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

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ABSTRACT

The study aimed to investigate whether the Egg White Hydrolysate (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: HgCl₂ (1st 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 behavioural experiments. Reactive Oxygen Species (ROS) in the hippocampus were determined by the 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 subsequent oxidative damage in the 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 the hippocampus. In conclusion, EWH prevents 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 metal 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 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 with 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 adult rats after chronic exposure to low Hg doses, similarly to human occupational exposure ([Mello-Carpes *et al.* 2013](#)). These effects were probably associated, at least in part, with 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 while synthetic antioxidants can help to eliminate the free radicals generated ([Haber and Gross 2015](#)). However, the toxicity of these chemical compounds limits their 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 been demonstrated to possess antioxidant action and beneficial functions in human health ([Sun *et al.* 2014](#)). Previously, we described that egg white hydrolysate protein (EWH) possesses 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 have been no studies reporting the effects of bioactive peptides from egg proteins as an antioxidant therapeutic alternative for this metal exposure. Thus, the aim of our study was to investigate whether dietetic supplementation with EWH is able to prevent 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 pasteurised 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°C), 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 a 12 h light/dark cycle, 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 (HgCl₂) 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 previously described model ([Wiggers *et al.* 2008](#)) and tap water by gavage; and d) Hydrolysate plus Mercury: received both treatments, HgCl₂ by intramuscular injections and EWH by gavage.

During the treatment, 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 behavioural 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 anaesthetised with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *i.p.*), and euthanised by decapitation.

Subsequently, the hippocampus of some animals was excised from the 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 the 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 minimise 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 exposure and analyse the possible protection promoted by EWH on the short (STM) and long-term recognition memory (LTM), an OR task involving exposure to two different stimuli objects was performed. For this, an open-field arena (50 cm × 50 cm × 50 cm) composed of polyvinyl chloride plastic, plywood and transparent acrylic was used. All of the OR procedures were performed in the light period in the absence of any specific behavioural stimulus, as previously described ([Myskiw *et al.* 2008](#)). Animals were first habituated to the open-field apparatus for 20 min per day during 4 days before the training. After habituation, on 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 behaviour). The object and the arena were cleaned with 70% ethanol after the testing of each animal to avoid confusion due to lingering olfactory stimuli and preferences. The experiments were performed by an observer blind to the treatment condition of the animals. To statistically analyse the data from OR tasks, they were converted into the percentage of total exploration time (Mello-Carpes and Izquierdo 2013).

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

To confirm that memory experiments did not suffer interference from possible behavioural 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 behaviour and pain sensibility. For the OF test, at the end of the treatment, rats were placed on the left quadrant of a 50 cm $\times$ 50 cm $\times$ 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 $\times$ 5 cm), two open arms (50 cm long $\times$ 10 cm wide, 0.5 cm high borders) and two enclosed arms (50 cm deep $\times$ 10 cm wide, with 10 cm-high walls), elevated 50 cm above the ground. The animal was placed in the centre of the

apparatus facing the open arm and its locomotion was observed for 5 min. The total number of entries in the open and closed arms and time spent in each was recorded via infrared sensors over a 5 min session. The pain threshold at the end of the treatment was determined using the TF test, as described previously ([Tjolsen *et al.* 1989](#)). For the TF test, pain was induced by focusing infra-red light on the tail of the mice 5 cm 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 homogenised 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 the 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 DCFH-DA to fluorescent dichlorofluorescein (DCF) was measured as a method of detecting intracellular ROS. The formation of the oxidised 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 the 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 sulphate solution (8%) at 95°C for 1 h, and the colour 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 FeCl₃ (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 Fe³⁺-TPTZ complex to a coloured Fe²⁺-TPTZ complex was read against a blank reagent (1 mL FRAP reagent + 10 µL distilled water) at 593 nm (Spectramax M5 Microplate/Cuvette Reader, Molecular Devices, Pennsylvania, USA). The standard dose-response curve of Trolox (50-1000 µM – water soluble analogue of vitamin E) was performed and the 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 analyser (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 vapour instead of the chemical reduction step used in traditional liquid-based analysers. Samples were weighed directly in an 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 $\pm$ SEM. The OR task results were converted to a percentage of total exploration time and were analysed using a one-sample t-test considering a theoretical mean of 50%. The OF, PM and TF tests data were analysed 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 or in those groups that received the EWH co-treatment. Body weight was also similar between the experimental groups ($P > 0.05$; data not shown).

