory, and they are not a result of anxiety, elevated pain threshold and/or affected locomotor or exploratory activity.

(TABLE 2)

3.4. Hippocampal ROS levels, lipid peroxidation and total antioxidant capacity

The levels of ROS were significantly elevated in hippocampus of Hg-treated rats compared to untreated rats ($P < 0.002$, Figure 2A). However, Hg intoxication did not change the MDA levels/lipid peroxidation in this tissue ($P = 0.11$, Figure 2B). Co-treatment with EWH caused a significant reduction in ROS levels ($P < 0.002$, Figure 2A), suggesting that it was able to prevent the oxidative stress caused by long-term Hg exposure. Regarding hippocampal total antioxidant capacity, the results showed that antioxidant capacity was not affected by Hg-treatment in this tissue ($P = 0.80$), while EWH intake caused a reduction of the antioxidant capacity power in hippocampus of rats exposed chronically to low doses of $HgCl_2$ ($P < 0.0001$, Figure 2C).

(FIGURE 2)

3.5. Hg levels in brain and hippocampus

Rats from Hg group exhibited a significant increase of Hg levels in brain and hippocampus after 60 days of treatment ($P < 0.0006$, Figure 3). However, the metal levels in the group that received the co-treatment of EWH were similar to untreated group ($P = 0.70$) and significantly reduced in comparison to Hg group ($P < 0.0006$, Figure 3). These data show that Hg accumulates on brain and hippocampus and suggest that the EWH was able to prevent the metal deposition in this tissue.

(FIGURE 3)

4. DISCUSSION

In the present study we demonstrated for the first time that EWH treatment is able to prevent STM and LTM impairments induced by low Hg concentration chronic exposure. In addition, we suggested that this memory deficit may be related to the metal deposition and the oxidative damage in hippocampus of adult rats exposed to this metal.

Hippocampus is involved in the learning and memory functions and plays a critical role in the process of forming and recovering certain types of memory (Squire 2004; Winocur *et al*. 2006). However, it is one of the brain areas more affected by environmental injuries. Despite the protection provided by the Blood-Brain Barrier (BBB), a significant amount of neurotoxicant agents have the ability to penetrate it and cause damage to the CNS (Aggleton and Pearce 2001; Abo El-Khair *et al*. 2014). The effects of the Hg as a neurotoxicant agent are well established and learning and memory impairments have been described even at environmentally relevant levels of this metal (Tofighi *et al*. 2011; Bernhoft 2012; Chehimi *et al*. 2012).

Decrease in memory and cognitive functions related to damage at hippocampal structure and reduction in the number of hippocampal neurons were observed in rats after a chronic treatment with low doses of organic Hg (Wu *et al*. 2016a). Despite its low liposolubility, inorganic Hg also demonstrated to induce STM and LTM impairments and behavioral changes associated to content of Hg in the hippocampus (Teixeira *et al*. 2014), cerebrum and cerebellum (Moraes-Silva *et al*. 2014). In accordance with these findings, previously we described in an adult animal experimental model of chronic Hg exposure, that simulates common human professional exposition to the metal (Wiggers *et al*. 2008), aversive and recognition

memory injury (Mello-Carpes *et al*. 2013). Although its mechanisms of action have not been elucidated in that situation, the mentioned study was important to show that even lower concentrations than those previously studied, which are within the limits set by regulatory agencies, also promote impairment in the CNS at a long-term.

In this current work we showed that the EWH intake was able to prevent the deficits on STM and LTM object recognition promoted by Hg at low concentrations. Bioactive peptides isolated from protein hydrolysis exhibited various biological activities such as antihypertensive (Kim *et al*. 2001), hypocholesterolemic (Kashima *et al*. 2014), metal-chelating, free radical scavenging and antioxidant activities (Homayouni-Tabrizi *et al*. 2015), acting on cardiovascular, metabolic and neurologic disorders (Gallegos-Tintore *et al*. 2011).

