EGG WHITE HYDROLYSATE PROMOTES NEUROPROTECTION FOR NEUROPATHIC DISORDERS INDUCED BY CHRONIC EXPOSURE TO LOW CONCENTRATIONS OF MERCURY

Egg white hydrolysate and neuroprotection for Hg

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ABSTRACT

This study aims to investigate whether the egg white hydrolysate (EWH) acts on the neuropathic disorders associated with long-term Mercury (Hg) exposure in rats. 8-week-old male Wistar rats were treated for 60 days with: a) Control - saline solution (*i.m.*); b) Mercury - $HgCl_2$ (1^{st} dose 4.6 μg/kg, subsequent doses 0.07 μg/kg/day, *i.m.*); c) Hydrolysate - EWH (1 g/kg/day, gavage); d) Mercury and Hydrolysate. Mechanical allodynia was assessed using Von Frey Hairs test; heat hyperalgesia by the plantar test; catalepsy by a modification of the "ring test" and spontaneous locomotor activity by a photocell activity chambers. Analyses were performed at 0, 30 and 60 days of treatment. Brain and plasma MDA, plasma NPSH and TNF-α determination and skin immunohistochemistry were performed at 60 days. Hg induced a reduction in mechanical sensitivity threshold at 30 and 60 days and in thermal sensitivity threshold at 60 days. At the end of treatment catalepsy was developed, but there was not significant alteration in spontaneous locomotor activity. Hg also increased brain and plasma MDA, plasma NPSH and TNF- α levels and the number of Merkel cell–neurite complex in the skin. EWH prevented the development of mechanical allodynia, thermal hyperalgesia and catalepsy induced by Hg and the increase in MDA concentration in brain and plasma and in the number of Merkel cell–neurite complex in the skin. In conclusion, EWH promotes neuroprotection against the toxic effects caused by Hg, demonstrating a beneficial therapeutic potential.

Keywords: Mercury; Peripheral Neuropathy; Motor Behavioral Disorders; Oxidative Stress; Egg White Hydrolysate.

1. INTRODUCTION

Mercury (Hg) is a toxic xenobiotic compound actually considered one of the major environmental pollutants (Park & Zheng, 2012). Often human exposure to Hg occurs in a chronic manner, by its contact over the years at low concentrations in situations such as ocupational exposure, dietary (mainly fish intake) and use or handling dental amalgam (Chen et al., 2005). In these conditions, metal exposure can promote human intoxication by organic (from food) (Montgomery et al., 2008), inorganic (from industrial activity) (Teixeira et al., 2014; Moraes-Silva et al., 2014) and elementar forms (from dental amalgam restorations) (Echeverria et al., 2005).

The main target organs for Hg toxicity are brain (Yoshida et al., 2014), myocardium (Vassallo et al., 2011), liver and kidney (Joshi et al., 2014), skin (Moody et al., 2009), lung (Lim et al., 1998), testis and prostate (Martinez et al., 2014), which are associated to dysfunction after acute and chronic exposure. Studies about Hg damage were initiated after environmental disasters involving methylmercury exposure as occurred in Minamata (1953), Niigata (1960) and Iraq (1971) (Clarkson & Strain, 2003). In all cases, negative consequences for health in the residents followed for several years after disasters, manifested mainly through neurological diseases (Bernhoft, 2012).

Although there is a strong association between exposure to organic mercury and the development of neurological disorders, there is a growing number of studies showing that exposure to inorganic mercury can also promote damage to the central and peripheral nervous system (Chehimi et al., 2012). Inorganic Hg is a powerful pro-oxidant element and it presents strong affinity for selenium. Due to a higher biological half-life, which is estimated to be about 60 days, the long-term retention of this metal

may play a role in inducing or promoting oxidative stress, neurodegenerative diseases and memory impairment (Wiggers et al., 2008b; Mello-Carpes et al., 2013).

