Potential benefits of egg white hydrolysate in the prevention of Hg-induced dysfunction on adipose tissue

Egg white hydrolysate on Hg-induced adipose tissue damage

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ABSTRACT

Aim: To investigate the effects of egg white hydrolysate (EWH) on the lipid and glycemic metabolism disruption in the white adipose tissue (WAT) dysfunction induced by mercury (Hg). Experimental: Wistar rats were treated for 60 days: Control (saline, intramuscular - i.m.); Hydrolysate (EWH, gavage, 1 g/kg/day); Mercury ($HgCl_2$, i.m., 1st dose 4.6 µg/kg, subsequent doses 0.07 µg/kg/day) and Hydrolysate-Mercury (EWH-$HgCl_2$). Hg levels and histological analyses were performed in epididymal WAT (eWAT), pancreas and liver. GRP78, CHOP, PPARα, PPARγ, leptin, adiponectin, and CD11 mRNA expressions were analyzed in eWAT. Plasma lipid profile, glucose, and insulin levels were measured. Antioxidant status was also evaluated in plasma and liver. Results: EWH intake prevented the reduced eWAT weight, adipocyte size, insulin levels, antioxidant defenses and the increased glucose and triglycerides levels induced by Hg exposure; hepatic glutathione levels were higher in rats co-treated with EWH. The increased mRNA expression of CHOP, PPARα, and leptin induced by Hg was reduced in co-treated rats. EWH did not modify the elevated mRNA expression of GRP78, PPARγ and adiponectin in Hg-treated rats. Increased levels of Hg were found in liver; the co-treatment did not alter this parameter. EWH prevented the morphological and metabolic disorder induced by Hg, by improving antioxidant defenses, inactivating pro-apoptotic pathways and normalizing mRNA expression of PPARs and adipokines. Its effects enabled an increase in insulin levels and a normal balance between the fat storage and expenditure mechanisms in WAT. Conclusions: EWH may have potential benefits in the prevention and management of Hg-related metabolic disorders.

Keywords: Egg White Hydrolysate; Functional Food; Bioactive Peptides; Mercury; Lipid and Glucose Metabolism; White Adipose Tissue.

1. INTRODUCTION

Adipose tissue plays a central role in the development of metabolic-related chronic diseases like diabetes mellitus (DM) and metabolic syndrome (MS). White Adipose Tissue (WAT) exerts an essential function in energy homeostasis since it secretes several adipokines interacting with central and peripheral organs. This insulin-sensitive tissue influences metabolic processes, including carbohydrate and lipid metabolism, inflammation, blood pressure, energy expenditure and feeding behavior.[1,2] Dysfunctional WAT can alter adipocytes size, insulin sensitivity and insulin-sensitizing hormone secretion mediated by oxidative stress, systemic inflammation, cell apoptosis, and adipokine deregulation.[3,4]

Apart from exerting an endocrine and energy storage function, WAT appears to act as a depot for circulating toxic lipophilic compounds, such as heavy metals. Although the accumulation of heavy metals in WAT might protect other more sensitive vital organs, accumulated pollutants in this tissue may induce harmful effects, disrupting WAT homeostasis and increasing pro-oxidative and pro-inflammatory process, known risk factors for several chronic conditions, including DM.[3,4,5]

Mercury (Hg) is a common non-essential heavy metal found in the environment and released through industrial and human activities. This metal can accumulate throughout the ecological food chain and affect the human health mainly by dietary sources.[6,7,8] The relationship between Hg exposure and DM is unclear. However, it is known that Hg can impair the antioxidant defense system, inducing the oxidative stress and apoptosis of pancreatic islet β-cells, which results in hyperglycemia.[9,10,11]

Recently, we have demonstrated that long-term Hg exposure to low doses induced metabolic effects in 60-day treated rats, which showed reduced adipocytes size and plasma insulin levels, in addition to hyperglycemia and hyperlipidemia. These

disturbances were related to the increased oxidative stress, endoplasmic reticulum (ER) stress and the disrupted peroxisome proliferator-activated receptors (PPARs) and adipokines mRNA expressions induced by the metal in the WAT, which possibly induced a lipotoxic effect.[12]

Although they have side and adverse effects, antidiabetic agents and insulin are the primary therapy available for DM. Thus, currently, multiple studies have emphasized the possibility of using food-derived compounds to control metabolic complications related to DM.[2] Evidence shows that enzymatic hydrolysis of proteins produces bioactive peptides that, in addition to increasing the nutritional value of the food, provide health benefits, due to its biological properties.[13,14]

Our research group developed an egg white hydrolysate (EWH) obtained after enzymatic hydrolysis from pepsin for 8 h, which derived peptides have demonstrated multifunctional *in vitro* properties, including angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP-IV) inhibition, antioxidant, anti-inflammatory and hypocholesterolemic activities. Moreover, *in vivo* studies showed that EWH improved the pro-inflammatory and oxidative status on Zucker fatty rats (ZFR) and high-fat/high-dextrose fed rats, reducing adipose tissue accumulation, hepatic steatosis, decreasing the plasmatic concentration of free fatty acids and improving the glucose metabolism.[15,16,17] However, to date, there are no studies that demonstrate the effects of this EWH on WAT dysfunction and metabolic disorders caused by exposure to toxic pollutants, such as Hg.

Therefore, we hypothesized that the EWH might reduce hyperglycemia and hyperlipidemia, and it can protect the WAT against Hg-related metabolic toxicity. In this context, we investigated the effects of EWH in rats chronically exposed to Hg at low doses and explored whether the EWH-derived bioactive peptides could protect Hg-related dysfunction in WAT.

2. EXPERIMENTAL

2.1 EWH preparation

The commercial pasteurized egg white was hydrolyzed for 8 h with pepsin as previously described.[15] After, the hydrolysate was centrifuged at 2500g for 15 min and the supernatant was frozen and lyophilized until used. The peptide profile and the degree of hydrolysis of EWH, whose peptide sequences have been previously identified (FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, IVF, YQIGL, SALAM, FSL), were checked by RP-HPLC.[18]

2.2 Animals care and general protocol

Eight-week-old male Wistar rats (~250 g, n=32) 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*. Four groups (n=8/each) were treated for 60 days with: a) saline solution 0.9% by intramuscular injections (i.m.) and tap water by gavage (Control); b) saline solution 0.9% (i.m.) and EWH from pepsin for 8 h diluted in tap water (1 g/kg/day) by gavage[19] (EWH); c) mercury chloride – $HgCl_2$ (i.m.), the 1st dose of 4.6 µg/kg, and subsequent doses of 0.07 µg/kg/day to cover daily loss[20] and tap water by gavage ($HgCl_2$) and d) both treatments (EWH-$HgCl_2$). Appropriate safety measures were adopted for handling the animals during the treatment period. General health, body weight and consumption of water and food by animals were recorded weekly during the treatment period.

2.3 Blood and organ collection

After ending the treatment period and overnight fasting, rats were deeply anesthetized (ketamine, 87 mg/kg, and xylazine, 13 mg/kg, i.p.) and after the loss of the righting reflex, they were submitted to an aortic artery puncture. Blood was collected into tubes containing lithium heparin as an anticoagulant, centrifuged at 2500 g for 20 min at

4°C, divided into aliquots, and kept frozen at -80°C until assayed for biochemical experiments (glucose, insulin, triglycerides, total cholesterol, and antioxidant capacity). After that, rats were euthanized by decapitation, and the epididymal white adipose tissue (eWAT), pancreas, and liver were carefully removed, cleaned, weighed, and processed for histological, biochemical, and molecular studies. The ratio of organ-weight to tibia length was calculated for each organ.

2.4 Hg quantification in tissues

Total Hg concentration was determined in eWAT and liver samples by a Hg analyzer (SMS 100, PerkinElmer, Inc., Shelton, CT) in the Atomic Spectrometry Service at the Universidad de Málaga, Spain, according to thermal decomposition, amalgamation and atomic absorption principles described in the EPA Method 7473 (DT-CVAAS).[21] In this protocol a decomposition furnace is used 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. A calibration line was performed for determination of total Hg, with a range of 8 to 10 points from an Hg pattern of 100 ppm. The concentration values obtained corresponded to wet tissue. Data were presented as total Hg (ng/g of tissue).

2.5 Plasma analytical procedures

2.5.1 Glucose and insulin concentration

Fasting plasma glucose and plasma insulin levels were measured by a glucose-oxidase enzymatic test (commercial kit, Ref. 41012; Spinreact SAU, Girona, Spain) and an ultrasensitive rat Insulin ELISA kit (Ref. RAB0904; Sigma-Aldrich, St Louis, MO, USA), respectively. Both measures were spectrophotometrically determined at 505 nm and 405 nm, respectively, using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Values of glucose concentration were expressed as milligrams/dL of plasma

and insulin value was expressed as the micromol of insulin/L of the plasma. Plasma concentrations of both fasting glucose and insulin were used to predict the secretory capacity of pancreatic β-cells [HOMA-β] and calculate insulin resistance [homeostasis model assessment (HOMA)-IR] indexes with the following formulas: HOMA-β = 20 × fasting insulin (μU/mL) / [fasting glucose (mM) − 3.5]; HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mM) / 22.5.[22]

2.5.2 Triglycerides and total cholesterol levels

Colorimetric methods were used to measure triglycerides and total cholesterol levels, through commercial kits (Ref. 41032 and Ref. 41022; Spinreact S.A/S.A.U, Spain). The concentrations were spectrophotometrically determined at 505 nm (BioTek Instruments, Inc., Winooski, VT, USA). Values were expressed as milligrams/dL of plasma.