Previously a study showed that hydrolysates from porcine cerebral protein have the ability to protect against Pb^{2+}-induced learning and memory deficits and oxidative stress in developing mice (Zou *et al*. 2015). Hydrolysate of polygalasaponins also demonstrated to improve cognitive deficits induced by intrahippocampal injection of aged $A\beta_{25-35}$ in mice (Xu *et al*. 2011). Regarding to egg proteins, the EWH showed *in vitro* antioxidant capacity. The EWH with pepsin tood out for their peroxyl radical-scavenging activity (574 μmol Trolox/g protein) and for reducing the intracellular ROS levels in t-BOOH challenged RAW 264.7 macrophages, without any effect on cell viability, which suggests that the EWH could be useful to improve oxidative stress related pathologies, including neurodegenerative diseases (Davalos *et al*. 2004; Miguel *et al*. 2004).

When analyzed *in vivo*, the EWH treatment demonstrated to reverse the hypertension in SHR and Zucker rats due to its antioxidant and antihypertensive properties (Miguel *et al*. 2006; Pokora *et al*. 2014; Garces-Rimon *et al*. 2016). In

addition to these effects on cardiovascular system previously observed, in this study we demonstrated for the first time the beneficial effect of EWH also on CNS, protecting the hippocampus against memory impairments caused by Hg exposure.

Some of neuronal dysfunctions are related to oxidative stress in hippocampus promoted by the imbalance of ROS and antioxidants enzymes (Zhang *et al*. 2016). In the present study, we observed that the administration of Hg significantly increased hippocampal ROS formation and suggested that the memory impairment observed is associated with oxidative stress promoted by this metal. This result is in agreement with previous that reports increased ROS production in brain and mitochondria after chronic Hg exposure (Kim *et al*. 2015). We have also described in this model of exposure increased plasmatic and vascular ROS production and MDA levels (Rizzetti *et al*. 2013). Despite this evidence of lipid peroxidation in the cardiovascular system, in the current study no differences were found in MDA levels in hippocampus after Hg exposure compared to untreated rats. It suggests that in this level of exposure, is not possible to observe membrane damage and lipid peroxidation in this brain area, which usually occurs after a certain period of exposure to ROS.

The EWH intake was able to prevent the increase in the ROS production by Hg exposure in hippocampus, evidencing antioxidant activity also *in vivo.* Biological activities of protein hydrolysates are related to the composition and sequence of the amino acids, as well as the size of the peptides (Rao *et al*. 2012). The antioxidant activity of some peptides is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have a metal chelation potential (Ding *et al*. 2015). The presence of Tyr and Phe amino acids in the peptides is related to scavenging free radicals property (Sun *et al*. 2014). In addition, the His residues are directly associated to metal chelating property

(Gallegos-Tintore *et al*. 2011). Prior studies also have described the production of chelating peptides hydrolysates with His, Tyr and Phe amino acids residues, which consequently exert antioxidant activity (Torres-Fuentes *et al*. 2014). Taking into account that the main components of the EWH peptides are Tyr, His, Pro, Phe and Leu amino acids, we can suggest that the antioxidant effect of the EWH on the oxidative stress observed in hippocampus in this study is probably due to its metal chelating and subsequent free radicals scavenging activity.

The decreased FRAP value observed only in the Hydrolysate-Mercury group could confirm the chelating activity of the EWH. This technique is based on the power of the sample of donating electrons to reduce the ferric ion added to the medium, and indicates the antioxidant capacity of the sample as a reducing agent. The decreased values in the group that received both treatments would indicate a possible bond between Hg and EWH present in the sample, avoiding the donation of electrons to the ferric ion.

Regarding Hg accumulation in brain and hippocampus, studies related memory and behavior impairments after a chronic $HgCl_2$ exposition with a metal concentration in hippocampus ranging between 0.04 μg/g (Teixeira *et al*. 2014) and 0.4 μg/g of tissue (Moraes-Silva *et al*. 2014). In the present study we observed memory deficits with Hg hippocampus level of approximately 1 ηg/g which is considered lower than the previously described but suitable for simulating a common environmental exposure to the metal. Furthermore, this finding suggests that the level of Hg accumulation may directly be associated with the neurotoxic effect of Hg.

The exact mechanism by which $HgCl_2$ can penetrate through the BBB is unclear. Prior work reported, after exposure to metallic Hg vapor, the presence of inorganic Hg in brain, probably by its bond to selenium (Friberg and Mottet 1989). Recently

evidences suggest a disruption in Na/K ATPase activity in the cerebral vessels and a Ca^{2+}-mimetic action of the metal as the two possible pathways for inorganic Hg absorption by the CNS (Choi *et al*. 2011). Additionally, we demonstrated that EWH prevented the Hg accumulation in hippocampus, suggesting that EWH could interact peripherally to sequester Hg and prevent its uptake into the brain or could act directly across the BBB. Despite its mechanisms is poorly studied, the presence of peptides with Tyr residues at the N-terminal region of the amino acids sequence is related to the ability of the peptides cross the BBB (Teschemacher 2003).