In recent years, the use of natural substances considered antioxidants or chelating agents has been widely studied in poisoning by Hg and other heavy metals, which showed protective effect with the neutralization of reactive oxygen species (ROS) and increase of antioxidant functions (Cordero-Herrera et al., 2013). In this context, various bioactive peptides from food proteins have shown biological activities such as antioxidant, antihypertensive, inmunomodulating, hypocholesterolemic or antimicrobial (Freitas et al., 2013). Egg white is a very valuable source of proteins for human nutrition, due to their variety and functional capacity, and recently bioactive peptides derived from egg proteins have been described. Our research group had developed an egg white hydrolysate from pepsin hydrolysis whose peptides components, previously identified (Miguel et al., 2004), possess *in vitro* (Miguel et al., 2006) and *in vivo* (Moreno et al., 2015) antioxidant properties, reducing some complications related with pathologies associated with oxidative stress conditions. Thus, the aim of our study was to investigate whether this egg white hydrolysate (EWH) is able to act on the peripheral neuropathy and motor behavioral disorders associated with long-term Hg exposure in rats.

2. RESULTS

2.1. Food and drink intake and body weigh

Daily basal food and water intake of the rats were similar between the different groups at the end of the treatment (Food intake, in g/day – Control: 22.2±1.1, Mercury: 21.1±1.1, Hydrolysate: 21.1±1.0, Hydrolysate-Mercury: 21.4±1.1; Water intake, in

ml/day – Control: 42.9±1.4, Mercury: 42.6±1.3, Hydrolysate: 39.9±1.0, Hydrolysate-Mercury: 42.6±1.7, n=8, One-Way ANOVA, $p>0.05$). The rate of body weight gain was also similar among all the experimental groups (Total weight gain, in g, – Control: 187.7±11.8, Mercury: 164.4±7.2, Hydrolysate: 174.2±9.6, Hydrolysate-Mercury: 173.2±5.8, n=8, One-Way ANOVA, $p>0.05$).

2.2. Peripheral Neuropathy and Motor Behavioral Measures

Chronic exposure to low concentrations of Hg produced a significant reduction in the mechanical sensitivity threshold after 30 and 60 days of Hg treatment when compared this variable to control group, demonstrating the presence of mechanical allodynia. Interestingly, the EWH administration prevented the reduction of mechanical sensitivity induced by Hg exposure (Figure 1A, two-way ANOVA, n=8, $^{*}p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury). The heat hyperalgesia was evident after 60 days of Hg exposure and it was demonstrated by a significant decrease in the thermal sensitivity threshold in the mercury treated group when compared to control group. This decrease was parcially prevented when EWH was co-adminisered. EWH group showed similar values to control group in the mechanical sensitivity threshold after 30 and 60 days of the experimental study (Figure 1B, two-way ANOVA, n=8, $^{*}p<0.05$ vs Control).

(FIGURE 1)

In addition to peripheral neurologic harmful demonstrated by Hg exposure, it was observed catalepsy development in Hg-treated rats at the end of the treatment, suggesting the presence of motor behavioral disorders after long-term Hg exposure. The EWH administration totally prevented the catalepsy development (Figure 2A, two-way ANOVA, n=8, $^{*}p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury). Spontaneous motor activity was not significantly modified in the different experimental groups (Figure 2B).

Taken together, these findings suggest the presence of peripheral neuropathy and motor behavioral disorders in Hg-treated experimental group and evidence the potential of EWH to act against the neurologic damage promoted by the metal.

(FIGURE 2)

2.3. Biochemical and Immunohistochemical Measures

Chronic treatment with low doses of Hg demonstrated a significant increase in plasma and brain levels of MDA and plasma NPSH groups when compared these parameters to control group. EWH co-treatment prevented in both samples the increase in MDA levels promoted by the metal exposure (Figure 3A, one-way ANOVA, n=8, $^{*}p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury; Figure 3B, one-way ANOVA, n=8, $^{*}p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury). Increased plasma levels of NPSH groups also were observed in the group of the rats that received only EWH. However, EWH co-tretament did not modify the increase of plasma thiol groups resulting from exposure to the metal (Figure 3B, one-way ANOVA, n=8, $^{*}p<0.05$ vs Control). Regarding TNF-α plasma levels, Hg-treated rats showed higher levels than in control and EWH-treated rats. However, these values remained higher in Hg rats co-treated with EWH (Figure 4, one-way ANOVA, n=8, $^{*}p<0.05$ vs Control). These findings suggest that oxidative stress and inflammation factors are related to the neuropathic and motor behavioral disorders presents in inorganic Hg intoxication and evidence that EWH could acts on the oxidative stress in the nervous system but not on the inflammatory process caused by Hg exposure.