2.5.3 Antioxidant capacity

The antioxidant capacity of plasma was measured by oxygen radical absorbance capacity (ORAC) assay, as previously described[23] by a fluorimeter method (BMG Labtechnologies GmbH, Germany) and expressed as the micromol of Trolox (Sigma-Aldrich, St Louis, MO, USA) equivalent/μL of the plasma.

2.6 Liver glutathione determination

The supernatant of liver samples homogenate (PBS: 0.01 M PBS, 0.15 M NaCl, pH 7.4) was used for the evaluation of reduced glutathione, whose levels were determined by fluorimetric method[24] (BMG Labtechnologies GmbH, Germany), with excitation at 380 nm and emission at 470 nm. Bovine serum albumin was used for protein content determination (Bio-Rad Laboratories, Hercules, CA, USA), using and values were expressed as micromol of glutathione/g of protein.

2.7 RNA isolation and gene expression analysis

Total RNA for GRP78, CHOP, PPARα, PPARγ, leptin, adiponectin, and CD11 genes was isolated from eWAT using TriReagent (Sigma-Aldrich, St Louis, MO, USA) and quantified by Nanodrop 1000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA), as previously described.[12] Adequate dilution of cDNA was used as a template for different genes (Table 1 for SYBR Green primers and TaqMan probes), and 18S was used as the housekeeping gene. The amplification was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The relative quantity of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method was used.

2.8 Histopathological analysis

eWAT, pancreas, and liver were fixed in 10% formaldehyde for 1–2 days and then embedded in paraffin, sectioned at 5 μm and stained with hematoxylin/eosin, according to previously reported.[12] Zeiss Axioskop 2 microscope was used to morphometric analysis (Zeiss, Jena, Germany). Image J 1.45 software (National Institutes of Health, Bethesda, MD, USA) and Adiposoft program (http://fiji.sc/Adiposoft) were used to quantify adipocytes area and Zeiss Axioskop 2 microscope (image analysis software package AxioVision 4.6) to measure pancreatic islets area. The histological damage score assay in liver was based on a semiquantitative scoring system.[25] The total score of individual rats was expressed as the average of the sum of the different histological subscores.

2.9 Ethical approval

All experiments were carried out in strict accordance with the recommendations for biomedical research as stated by the Brazilian Societies of Experimental Biology, the guidelines for ethical care of experimental animals of the European Community, the current Spanish and European laws (EU Directive 2010/63/EU for animal experiments; R.D. 53/2013), and the International Guiding Principles for Biomedical Research

211 Involving Animals. Ethical Commission for the Use of Animals of Universidade Federal
212 do Pampa, Brazil, approved all experiments (institutional review board 05/2014), and by
213 the Ethical Committee of Research of the Universidad Rey Juan Carlos, Madrid, Spain
214 (institutional review board 39/2012). The experiments minimize the number of animals
215 used and their suffering during the execution of the protocols.

216 2.10 Statistical analysis

217 Data are expressed as mean ± SEM (Standard Error of the Mean) of the number
218 of animals used in each experiment. Statistical analyses were performed with GraphPad
219 Prism 6 Software (San Diego, CA, USA). In the histopathological analysis, once the areas
220 of the adipocytes were determined, a frequency distribution was calculated. The number
221 of total adipocytes within the distribution was subsequently calculated and used to convert
222 the frequency to a percentage of total adipocytes counted (percent relative cumulative
223 frequency - PRCF). The results were analyzed using two-way ANOVA followed by a
224 Bonferroni post hoc analysis, and differences were considered statistically significant at
225 $P<0.05$.

226 3. RESULTS

227 3.1 Food and liquid intake, body and tissues weight

228 No significant differences were observed in food and water daily intake between
229 the groups throughout the study (Food intake, in g/day – Control: 22.2 ± 1.1, EWH: 21.1
230 ± 1.1, $HgCl_2$: 21.1 ± 1.1, EWH-$HgCl_2$: 21.4 ± 1.1; Water intake, in mL/day – Control:
231 42.9 ± 1.4, EWH: 39.9 ± 1.0, $HgCl_2$: 42.6 ± 1.3, EWH- $HgCl_2$: 42.7 ± 1.7). Moreover, the
232 body weight gain was also similar between the groups at the end of the treatments (Table
233 2). However, EWH prevented the decreased absolute and relative eWAT weights induced
234 by chronic exposure to $HgCl_2$. No significant difference in pancreas and liver weights
235 was observed among the groups (Table 2).

236 3.2 Hg quantification in tissues

237 $HgCl_2$-treated rats showed higher concentrations of this metal in the liver when
238 compared to the control and EWH groups, and such values remained high in the group of
239 animals that received both treatments (Total Hg concentration, in ng/g – Control: 0.43 ±
240 0.07, EWH: 0.38 ± 0.05, $HgCl_2$: 4.30 ± 0.52*, EWH-$HgCl_2$: 5.09 ± 0.46*; *vs. Control).
241 Animals of all groups showed similar values of Hg levels in eWAT (Total Hg
242 concentration, in ng/g – Control: 0.59 ± 0.07, EWH: 0.41 ± 0.01, $HgCl_2$: 0.65 ± 0.03,
243 EWH-$HgCl_2$: 0.60 ± 0.04), indicating no further deposition of the metal in this tissue as
244 a result of this exposure.

245 3.3 Plasma glucose, plasma insulin, and indexes of insulin disorders

246 The EWH co-treatment prevented the increased fasting glucose and the decreased
247 fasting insulin levels in plasma from $HgCl_2$-treated rats. Rats receiving EWH alone had
248 similar fasting glucose and insulin concentrations than the control group (Figure 1A, B).
249 Corroborating with these data, no differences among the groups were found concerning
250 the HOMA-IR index (HOMA-IR – Control: 0.023 ± 0.002, EWH: 0.028 ± 0.007, $HgCl_2$:
251 0.022 ± 0.002, EWH-$HgCl_2$: 0.025 ± 0.005). However, $HgCl_2$-exposed rats showed a
252 significant lower level of HOMA-β index than the control group, which suggests that
253 metal exposure could induce pancreatic islet β-cell dysfunction. The EWH intake
254 prevented this harmful effect, as shown by the results of the EWH-$HgCl_2$ group, which
255 presented HOMA-β index similar to control group (HOMA-β – Control: 31.83 ± 0.50,
256 EWH: 29.78 ± 0.40, $HgCl_2$: 13.50 ± 1.06*, EWH-$HgCl_2$: 27.41 ± 0.57$^{\#}$ *vs. Control; $^{\#}$vs.
257 $HgCl_2$).

258 3.4 Lipid metabolic profile and oxidative stress markers

259 The EWH intake reversed the elevated plasma concentration of triglycerides
260 induced by the Hg, and the EWH-$HgCl_2$ group showed triglyceride levels similar to

unexposed rats, suggesting prevention in the lipid metabolic dysfunction (Figure 2A). No significant changes were observed in the total cholesterol levels among the different groups (Figure 2B).

Regarding the antioxidant status of the experimental animals, the EWH co-treatment avoided the decrease in the radical scavenging capacity of plasma induced by the $HgCl_2$ exposure (Figure 2C), suggesting a possible maintenance of some antioxidant defenses and, therefore, a better response of these animals to the increase in reactive oxygen species (ROS) generated by the metal. Moreover, EWH intake increased the hepatic glutathione concentration in the group that received both treatments when compared with control and $HgCl_2$ groups. However, there was no change in this parameter in animals receiving EWH alone (Fig 2D).

3.5 mRNA expression

The animals co-treated with EWH showed a reduction in the elevated mRNA levels for CHOP caused by the metal exposure in eWAT, suggesting that the consumption of hydrolysate may be able to prevent the expression of Hg-induced cellular apoptosis genes markers in this tissue. However, the GRP78 mRNA levels remained high at the end of the co-treatment with EWH, indicating the permanence of Hg-induced ER stress genes markers in the adipocytes (Figure 3A, B).

The EWH intake normalized the increased mRNA expression for PPARα, which probably indicates that the consumption of this hydrolysate prevents the disruption of gene markers of lipid metabolism in eWAT caused by Hg chronic exposure. Levels of PPARγ remained high in the group receiving both treatments, suggesting that EWH could improve adipogenesis-related gene markers in this affected tissue (Figure 3C, D). Besides, EWH-co-treated rats exhibited a reduction in the elevated leptin levels promoted by $HgCl_2$ exposure, and its value was similar to the control group. On the other hand,

adiponectin mRNA levels remained high in eWAT from rats that receiving both treatments. This finding reinforces the hypothesis that EWH prevents the Hg-induced injury to genes involved in adipokine production in this tissue (Figure 3E, F).

Finally, the real-time PCR analysis did not show a significant difference in CD11 mRNA levels in eWAT between the groups. The finding indicates that none of the proposed treatments interfere in gene markers related to this inflammatory pathway in eWAT of experimental rats (Figure 3G).