In summary, our results show for the first time that EWH prevents memory impairments induced by chronic exposure to low doses of Hg, through the chelating and antioxidant effects on the hippocampus. These findings may represent a good public health strategy since they indicate that EWH is a promising candidate as a new natural therapy for heavy metals intoxication.

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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.

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CONTRIBUTION STATEMENT

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

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TABLES

Table 1. Effect of chronic low doses of $HgCl_2$ and of treatment with EWH on water and food intake and body weight of rats.

	Untreated (n = 12)	Hydrolysate (n = 12)	Mercury (n = 12)	Hydrolysate-Mercury (n = 12)
Water Intake (ml/day)	42.91 ± 1.37	39.93 ± 1.00	42.59 ± 1.35	42.65 ± 1.67
Food Intake (g/day)	22.23 ± 1.14	21.07 ± 1.05	21.11 ± 1.06	21.40 ± 1.09
Initial Body Weight (g)	245.20 ± 2.90	245.50 ± 2.30	245.00 ± 1.70	245.40 ± 2.48
Final Body Weight (g)	432.90 ± 14.21	419.70 ± 10.58	409.40 ± 8.30	418.38 ± 6.94
Total Weight Gain (g)	187.70 ± 11.77	174.20 ± 9.61	164.40 ± 7.22	173.25 ± 5.80

One-Way ANOVA, P>0.05.

Table 2. Effect of chronic low doses of $HgCl_2$ and of treatment with EWH on control

behavioral tests of rats.

	Untreated (n = 12)	Hydrolysate (n = 12)	Mercury (n = 12)	Hydrolysate-Mercury (n = 12)
Open Field				
Crossings (n)	60.13 ± 3.76	59.67 ± 7.32	52.88 ± 6.21	63.43 ± 4.80
Rearings (n)	21.29 ± 2.27	26.83 ± 4.36	24.63 ±2.88	20.29 ± 2.80
Elevated plus maze – time spent in open arm (s)	12.24 ± 5.39	17.52 ± 3.20	17.44 ± 7.45	16.01 ± 4.49
Tail flick – latency (s)	10.71 ± 2.15	9.33 ± 1.02	10.13 ± 1.88	9.42 ± 1.06

One-Way ANOVA, P>0.05.

FIGURE LEGENDS

Figure 1. Effects of treatment with EWH on object recognition short- and long-term memory of rats exposed to low doses of $HgCl_2$ for 60 days. A. The animals were trained on OR task and tested 3 h later to evaluate STM. In the training session the animals were exposed to objects X and Y. In the test session the rats were exposed to a familiar (X) and to a novel object (W). B. The animals were trained on OR task and tested 24 h after training to evaluate LTM. In the training session the animals were exposed to objects X and Y. In the test session the rats were exposed to a familiar (X) and to a novel object (Z). Data are expressed as mean ± SEM of the percent of total exploration time; * $P < 0.05$ in one-sample t-test, considering a theoretical mean of 50%; n = 12 per group.

Figure 2. Effects of treatment with EWH on ROS levels, lipid peroxidation and total antioxidant capacity in hippocampus of rats exposed to low doses of $HgCl_2$ for 60 days. A. Levels of ROS in the hippocampus measured by DCF fluorescent intensity. B. TBARS levels measured by MDA in hippocampus. C. Total antioxidant capacity of hippocampus measured by FRAP. Data are expressed as mean ± SEM; * $P < 0.05$ compared to untreated group; # $P < 0.05$ compared to Hg group; one-way ANOVA followed by Bonferroni post-hoc; n = 6 per group.

Figure 3. Effect of treatment with EWH on Hg levels in brain (A) and hippocampus (B) of rats exposed to low doses of $HgCl_2$ for 60 days. Data are expressed as mean ± SEM; * $P < 0.05$ compared to untreated group; # $P < 0.05$ compared to Hg group; one-way ANOVA followed by Bonferroni post-hoc; n = 6 per group.