(FIGURE 3)

(FIGURE 4)

To analyze the presence of low threshold mechanoreceptors observed by Hg exposition, we performed an immunohistochemistry of the skin with UCH-L1 antibody. We found an increase in the number of Merkel cell–neurite complex in the Hg-treated rats compared to control rats, while the groups that received just hydrolysate or it associated with Hg were similar to control group (Figure 5, one-way ANOVA, n=8, $^{*}p<0.05$ vs Control). These findings can confirm the presence of neuropathic disorders by Hg-induced oxidative stress in this model of chronic exposure at low levels and suggest a probable role of EWH on the ROS generation by the metal.

(FIGURE 5)

2.4. Brain Mercury Quantification

Mercury levels in brain exhibited a significant increase after 60 days of $HgCl_2$ treatment when compared to control group. This tissue in Hydrolysate-Mercury group had significant lower concentrations of Hg compared to those of Hg-treated rats. The animals treated only with EWH showed similar values of mercury levels in the brain that control group (Total Hg concentration, in μg/g – Control: 0.6±0.2; Mercury: 2.1±0.3*; Hydrolysate: 0.2±0.0; Hydrolysate-Mercury: 0.8±0.2$^{\#}$; n=8, One-Way ANOVA, $^{*}p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury). These data show that inorganic mercury is also able to accumulate on structures of the central nervous system and suggest that the EWH prevented the deposition of Hg in the brain.

3. DISCUSSION

This is the first study, to the best of our knowledge, which shows that long-term exposure to inorganic Hg at low doses produces peripheral neuropathy and motor

behavioral disorders in adult rats, which are associated to oxidative stress and pro-inflammatory factors generated by metal exposure. In addition, we demonstrated that EWH intake during the same period of exposition to Hg can prevent the appearance of neuropathic dysfunctions acting against the oxidative stress and as a chelating compound.

Neural dysfunction and cognitive deficits are well established as some of the several consequences of Hg toxicity (Montgomery et al., 2008). Indeed, inorganic Hg is able to induce distinct neurotoxic effects which depend on its concentration and time exposure. Previously we described an experimental animal model of long-term exposition to low inorganic Hg doses, similar to human exposure, which showed Hg-induced oxidative stress and memory impairments after 30 and 60 days of metal exposure in adult rats (Wiggers et al., 2008b; Mello-Carpes et al., 2013).

In our study, we observed the development of sensorial peripheral neuropathy in Hg-treated rats after 60 days of metal exposition which was evidenced by the reduced mechanical and thermal sensory thresholds and the presence of mechanical allodynia and heat hyperalgesia. These findings suggest as a mechanism of involvement the damage of both thinly myelinated and unmyelinated fibers (Aδ and C types), which are more sensitive to thermal sensations, and large myelinated fibers (Aβ and Aγ types), responsibles for touch and pressure sensations (Xu et al., 2014). In accordance with the functional findings, the immunohistochemistry demonstrated in this study an increase in the number of Merkel cells in skin hindpaw of rats chonically exposed to inorganic Hg. Changes of these mechanoreceptors confirm the sensory involvement in peripheral neuropathy promoted by the Hg through the damage of large myelinated fibers Aβ type (Alsunousi & Marrif, 2014). In fact, althoug there are few studies that have focused on peripheral nerve impairment among persons after low-level Hg exposure, it has been

documented, a decrease in nerve conduction velocity, axonal degeneration and demyelinating changes in peripheral neuropathy (Kingman et al., 2005). These effects are related partly to metal binding to sulfhydryl groups and oxidative stress promoted by Hg (Clarkson & Strain, 2003). However, the mechanisms involved in the pathogenesis of this toxic axonopathy remain unclear.

Regarding Hg-induced oxidative stress, we found an increase in MDA levels in plasma and brain, which related to increased lipid peroxidation, and an increase in plasma non-proteic thiol groups, which represent the non-enzymatic antioxidant defenses (cysteine and glutathione) in rats after 60-days of Hg treatment. Lipid peroxidation have been proposed for the neurotoxicity induced by $HgCl_2$ (Mahboob et al., 2001) and evidence that reactive oxygen species (ROS) generation is a mediator of brain and nerves injury in several animal models of $HgCl_2$-induced toxicity (Sener et al., 2007). Previous findings of our group also showed that exposure to low doses of $HgCl_2$ during 30 days increases plasma thiol (SH) levels and lipid peroxidation suggesting that Hg can activate a variety of pro-oxidant factors as NADPH oxidase enzyme and produce a compensatory mechanism involving the endogenous antioxidants peptides in rats (Rizzetti et al., 2013). Our findings are in accordance with decribed previously for 30 days to Hg exposure and suggest that oxidative stress induced by inorganic Hg is related to neurophatic changes observed after a long-term Hg exposition.