3.6 Histology of adipose, pancreas and liver tissues

The histological study of the eWAT indicated that EWH intake prevented the marked reduction in adipocyte size induced by the Hg chronic exposure, the adipose tissue from rats of the EWH-$HgCl_2$ group showed similar morphology to one of the Control rats' group (Figure 4A, B, C, D, E). Moreover, in EWH co-treatment rats', there was a predominance of adipocytes larger than 3000 μm^2 (Figure 4F), indicating the prevention of the Hg-induced storage lipids disruption.

No changes were observed in the histological structure of the pancreas, and the pancreatic islet size was similar in all experimental animals (Figure 5). The histological analysis of liver tissue also revealed no damage in any of the groups analyzed (Figure 6), suggesting that neither Hg nor EWH promotes histopathological changes, as marked steatosis, fibrosis, fat microvesicles or lymphocyte infiltration according to score system used.

4. DISCUSSION

Our study has evidenced that EWH intake prevents the reduced eWAT weight, adipocyte size, plasma insulin levels, some antioxidant defenses, and the increased plasma glucose and triglycerides levels induced by Hg exposure, similar to which is found in exposed humans in occupational conditions. Furthermore, the increased expression of

311 CHOP, PPARα and leptin mRNA induced by the metal was reduced in rats co-treated
312 with EWH.

313 These results suggest that the EWH ameliorates the WAT plasticity disturbance
314 and the glycemic and lipid metabolism disorder induced by the Hg, mainly by improving
315 some antioxidant defenses markers and circulating insulin levels to avoid the activation
316 of pro-apoptotic pathways, PPARs and adipokines mRNA expression disruption induced
317 by metal exposure. Additionally, EWH appears to promote an average balance between
318 fat storage and expenditure mechanisms in WAT, preventing metabolic-related disorders
319 in Hg-exposed rats.

320 Peptides with Phe and Arg amino acids in the C-terminal position have been
321 related to DPP-IV inhibitory activity.[26] Moreover, amino acid sequences with Tyr and
322 Phe in C-terminal residue are involved in scavenging free radicals and antioxidant
323 activities.[27] Due to these properties, the EWH previously ameliorated the blood lipid
324 profile, oxidative status[23,28] and improved MS and obesity-related complications in
325 genetic and diet-induced obesity rats.[15,16,17] However, to our knowledge, it is the first time
326 that a study shows the improvement of lipotoxicity and metabolic disorders in WAT from
327 an animal model of chronic exposure to Hg after the EWH treatment.

328 Previously, we demonstrated the beneficial effects of the EWH consumption on
329 Hg-induced damage in reproductive,[29] central and peripheral nervous[30,31] and
330 cardiovascular systems.[32,33] These effects were mainly associated with the antioxidant,
331 anti-inflammatory and vasodilatory properties present in EWH.

332 A significant amount of data support that the Hg-induced WAT disruption, such
333 as altered adipocytokine secretion, disorders in metabolic activity and irregular adipocyte
334 size, is strongly related to DM development and progression. Previous studies have shown
335 decreased plasmatic insulin, increased blood glucose and oxidative stress markers in Hg-

exposed mice.[9,10,11,34] We previously demonstrated that of $HgCl_2$ at low doses induced hyperglycemia and hyperlipidemia related to oxidative damage in WAT from exposed rats[12] and, considering significant damage to all body systems promoted by Hg, mainly due to the induction of oxidative stress, there is a growing concern in developing alternative therapies to avoid or mitigate this damage.

The EWH co-treatment prevented the increased fasting glucose, the decreased fasting insulin levels and avoided the reduction in HOMA-β index in Hg-treated rats. These findings could indicate that EWH intake can ameliorate the disturbance in the pancreas' secretory activity and the Hg-induced β-cell dysfunction. In the same direction, herring milt protein hydrolysate increased HOMA-β index in obese and insulin-resistant mice, proving an improvement in β-cell function after these bioactive peptides' intake.[35] A short peptide of potato protein hydrolysate had also demonstrated antidiabetic potential, and it was highly efficient in maintaining the insulin-secreting β-cell population in Streptozotocin (STZ)-induced diabetic mice.[13,14]

It has been proposed that Hg exposure reduces insulin-mediated glucose uptake and decreases β-cell viability through increased ROS content.[34] One of the leading hypotheses is ROS production, which can cause oxidative damage in the β-cells, leading to cell apoptosis, low insulin production and hyperglycemia. The pancreas is sensitive to oxidative stress due to the low concentration of antioxidant defenses.[9,10,11,36] In this sense, we demonstrated that EWH intake avoided reducing systemic antioxidant defenses in Hg-treated rats. Thus, the decreased blood glucose and the increased serum insulin levels observed in our model may be due to the protection of pancreatic β-cells from oxidative damage. Similar results were found in a study that used whey peptides to treat STZ-induced diabetic rats, which verified that whey peptides' antioxidant activity was

360 responsible for decreasing the oxidative cytotoxic status in β-cells, improving the insulin
361 deficiency.[37]

362 Besides, increased levels of GSH found in the liver of animals exposed to Hg may
363 be associated with a defense mechanism against oxidative stress, as seen in other
364 experimental models.[17] Furthermore, the increase in GSH levels was even significantly
365 higher in EWH co-treated rats, which would indicate that it produced a greater response
366 than necessary to neutralize the metal and increase the body's non-enzymatic antioxidant
367 defense in metabolic disorders associated with oxidative stress. Histological studies in the
368 liver did not reveal significant differences between the experimental groups, which
369 suggest that the increase in antioxidant defenses observed in this organ could be
370 protecting its morphological structure against toxic damage caused by Hg.

371 Regarding the deposition of metal in the liver, the chronically exposed animals
372 showed high Hg levels in this organ, and this concentration remained high in the EWH
373 co-treated rats. As previously reported, the GSH-Hg interaction displays a crucial role in
374 decreasing Hg deposition in tissues and increasing Hg excretion in the bile of Hg-exposed
375 rats, indicating a protective role of GSH against Hg-toxicity.[38] Thus, increased GSH
376 levels found in the liver of the Hg-treated rats could indicate that possibly the metal
377 complexed to glutathione was being transported to the liver to be excreted via bile, and
378 the EWH would be stimulating this mechanism of metal chelation.

379 The deficit of insulin production or tissue sensitivity to insulin impairs adipocyte
380 functions and lipid storage in WAT. The incapacity of WAT to properly expand in the
381 presence of nutrients promotes ectopic lipid accumulation, known as lipotoxicity, in
382 tissues such as the liver.[37,39] In the current study, we observed an increase in plasma
383 triglycerides level and reduced the adipocyte size in eWAT of Hg-rats. This eWAT
384 dysfunction may be related to the reduced plasma insulin levels, which impaired

adipocytes from properly storing triglycerides, increasing circulating triglyceride levels in Hg-exposed rats.

Enhanced adipogenesis by using compounds mimicking insulin effects or improving insulin sensitivity or production provides a novel strategy for controlling metabolic diseases' complications.[2,40] In this sense, insulin induces adipocytes differentiation in WAT, a process accompanied by the incorporation of lipid droplets and regulated by a family of immunomodulatory proteins like PPAR.[41] PPAR isoforms (α, β, and γ) are expressed in many tissues and play an essential role in regulating lipid metabolism, insulin sensitivity, cell differentiation, and adipokine secretion.[42] PPARα has been involved in regulating lipid oxidation and energy expenditure, and PPARγ is an essential regulator of adipocyte differentiation and energy storage.[43] Our study evidenced that EWH promoted adipocyte expansion and increased lipid accumulation, accompanied by increased adiponectin and PPARγ mRNA expression in eWAT. In addition to these enhanced insulin effects on the upregulation of fat store mechanisms, EWH treatment decreased lipid oxidation in eWAT, evidenced by the reduction in PPARα mRNA expression, downregulating Hg-induced fat expenditure pathways in exposed-rats.

Bioactive peptides from EWH are potentially responsible for their beneficial effects on adipogenic differgenes markers entiation, insulin signaling, and antioxidant responses in adipocytes. A similar finding was reported previously using another EWH that presented insulin-sensitizing and mimetic properties in 3T3-F442A preadipocytes, promoting adipogenic action by PPARγ upregulation.[2] Other authors showed that synthetic lactotripeptides from casein upregulated PPARγ levels and lipid storage in 3T3-F442A cells.[44] A bioactive peptide mixture from whey protein also induced effects on PPARγ, supported by increased PPARγ protein levels, upregulation of PPARγ-sensitive genes, and increased triglycerides accumulation in WAT.[37]