3.2. Short and Long Term memory

During the training session, rats from all treatment groups explored the two objects (X and Y) for a similar percent of total exploration time. 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 ($66.96 \pm 7.69\%$; $P < 0.0001$ for STM and $69.31 \pm 10.99\%$; $P < 0.0005$ for LTM). However, Hg-treated rats spent about 50% of the 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 ($55.29 \pm 22.09\%$; $P = 0.39$ for STM, and $53.80 \pm 16.00\%$; $P = 0.47$ for LTM). Rats that received both treatments, Hg and EWH, spent more than 50% of the total exploration time exploring the new objects ($73.55 \pm 9.77\%$; $P < 0.0001$ for STM and $61.47 \pm 2.63\%$; $P < 0.0001$ for LTM), indicating that EWH intake prevented the recognition memory deficits induced by the metal (Figure 1A and 1B).

3.3. Control behavioural experiments

None of the treatments altered the number of crossings and rearings during the 5 min long free OF exploration session (Crossings, n – Untreated: 60.13 ± 3.76 ; Hydrolysate: 59.67 ± 7.32 ; Mercury: 52.88 ± 6.21 ; Hydrolysate-Mercury: 63.43 ± 4.80 ; Rearings, n – Untreated: 21.29 ± 2.27 ; Hydrolysate: 26.83 ± 4.36 ; Mercury: 24.63 ± 2.88 ; Hydrolysate-Mercury: 20.29 ± 2.80 ; $n = 12$, one-way ANOVA, $P > 0.05$).

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 (Time spent in open arm on PM, s – Untreated: 12.24 ± 5.39 ; Hydrolysate: 17.52 ± 3.20 ; Mercury: 17.44 ± 7.45 ; Hydrolysate-Mercury: 16.01 ± 4.49 ; Latency on TF, s – Untreated: 10.71 ± 2.15 ; Hydrolysate: 9.33 ± 1.02 ; Mercury: 10.13 ± 1.88 ; Hydrolysate-Mercury: 9.42 ± 1.06 ; $n = 12$, one-way ANOVA, $P > 0.05$). These results confirm that the results observed for the OR task are related to HgCl_2 chronic exposure effects on memory, and are not a result of anxiety, elevated pain threshold and/or affected locomotor or exploratory activity.

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).

3.5. Hg levels in brain and hippocampus

Rats from the Hg group exhibited a significant increase of Hg levels in the 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 those in the

untreated group ($P = 0.70$) and significantly reduced in comparison to the Hg group ($P < 0.0006$, Figure 3). These data show that Hg accumulates in the brain and hippocampus and suggest that EWH was able to prevent metal deposition in this tissue.

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 chronic exposure to low Hg concentrations. In addition, we suggested that this memory deficit may be related to the metal deposition and oxidative damage in the hippocampus of adult rats exposed to this metal.

The hippocampus is involved in 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 commonly 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 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](#)).

Decreases in memory and cognitive functions related to damage to the hippocampal structure and a reduction in the number of hippocampal neurons were observed in rats after chronic treatment with low doses of organic Hg ([Wu *et al.* 2016a](#)). Despite its low liposolubility, inorganic Hg was also demonstrated to induce STM and LTM impairments and behavioural changes associated with the content of Hg in the

hippocampus ([Teixeira et al. 2014](#)), cerebrum and cerebellum ([Moraes-Silva et al. 2014](#)).