Indeed, oxidative stress is associated with damage in peripheral nerve conduction and axonal degeneration in others diseases, as diabetes-induced peripheral neuropathy (Shun et al., 2004). In this condition, skin immunohistochemistry revealed that neurotrophin-3 (NT-3), an essential molecule for the development of Merkel cell afferent nerve fibres, was significantly higher in affected skin biopsies from patients with diabetic neuropathy (Kennedy et al., 1998) and was attributed to the severity

degree of skin denervation caused by oxidative stress in diabetic neuropathy. In view of this finding, we suggest in our study that the changes in the skin nerves observed in Hg treated rats is associated with oxidative stress induced by long-term metal exposure, which promotes an increase in Merkel cells mechanoreceptors as consequence of a compensatory mechanism due to innervation degeneration.

In addition to oxidative stress induced by inorganic Hg, our study also evidenced an increase in plasma TNF-α level, which is an important proinflammatory biomarker and a neurological injury indicator (Woodcock & Morganti-Kossmann, 2013). A few authors have decribed association between inorganic Hg exposure and increase in immune cell release of the proinflammatory cytokines (Gump et al., 2014; Pecanha et al., 2010). These studies suggest that ROS generation from Hg-induced pro-oxidant enzymes may be responsible for over expression of proinflammatory mediators and in consequence the association between oxidative stress and inflammation in Hg intoxication.

Despite the evident damage caused by Hg on the nervous system, EWH was able to normalize the neurofunctional parameters altered by Hg exposure assessed in 30 and 60 days of treatment, which was related to action on the oxidative stress and the alteration in the Merkel cells-complex number promoted by Hg exposure, suggesting its powerful antioxidant potential. However, since our findings reveal that EWH prevented the increase in MDA levels but did not alter the increment in TNF-α levels, we suggest that $HgCl_2$ could induce neuropathic injury through two pathways, by activating pro-oxidant factors and promoting oxidative damage and stimulating proinflammatory mediators release.

Previously we demonstrated that hydrolysis of egg white proteins with pepsin enzyme produces hydrolysates with *in vitro* and *in vivo* antihypertensive and antioxidant

capacities (Manso et al., 2008). Recently, a study involving egg protein hydrolysate intake showed a beneficial effect *in vivo* by attenuating renal damage development and preventing aortic endothelial dysfunction, demonstrating an antiinflammatory potential (Wang et al., 2012). In this study we suggest that antioxidant capacity of the EWH is the main mechanism of action on oxidative damage induced by Hg in nervous system. In fact, the potential antioxidant capacity of egg proteins depends of type and position of amino acid residues contained in the structure of the peptides (Zambrowicz et al., 2015). Some studies demonstrated that the antioxidant property of egg yolk hydrolysates is determined by the presence of peptides composed of the amino acid leucine at their N-terminal position due to the ability of this amino acid to readily cleave hydrogen (Eckert et al., 2014). Interstingly, the EWH posses the amino acid leucine at N-terminal position in several peptides indentified (Miguel et al., 2004), demonstrating that its therapeutic potential is associated to a powerful antioxidant effect.

Regarding motor behavioral disorders observed in this study we also evidence deficits in the central nervous system and motor behavior changes, with development of catalepsy, however, without changes in motor activity. These results agreed with other studies that showed changes in catalepsy related to cortical and cerebellar Hg content in rats and consequent acetylcholinesterase activity and brain dopamine system alterations (Olczak et al., 2001). We also observed Hg deposition in brain tissue suggesting damage at central structures, as the basal ganglia, by Hg accumulation and and subsequent oxidative stress as a possible mechanism for motor behavioral disorders here presented. In this study we also observed that the EWH prevented Hg accumulation in the brain, suggesting a possible chelating effect at the Central Nervous System (CNS). Since the EWH presents some peptides composed by a tyrosine residue at the N-terminal region in amino acids sequence which is related to the ability of the peptide