410 The adipokines produced in WAT can be affected by metabolic diseases. Thus,
411 impaired secretion of insulin-sensitizing adipokines, such as leptin and adiponectin, is
412 involved in WAT dysfunction.[45] Leptin controls the amount of fat stored in the body.
413 Studies suggest an inverse relationship between insulin secretion and leptin levels.[46] Our
414 study showed that rats exposed to Hg for a long time showed impaired insulin secretion
415 and increased leptin mRNA expression in eWAT, contributing to the adipocytes' reduced
416 storage of triglycerides. Interestingly, plasma insulin levels were normalized after EWH
417 co-treatment, resulting from restoring leptin action to normal in Hg-exposed rats. In
418 agreement with our findings, the authors have reported the use of leptin as an attractive
419 candidate for the treatment of obesity due to potent effects on loss adiposity in rodents.[47]
420 Adiponectin is responsible for increasing insulin sensitivity in WAT, liver,
421 skeletal muscle, and improving adipocyte lipid storage, preventing ectopic lipid
422 accumulations. PPARγ modulates these insulin-sensitizing effects.[35] Our findings
423 revealed increased adiponectin mRNA levels in eWAT of Hg-treated rats. This
424 unexpected adiponectin increase has also been observed in palatable diet-fed C57BL/6
425 mice and ZFR, and it was associated with adiponectin resistance.[15,16,17] The increased
426 adiponectin mRNA expression could be related to the rise in PPARγ mRNA expression
427 also found in Hg-exposed rats. This mechanism may be implicated in the attempt to
428 normalize the adipocyte size in eWAT of the Hg-treated animals. Interestingly, the group
429 of animals that consumed both treatments concomitantly maintained high adiponectin and
430 PPARγ mRNA levels, which was related to ameliorative Hg-induced lipotoxicity and
431 lipid storage capacity in eWAT. A higher adiponectin expression level in the WAT was
432 also found in diabetic animal models after casein consumption, which pointed to the
433 increased insulin sensitivity in these animals.[48]

We also found that the EWH intake avoided the Hg-induced activation of pro-apoptotic pathways genes markers in exposed-rats, contributing to the morphofunctional normalization of eWAT. Extrinsic and intrinsic pathways have been proposed to induce cell apoptosis, which involves, respectively, the release of extracellular stimulators, such as tumor necrosis factor-α (TNF-α) or intracellular ROS production.[49] Furthermore, intracellular ROS production is involved in ER stress development, apoptosis, and metabolic-related disorders in animal models.[50] Tissues in which large amounts of secreted proteins are synthesized, like pancreatic β-cells and WAT, are susceptible to ER stress induction, potentially destroying the cells, resulting in β-cells or WAT dysfunction, and eventually causing DM.[50,51]

We observed that the consumption of EWH prevented the increase in apoptotic genes markers in eWAT from rats exposed to Hg, indicating an anti-apoptotic effect, even though the levels of the ER stress marker remain high after the consumption of the hydrolysate. This finding suggests that the cellular apoptosis pathways activated by the metal are independent of the increase in ER stress and were probably activated directly by the excess of ROS present in this tissue. Thus, EWH, acting as a potent antioxidant agent, can prevented cell apoptosis and normalized the size of adipocytes and their lipid storage function. In this context, previous studies have also described the anti-apoptotic effect of an egg-white peptide in HEK-293 cells against H_2O_2-induced mitochondrial-dependent cell apoptosis.[49]

The current study is the first step to elucidate the mechanism by which EWH acts to improve the plasticity of adipose tissue and metabolic parameters impaired by prolonged exposure to Hg. Future studies are needed to detail the effects of EWH more accurately on this experimental animal model, including functional analyses at the pancreas and small intestine level, to verify the digestion/absorption of glucose and lipids.

5. CONCLUSIONS

Our data suggest that EWH balanced the mechanisms involved in lipid store and expenditure in WAT, preventing the reduction of adipocyte size and adipocyte apoptosis and the consequent lipotoxicity induced by prolonged exposure to Hg. These effects appeared to improve blood glucose and lipid levels in animals that received both treatments, probably due to the increment in some antioxidant defenses and insulin production. Considering the fundamental role of WAT dysfunction in DM's pathogenesis, EWH may have potential benefits in preventing and managing Hg-related metabolic disorders.

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653 DVV, MM, GAW; Investigation: DAR, PC, JAUO, MM, GAW; Methodology and

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COMPETING INTERESTS

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

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668 FIGURE LEGENDS

669

670 Figure 1. Effects of EWH on fasting plasma glucose (A) and insulin (B) concentration of
671 rats exposed to low doses of $HgCl_2$ for 60 days; the results are expressed as the mean ±
672 SEM, n=8 each group, two-way ANOVA, followed by Bonferroni post hoc, *P<0.05 vs.
673 Control; #P<0.05 vs. $HgCl_2$. Units: μmol: micromol; mg: milligram; L: liter; dL: deciliter.

674

675 Figure 2. Effects of EWH on fasting plasma triglycerides (A), total cholesterol
676 concentration (B) and radical scavenging capacity of plasma (C) of rats exposed to low
677 doses of $HgCl_2$ for 60 days; the results are expressed as the mean ± SEM, n=8 each group,
678 two-way ANOVA, followed by Bonferroni post hoc, *P<0.05 vs. Control; #P<0.05 vs.
679 $HgCl_2$. Units: μmol: micromol; mg: milligram; dL: deciliter; mL: milliliter.

680

681 Figure 3. Effects of EWH on GRP78 (A), CHOP (B), PPARα (C), PPARγ (D), leptin (E),
682 adiponectin (F) and CD11 (G) mRNA expression levels in eWAT of rats exposed to low
683 doses of $HgCl_2$ for 60 days determined by quantitative RT-PCR and normalized as a ratio
684 to the corresponding 18S mRNA expression level; the results are expressed as the mean
685 ± SEM, n=8 each group, two-way ANOVA, followed by Bonferroni post hoc, *P<0.05
686 vs. Control.

687

688 Figure 4. Effects of EWH on adipocytes histology in eWAT of rats exposed to low doses
689 of $HgCl_2$ for 60 days. Typical morphologies of eWAT from Control (A), EWH (B), $HgCl_2$
690 (C), and EWH-$HgCl_2$ (D) groups are shown. Note the reduction in adipocyte size
691 observed in $HgCl_2$-treated rats compared to control and the normalization of adipocyte
692 size in rats co-treated with EWH. Adipocytes mean area (μm^2) (E) and percent relative

693 cumulative frequency distribution of adipocytes (%) (F) in Control, EWH, $HgCl_2$, and
694 EWH-$HgCl_2$ rats. Note the rate of the smallest adipocytes increased in $HgCl_2$ group and,
695 which was normalized in rats co-treated with EWH; the results are expressed as the mean
696 ± SEM, n=8 each group, two-way ANOVA followed by Bonferroni post hoc, *P<0.05
697 vs. Control; #P<0.05 vs. $HgCl_2$. Bar=50 μm.
698
699 Figure 5. Effects of EWH on pancreas histology of rats exposed to low doses of $HgCl_2$
700 for 60 days. Typical morphologies of the pancreas from Control (A), EWH (B), $HgCl_2$
701 (C), and EWH-$HgCl_2$ (D) groups are shown. Islets mean size (μm^2) (E) of Control, EWH,
702 $HgCl_2$, and EWH-$HgCl_2$ rats; the results are expressed as the mean ± SEM, n=8 each
703 group, two-way ANOVA, followed by Bonferroni post hoc, P>0.05. Bar=50 μm.
704
705 Figure 6. Effects of EWH on liver histology of rats exposed to low doses of $HgCl_2$ for 60
706 days. Typical morphologies of the liver from Control (A), EWH (B), $HgCl_2$ (C), and
707 EWH-$HgCl_2$ (D) groups are shown. Microscopic damage score (E) in the liver of Control,
708 EWH, $HgCl_2$, and EWH-$HgCl_2$ rats; the results are expressed as the mean ± SEM, n=8
709 each group, two-way ANOVA, followed by Bonferroni post hoc, P>0.05. Bar=50 μm.
710
711 Graphical Abstract. The chronic Hg exposure at low doses reduced the adipocytes size,
712 which was related to the decreased antioxidant defenses and circulating insulin levels,
713 and the increased leptin mRNA expression, activation of pro-apoptotic pathways and
714 altered lipolysis mechanisms in eWAT, leading to hyperglycemia and hyperlipidemia.
715 The EWH co-treatment prevented the reduced adipocytes size, the decreased antioxidant
716 defenses and insulin levels and the increased leptin mRNA expression and activated pro-

717 apoptotic pathways, improving the lipogenesis mechanisms in eWAT and normalizing
718 the glycemic and lipid profile.

TABLE

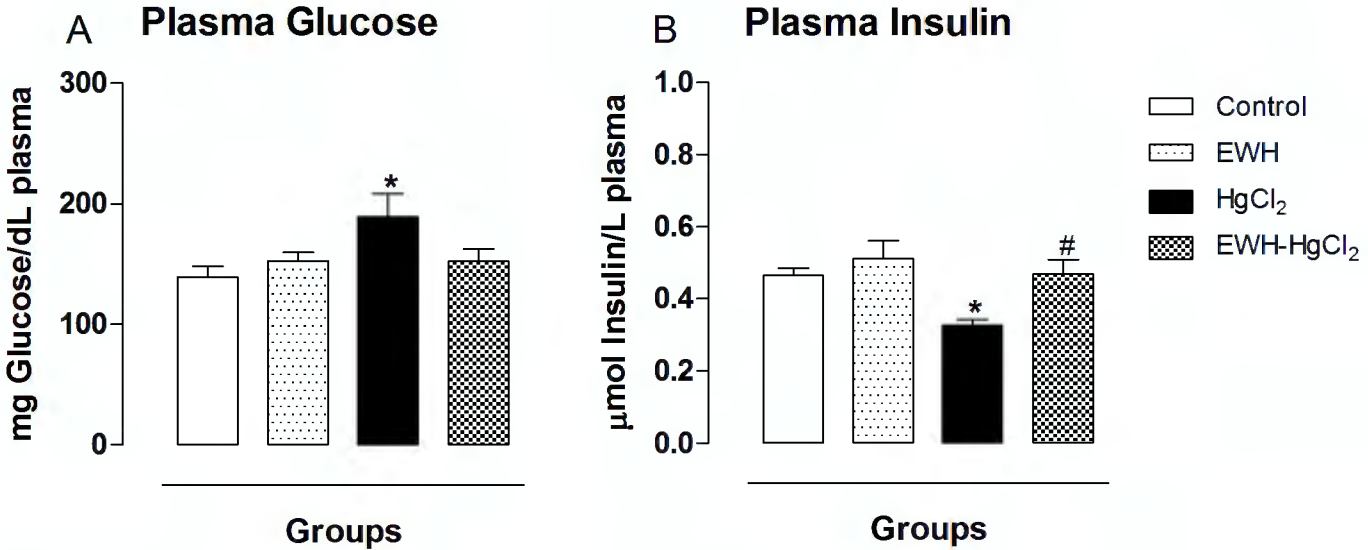
Table 1. Primer sequences for real-time quantitative PCR.