In accordance with these findings, we previously described aversive and recognition memory injury (Mello-Carpes *et al.* 2013) in an adult animal experimental model of chronic HgCl₂ exposure for 30 days that simulates common human professional exposition to Hg, with a blood Hg concentration about 8 ng/mL at the end of the treatment ([Wiggers et al. 2008](#)). Although its mechanisms of action have not been elucidated in that situation, the mentioned study was important as it showed that even lower concentrations than those previously studied, which are within the limits set by regulatory agencies, also promote impairment in the CNS in the long-term.

On the other hand, in the current study, the Hg concentration in the blood of HgCl₂-treated rats was approximately 3.04 ng/mL (unpublished data). This reduction in the Hg blood levels after a long-term exposure when compared with those observed after 30 days of HgCl₂ exposure suggests that Hg has probably left the bloodstream and deposited into the organs, as the brain and hippocampus.

In this current work, we showed that the EWH intake was able to prevent deficits in 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](#)), hypocholesterolaemic ([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 Pb²⁺-induced learning and memory deficits and oxidative stress in developing mice ([Zou et al. 2015](#)). Hydrolysate of polygalasaponins also

improved cognitive deficits induced by intrahippocampal injection of aged A β _{25–35} in mice (Xu *et al.* 2011). Regarding egg proteins, the EWH showed *in vitro* antioxidant capacity. The EWH with pepsin removed due to the peroxy radical-scavenging activity (574 μ mol 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 analysed *in vivo*, the EWH treatment was 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 previously observed effects on the cardiovascular system, 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 neuronal dysfunctions are related to oxidative stress in the 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 the brain and mitochondria after chronic Hg exposure (Kim *et al.* 2015). We have also described increased plasmatic and vascular ROS production and MDA levels in this model of exposure (Rizzetti *et al.* 2013). Despite this evidence of lipid peroxidation in the cardiovascular system, no differences were found in MDA levels in the hippocampus after Hg exposure compared to untreated rats in the current study. It suggests that it is not possible to observe

membrane damage and lipid peroxidation in this brain area following this level of exposure, which usually occurs after a certain period of exposure to ROS.

The EWH intake was able to prevent the increase in ROS production by Hg exposure in the hippocampus, proving antioxidant activity *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 donors, 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 radical properties (Sun *et al.* 2014). In addition, the His residues are directly associated with 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 acid 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 EWH on the oxidative stress observed in the hippocampus in this study is probably due to its metal chelating and subsequent free radical scavenging activity.

The decreased FRAP value observed only in the Hydrolysate-Mercury group could confirm the chelating activity of EWH. This technique is based on the power of the sample to donate electrons to reduce 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 indicates a possible bond between Hg and EWH present in the sample, avoiding the donation of electrons to the ferric ion.

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

The exact mechanism by which HgCl_2 can penetrate through the BBB is unclear. Previous work reported, after exposure to metallic Hg vapour, the presence of inorganic Hg in brain, probably due to its bond with selenium (Friberg and Mottet 1989). Recent evidence suggests 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 Hg accumulation in the 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 having been poorly studied, the presence of peptides with Tyr residues at the N-terminal region of the amino acid sequence is related to the ability of the peptides to 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, analysed 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 approved the final manuscript.

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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 $\pm$ 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 the 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 the hippocampus. C. Total antioxidant capacity of the hippocampus measured by FRAP. Data are expressed as mean $\pm$ 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 HgCl₂ for 60 days. Data are expressed as mean $\pm$ 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.

HIGHLIGHTS

Mercury (Hg) promotes Short- and Long-Term Memory impairments in adulthood rats.

Memory deficits appear to be related to the oxidative stress caused by the Hg.

Egg White Hydrolysate (EWH) prevents Hg-induced memory impairments.

EWH likely inhibits Hg-induced damage through its antioxidant and chelating effects.

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