cross the Blood-Brain Barrier (BBB) (Teschemacher, 2003), we suggest that EWH is able to cross, acting as a chelating compound and in consequence exerting an antioxidant effect in the CNS. Previous studies reported food products and metal lingant proteins as potent chelators for heavy metals, include in Hg exposure, reducing the brain and blood metal concentration (Klaassen et al., 2009). Natural compounds, especially peptides can reduce absorption or reabsorption of toxic metals and to support natural detoxification pathways. Its efficiency as a chelating compound is due to sulfur composition which have great affinity for heavy metals, increasing and improving its excretion (Sears, 2013).

In summary, our results suggest for the first time that EWH supplementation can exert a beneficial effect against peripheral neuropathic dysfunction and motor behavioral disorders promoted by chronic exposure to low concentrations of Hg for 60 days, acting as a chelating compound, preventing oxidative and neural damage development, showing to be a beneficial antioxidant therapeutic strategy.

4. EXPERIMENTAL PROCEDURE

4.1. Ethics Aspects

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). The experiments were 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.

4.2. Animals and Study Design

8-week-old male Wistar rats (200-250 g) were maintained under environmentally controlled 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 of eight rats each, which were treated for 60 days with: a) intramuscular injections (*i.m.*) of saline solution 0.9% and tap water by gavage (Control); b) *i.m.* injections of mercury chloride ($HgCl_2$), the 1st dose 4.6 μg/kg, and subsequent doses of 0.07 μg/kg/day, to cover daily loss, using model described previously (Wiggers et al., 2008a) and tap water by gavage (Mercury); c) *i.m.* injections of saline solution 0.9% and EWH from pepsin for 8 hours diluted in tap water (1 g/kg/day), by gavage, according to model describe by (Miguel et al., 2006) (Hydrolysate); d) both treatments (Hydrolysate – Mercury). 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.

4.3. Tactile sensitivity: Von Frey hair test

Mechanical sensitivity was assessed by measuring the withdrawal threshold to calibrated von Frey hairs (Bioseb Instruments, USA) (Vera et al., 2007). The test was realized at the start (0 day), in 30 and 60 days of treatment. Rats were placed individually on an elevated iron mesh in a clear plastic cage and the filaments were applied to the plantar aspect of each hindpaw, from below the mesh floor. Each stimulus was applied for approximately 1 second with an interstimulus interval of approximately

3 second. A significant decrease in von Frey hair threshold evoked by mechanical stimulus was defined as presence of mechanical allodynia.

4.4. Thermal sensitivity: plantar test

Responses to thermal stimuli were evaluated right after mechanical sensitivity using a plantar test apparatus (Ugo Basile, Comerio VA, Italy) (Bennett & Xie, 1988). During the testing days rats were placed within a plastic compartment on a glass floor and a light source beneath the floor was aimed at the mid plantar surface of the hindpaw. So, the withdrawal reflex interrupts the light and automatically turns-off the light and a timer. The withdrawal latency of each paw was measured during three trials at 2 min intervals and the mean of the three readings was used for data analysis.

4.5. Catalepsy

Catalepsy was measured using a modification of the "ring test" (Fox et al., 2001). Rats were hung by their front paws from a rubber-coated metal ring fixed horizontally at a height that allowed their hindpaws to just touch the bench. The time taken for the rat to move-off the ring was measured with a cut-off limit of 30s.

4.6. Spontaneous locomotor activity

Spontaneous locomotor activity was evaluated in 30 and 60 days of treatment using individual photocell activity chambers (Cibertec S.A., Madrid, Spain) (Vera et al., 2007). For this, rats were placed in the recording chambers and the number of interruptions of photocell beams was recorded over a 30-min period. Total number of activity counts throughout the 30 min of test duration was recorded. The mean number of crossings of the photocell beams was used for comparison.

4.7. Blood and Tissue Collection

At the end of the treatment period, after the behavioral assessment, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *i.p.*), and after loss of the righting reflex they were submitted to an aorta artery puncture and blood was subsequently collected to obtain plasma for the biochemical experiments. Thereafter, rats were euthanized by decapitation, and the brain and the plantar surface skin of the right hindpaw were carefully removed for biochemical and immunohistochemistry analysis.