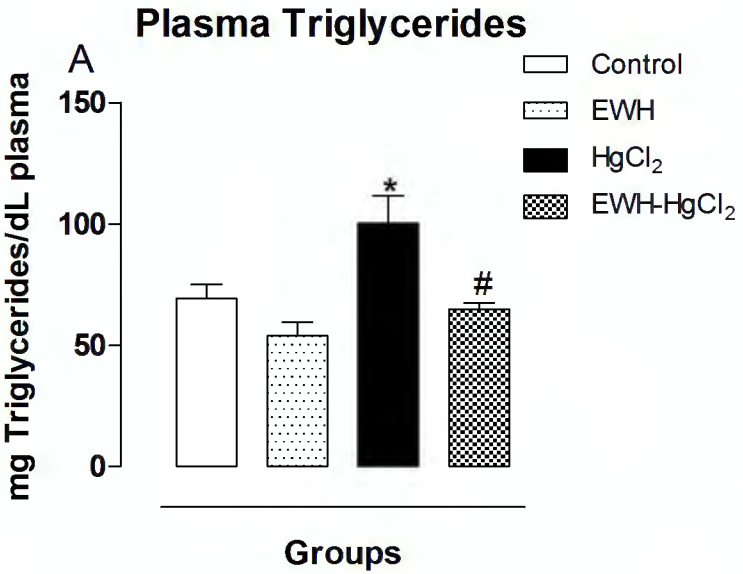
Genes		Primer Sequences
18s	Forward	CGGCTACCACATCCAAGGAA
	Reverse	GTCGGAATTACCGCGGCT
CHOP	Forward	CCACCACACCTGAAAGCAGAA
	Reverse	AGGTGAAAGGCAGGGACTCA
GRP78	Forward	GCCTCATCGGACGCACTT
	Reverse	AACCACCTTGAATGGCAAGAA
PPARα	Forward	CCTAGGGTACCACTAGGGAGT
	Reverse	GCCCGAATAGTTCGCCGAAA
PPARγ	Forward	GATGCACTGCCTATGAGCACTT
	Reverse	AGAGGTCCACAGAGCTGATTCC
Leptin	Forward	CCAGGATGACACCAAAACCCT
	Reverse	GCTGGTGAGGACCTGTTGAT
Adiponectin	Forward	CAGTGGATCTGACGACACCAA
	Reverse	TGGGCAGGATAAGAGGAACA
CD11	Forward	TGCCATAATGCAAGTTGCTG
	Reverse	ATCACCAGCAAAGTGGAAGC

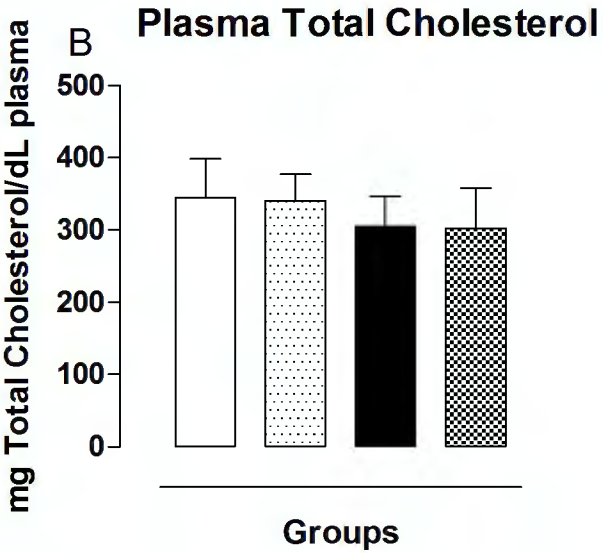
Table 2. Effect of EWH on body weight (g) and absolute and relative organs weight (g/cm of tibia length) of rats exposed to low doses of $HgCl_2$ for 60 days.

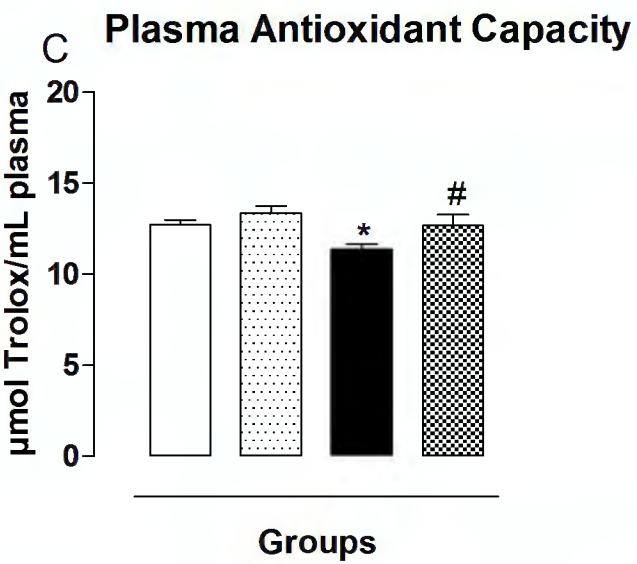
	Control (n=8)	$HgCl_2$ (n=8)	EWH (n=8)	EWH-$HgCl_2$ (n=8)
Initial Body Weight (g)	245.20 ± 2.90	245.00 ± 1.70	245.50 ± 2.30	245.40 ± 2.48
Final Body Weight (g)	432.90 ± 14.21	409.40 ± 8.30	419.70 ± 10.58	418.38 ± 6.94
Total Weight Gain (g)	187.70 ± 11.77	164.40 ± 7.22	174.20 ± 9.61	173.25 ± 5.80
Epididymal Adipose Tissue (g)	15.48 ± 1.22	11.18 ± 0.49*	13.06 ± 0.94	15.29 ± 0.93$^{\#}$
Epididymal Adipose Tissue (g/cm)	3.84 ± 0.30	2.71 ± 0.13*	3.13 ± 0.21	3.99 ± 0.20$^{\#}$
Pancreas (g)	0.57 ± 0.03	0.64 ± 0.03	0.73 ± 0.03	0.74 ± 0.08
Pancreas (g/cm)	0.14 ± 0.007	0.15 ± 0.007	0.18 ± 0.007	0.19 ± 0.03
Liver (g)	9.03 ± 0.28	9.30 ± 0.31	9.37 ± 0.30	9.16 ± 0.27
Liver (g/com)	2.24 ± 0.05	2.25 ± 0.07	2.26 ± 0.07	2.36 ± 0.06

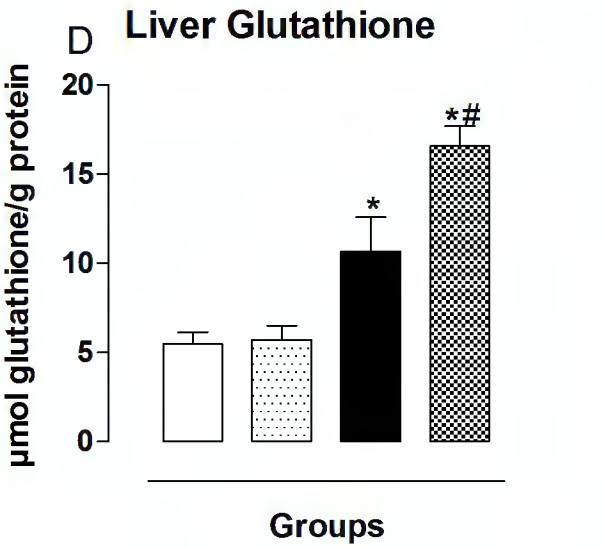
The results are expressed as the mean ± SEM, n of each group in parenthesis. The relative organ weight was calculated by use of the formula: organ weight/tibia length. Units: g: gram, cm: centimeter; two-way ANOVA followed by Bonferroni post hoc, $^{*}P<0.05$ vs. Control; $^{\#}P<0.05$ vs. $HgCl_2$.

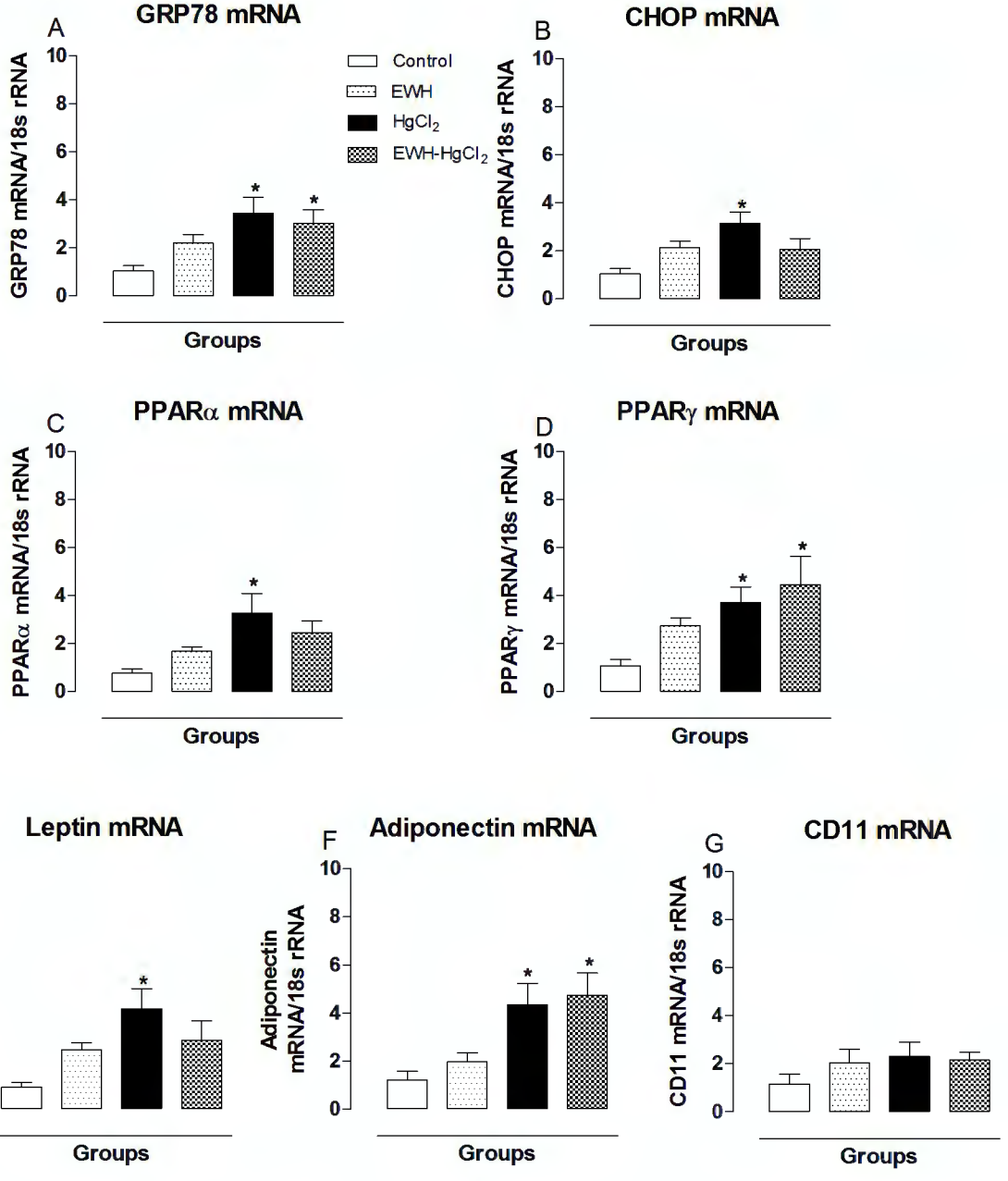

A
Plasma Glucose
mg Glucose/dL plasma
300
200
100
0
*
Groups
B
Plasma Insulin
µmol Insulin/L plasma
1.0
0.8
0.6
0.4
0.2
0.0
*
#
Groups
Control
EWH
HgCl2
EWH-HgCl2

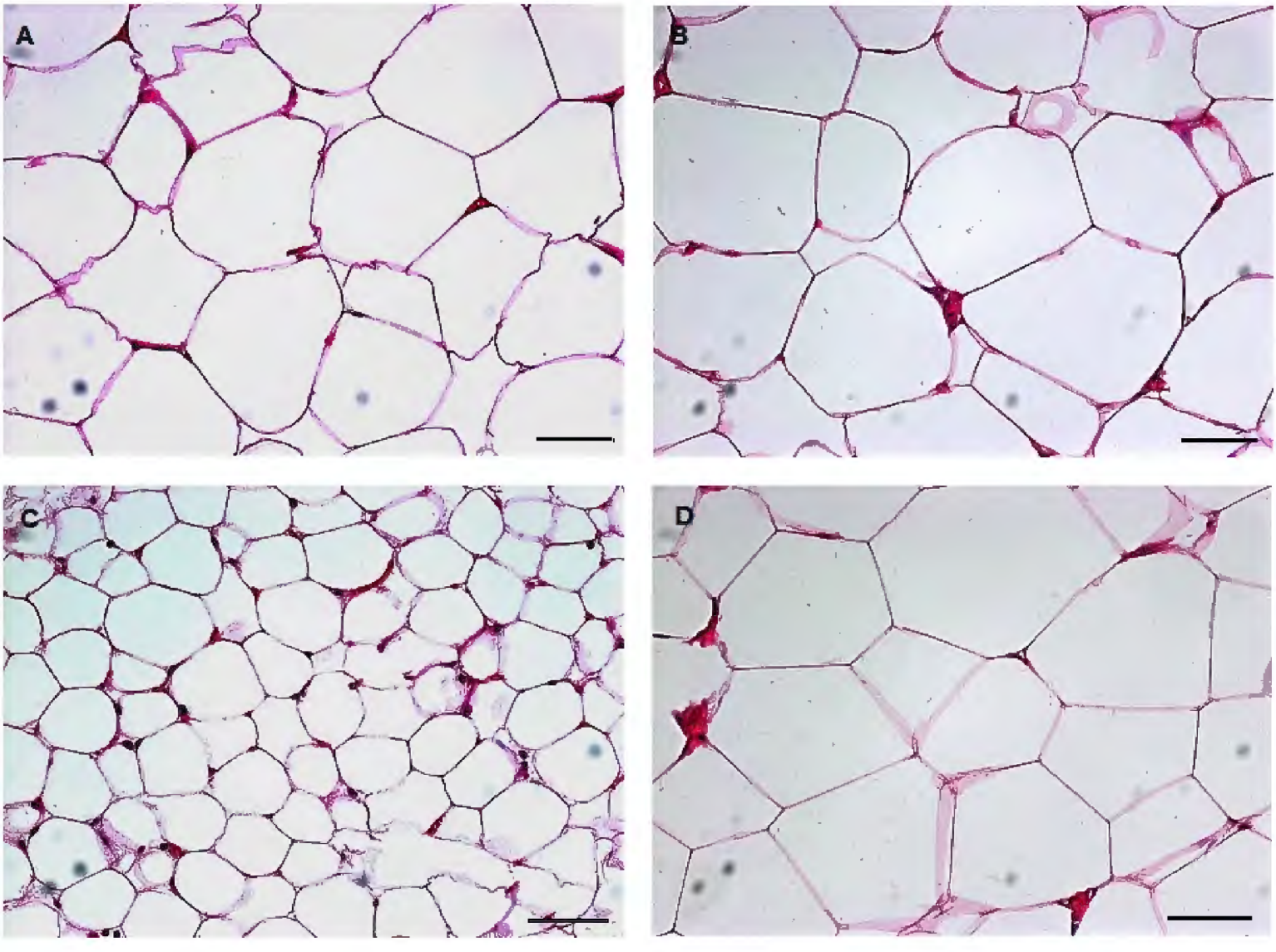

Plasma Triglycerides
A
Control
EWH
HgCl2
EWH-HgCl2
mg Triglycerides/dL plasma
150
100
50
0
*
#
Groups

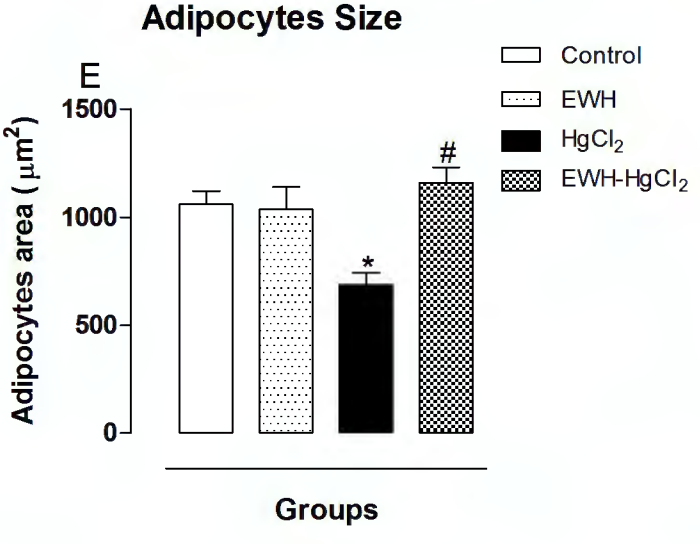

Plasma Total Cholesterol
B
mg Total Cholesterol/dL plasma
500
400
300
200
100
0
Groups