4.8. Plasma and brain malondialdehyde levels

Malondialdehyde (MDA) levels in plasma and brain was determined colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). To prepare the tissue samples, brain 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 and 4°C to yield a pellet that was discarded and a low-speed supernatant (S1) was used for the measure. Thus, an aliquot of plasma or S1 were incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4) or acetic acid buffer, and sodium dodecil sulphate 8.1% (SDS) at 95ºC for 60 min. The color reaction was measured at 532 nm against blanks (Spectrophotometer Femto 600 S, FEMTO, São Paulo, Brazil). The results were expressed as nanomoles of MDA / ml of plasma or nanomoles of MDA / mg of protein.

4.9. Plasma Non-Proteic Thiol Groups Levels

NPSH was estimated in plasma (Ellman, 1959) mixed with 10% trichloroacetic acid (TCA) and centrifuged. An aliquot of supernatant was added in potassium phosphate buffer 1M, pH 7.4, and the absorbance was measured at 412 nm against blank (Spectrophotometer Femto 600 S, FEMTO, São Paulo, Brazil). Then, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) 10 mM was added followed by incubation at 37°C for 60 min. After incubation, the absorbance of the sample was again measured at 412 nm. The result was expressed as nanomoles of thiol groups / ml of plasma.

4.10. Plasma TNF-α Determination

Tumor necrosis factor-α (TNF-α) concentration in plasma was determined using a rat TNF-α ELISA kit (Invitrogen, Waltham, MA USA). Spectrophotometric measurements were made at 450 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The plasma TNF-α value was expressed as picograms of TNF-α / ml of plasma.

4.11. Brain Mercury Quantification

Total Hg concentration was determined in brain samples by a mercury analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Servicio de Espectrometría Atómica de los Servicios Centrales de Investigación de la Universidad de Málaga 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 mercury 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 (ηg / g of tissue).

4.12. Skin Immunohistochemistry

Skin immunohistochemistry was performed on paraffin-embedded 4 μm tick sections. 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 10% (vol/vol) 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 mouse antibody against Ubiquitin Carboxyl-terminal Hydrolase-1 (UCH-L1) (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to stain the Merkel cell–neurite complex, which consist low threshold mechanoreceptors. 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). Quantification of positive Merkel cell–neurite complex was performed on 10 fields per sample.

4.13. Data analysis and statistics

Data are presented as the mean values ± SEM. Differences were analysed using unpaired one or two-way Analysis of Variance (ANOVA) followed by post hoc Bonferroni multiple comparison test. Values of $p<0.05$ were regarded as being significantly different.

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CONFLICT OF INTEREST

The authors state do not have any conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: DAR, JAUO, FMP, GV, DVV, MMC, GAW; performed the experiments: DAR, FF, SM, GV; analyzed the data: DAR, JAUO, FMP, GV, DVV, MMC, GAW; contributed reagents/materials/analysis tools: JAUO, GV, DVV, MMC, GAW; wrote the paper: DAR, JAUO, DVV, MMC, GAW.

FIGURE LEGENDS

Figure 1. Mechanical (A) and Thermal (B) sensitivity assessment. N of each group=8; error in bars indicate SEM; two-way ANOVA *$p<0.05$ vs Control and $^{\#}p<0.05$ vs Mercury.

Figure 2. Catalepsy (A) and Spontaneous Locomotor activity (B) assessment. N of each group=8; error in bars indicate SEM; two-way ANOVA *p<0.05 vs Control and #p<0.05 vs Mercury.

Figure 3. Plasma (A) and brain (B) MDA levels and plasma NPSH (C) levels. N of each group=8; error in bars indicate SEM; one-way ANOVA *p<0.05 vs Control and #p<0.05 vs Mercury.

Figure 4. Plasma TNF-α levels. N of each group=8; error in bars indicate SEM; one-way ANOVA *p<0.05 vs Control.

Figure 5. Merkel-cell neurite complex in skin (arrow) of control (A), mercury (B), hydrolysate (C) and hydrolysate-mercury (D) groups detected by immunohistochemistry, bar 50 μm. Quantification of Merkel-cell neurite complex in skin by immunohistochemistry (E). N of each group=8; error in bars indicate SEM; one-way ANOVA *p<0.05 vs Control.

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