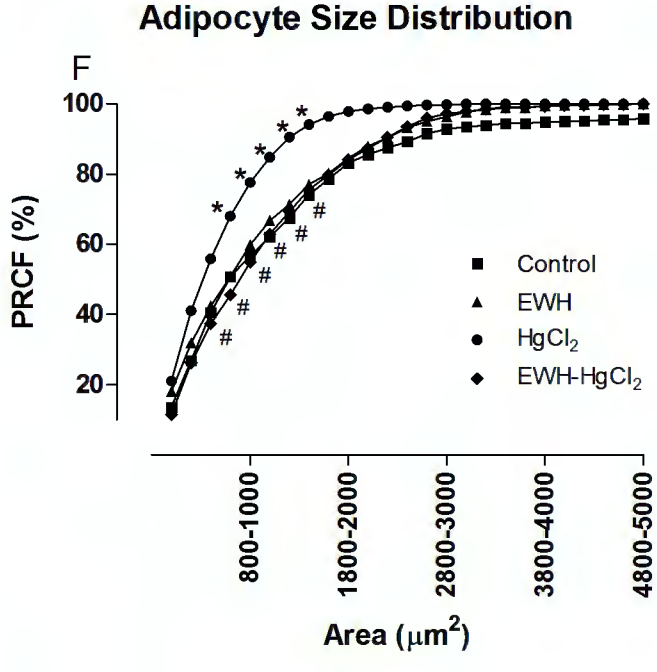

Plasma Antioxidant Capacity
C
µmol Trolox/mL plasma
20
15
10
5
0
*
#
Groups

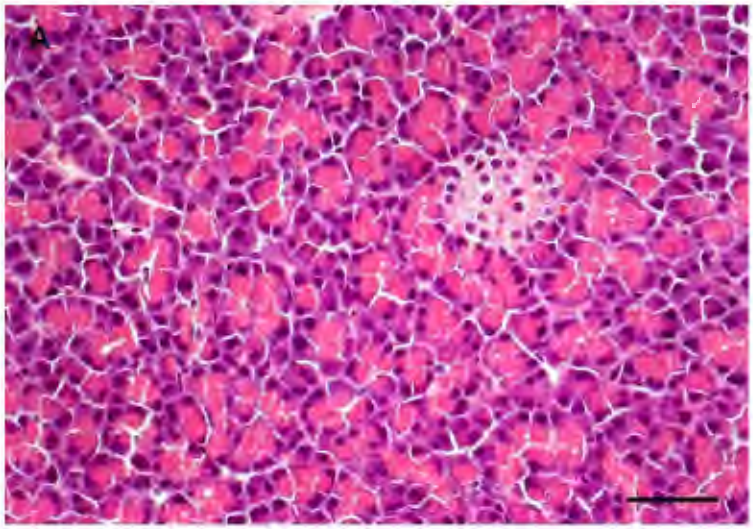

Liver Glutathione
D
µmol glutathione/g protein
20
15
10
5
0
*
*#
Groups

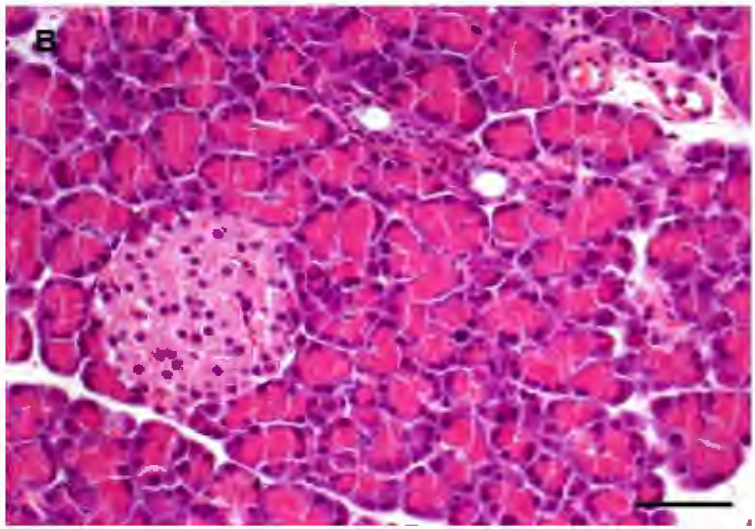

A
GRP78 mRNA
GRP78 mRNA/18s rRNA
Control
EWH
HgCl2
EWH-HgCl2
Groups
B
CHOP mRNA
CHOP mRNA/18s rRNA
Groups
C
PPARα mRNA
PPARα mRNA/18s rRNA
Groups
D
PPARγ mRNA
PPARγ mRNA/18s rRNA
Groups
E
Leptin mRNA
Leptin mRNA/18s rRNA
Groups
F
Adiponectin mRNA
Adiponectin mRNA/18s rRNA
Groups
G
CD11 mRNA
CD11 mRNA/18s rRNA
Groups

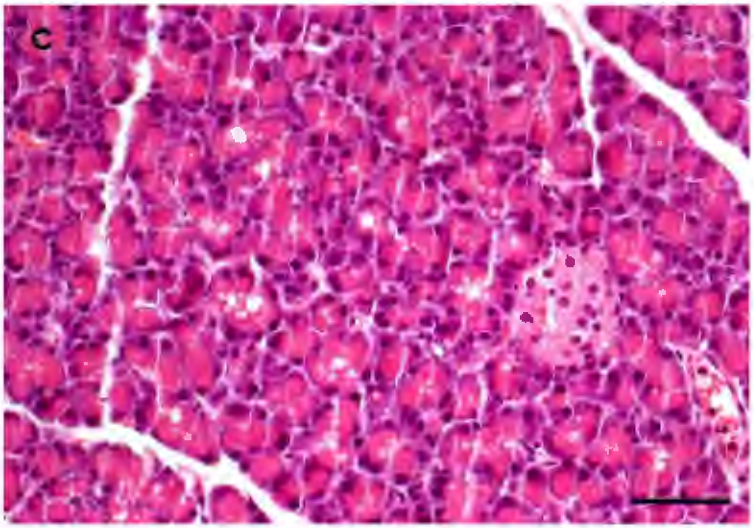

A
B
C
D

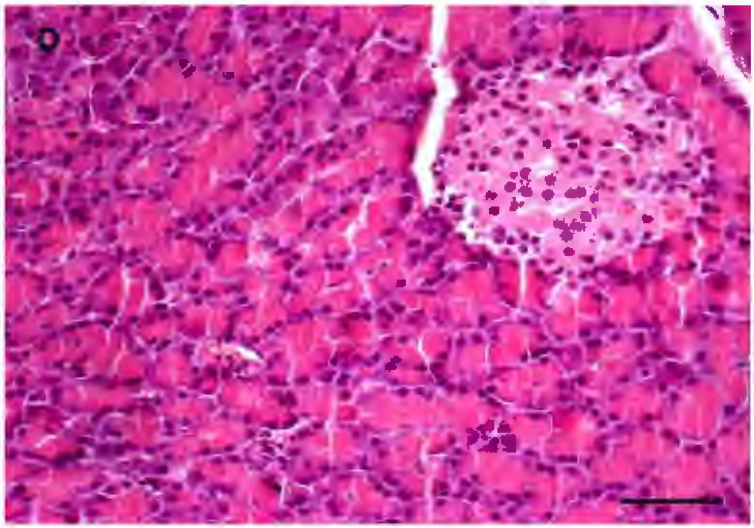

Adipocytes Size
E
Adipocytes area (μm²)
1500
1000
500
0
Control
EWH
HgCl2
EWH-HgCl2
*
#
Groups

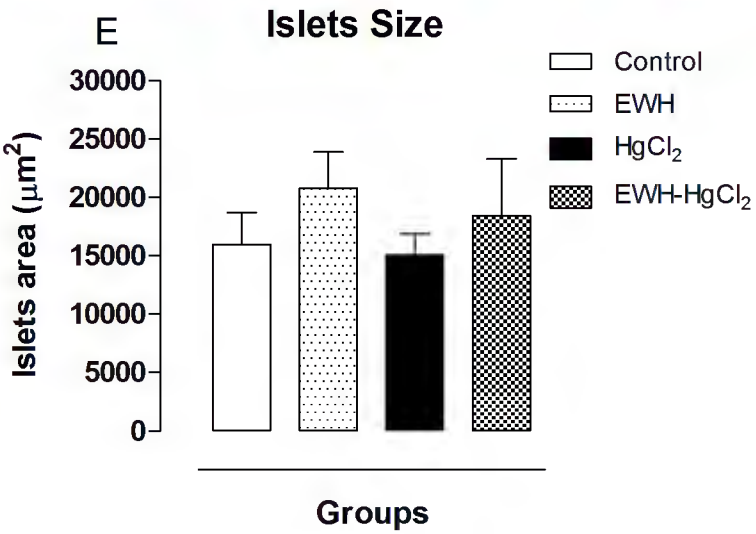

Adipocyte Size Distribution
F
PRCF (%)
100
80
60
40
20
Control
EWH
HgCl2
EWH-HgCl2
800-1000
1800-2000
2800-3000
3800-4000
4800-5000
Area (μm²)

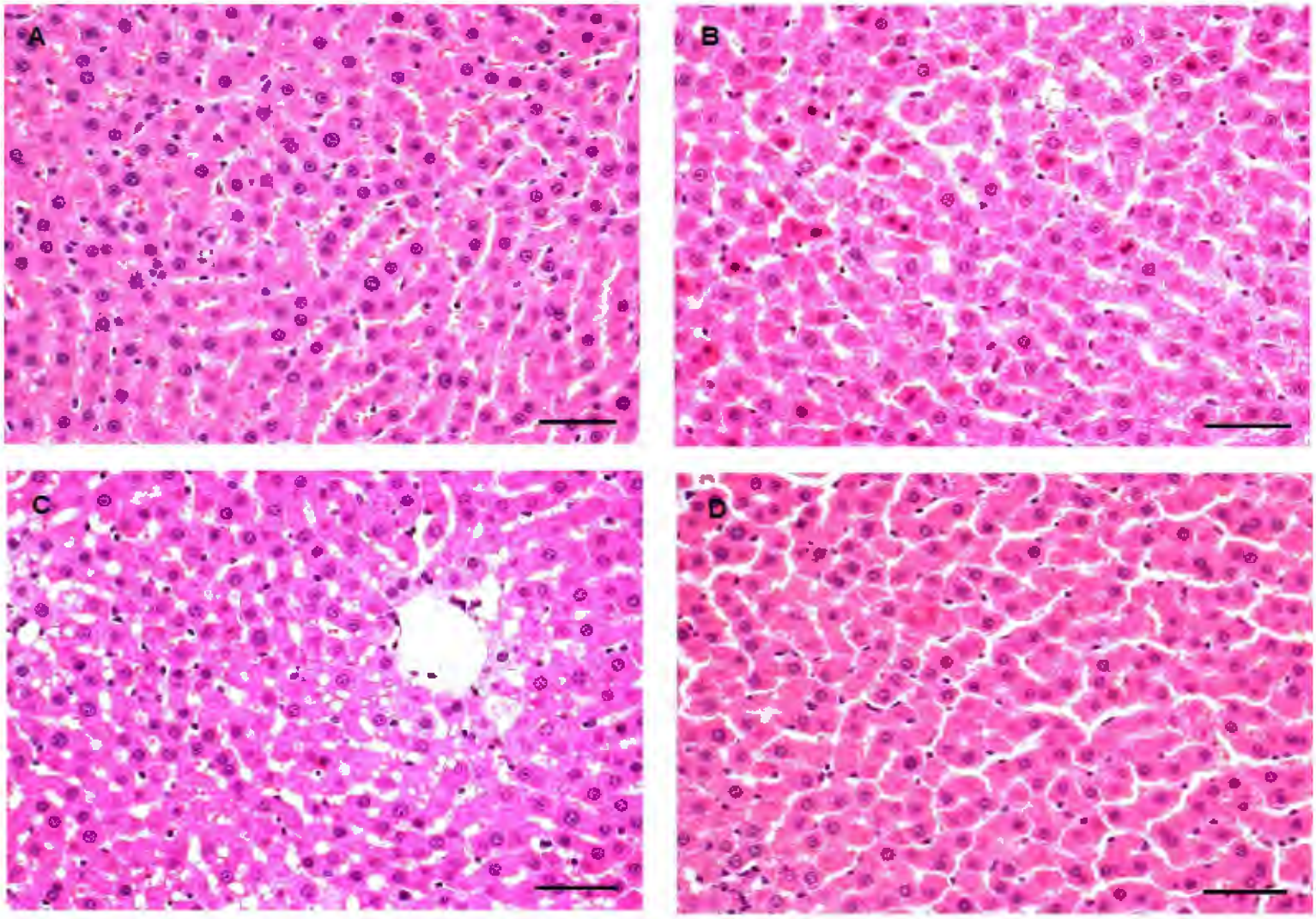

A

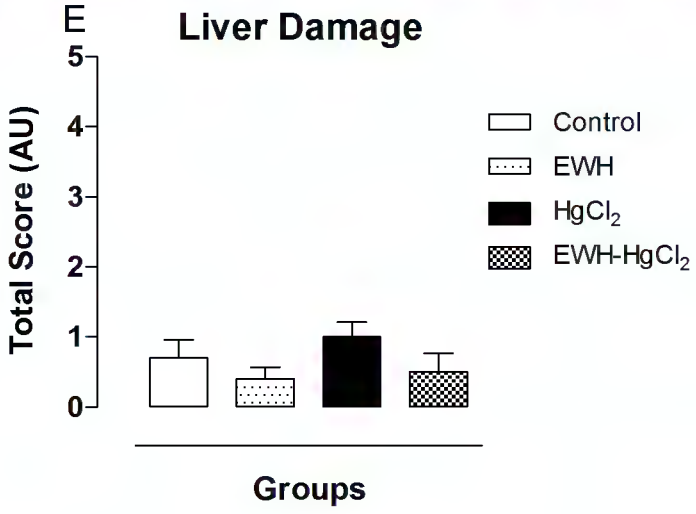

B

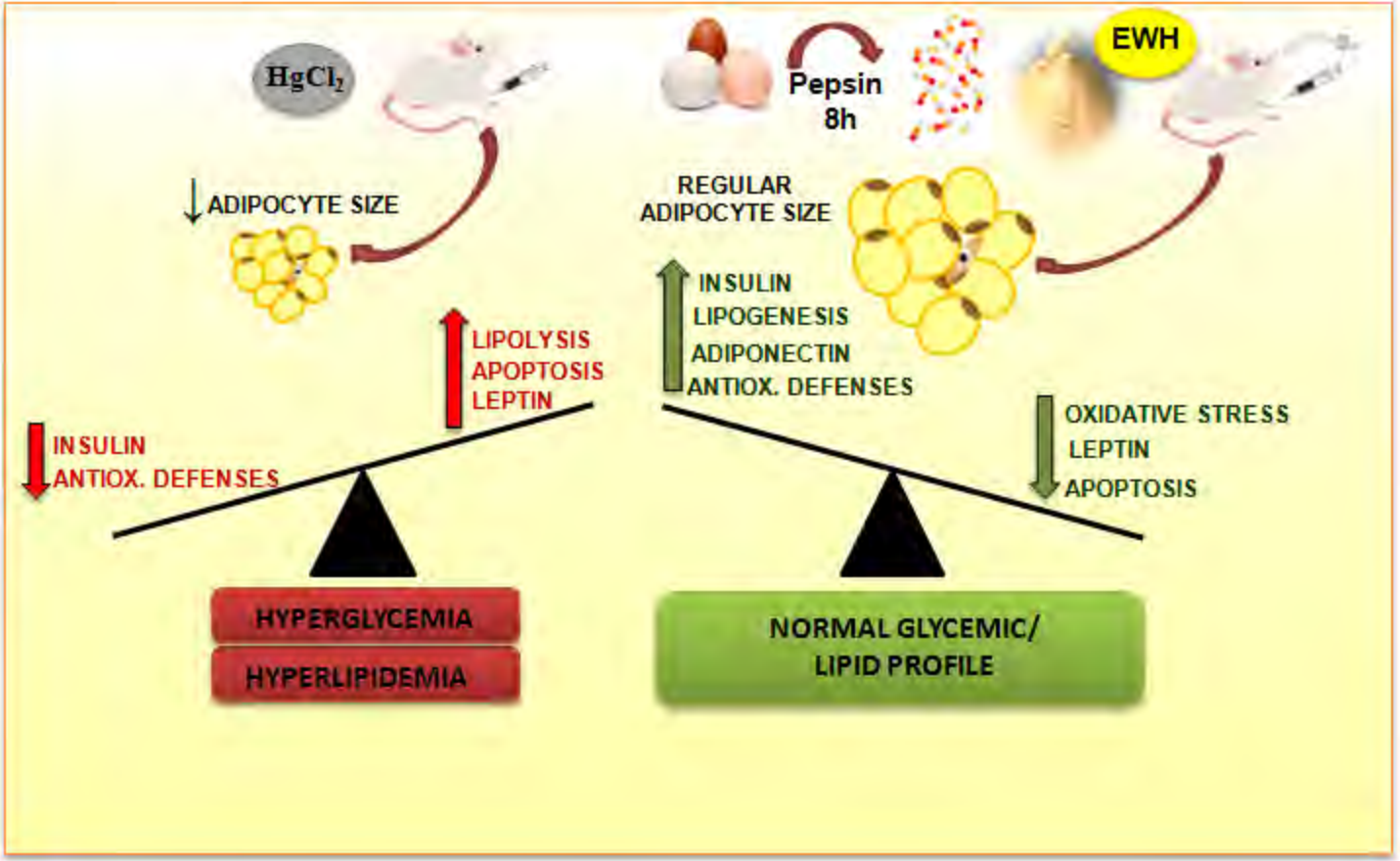

C


D


E
Islets Size
Islets area (µm²)
30000
25000
20000
15000
10000
5000
0
Control
EWH
HgCl2
EWH-HgCl2
Groups


A
B
C
D


E
Liver Damage
Total Score (AU)
0
1
2
3
4
5
Control
EWH
HgCl2
EWH-HgCl2
Groups


HgCl2
ADIPOCYTE SIZE
Pepsin
8h
EWH
REGULAR
ADIPOCYTE SIZE
INSULIN
LIPOGENESIS
ADIPONECTIN
ANTIOX. DEFENSES
LIPOLYSIS
APOPTOSIS
LEPTIN
INSULIN
ANTIOX. DEFENSES
OXIDATIVE STRESS
LEPTIN
APOPTOSIS
HYPERGLYCEMIA
HYPERLIPIDEMIA
NORMAL GLYCEMIC/
LIPID PROFILE