Egg white hydrolysates improve vascular damage in obese Zucker rats by its antioxidant properties

M. Garcés-Rimón,[a,c] C. González,[b,c] R. Hernanz,[b] E. Herradón,[b,c] A. Martín,[b] R. Palacios,[b] M.J. Alonso,[b] J.A. Uranga,[b,c] V. López-Miranda,[b,c] M. Miguel[a,c].

[a]*Instituto de Investigación en Ciencias de Alimentación (CIAL, CSIC-UAM), Madrid, Spain.*

[b]*Dpto. de Ciencias Básicas de la Salud. Facultad de Ciencias de la Salud. Universidad Rey Juan Carlos. Alcorcón, Madrid, Spain.*

[c] *Grupo de Investigación en Nutrición y Farmacología (URJC), Unidad Asociada I+D+i al Instituto de Investigación en Ciencias de la Alimentación (CSIC).*

Running Title: Egg white peptides on obesity-related cardiovascular complications

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Correspondence should be sent to:

Dra. Marta Garcés Rimón

Dpto. de Bioactividad y Análisis de Alimentos

Instituto de Investigación en Ciencias de Alimentación

28049 Madrid, Spain

E-mail: m.garces.rimon@gmail.com

Abstract

Metabolic syndrome (MS) is associated with increased risk of premature death as a consequence of cardiovascular complications development, among others. Dietary intervention has been suggested to be the safest and most cost-effective option for treatment of those alterations present in MS or to delay the pharmacological therapy in these diseases. The aim of this study was to analyze the potential valuable effects of two different egg white protein hydrolysates, namely HEW1 and HEW2, selected by their multifunctional *in vitro* properties, in obese Zucker rats (ZFR), an experimental model of MS, with emphasis on the development of hypertension and vascular complications. For this, ZFR were divided into three groups receiving from 8^{th} to 20^{th} week tap water or 750 mg/Kg/day HEW1 or HEW2 in drinking water. Direct blood pressure and heart rate, basal cardiac function in isolated hearts and reactivity in mesenteric resistance arteries were evaluated. Additionally, reactive oxygen species production by dihydroethidium-emitted fluorescence, NOX-1 mRNA levels by qRT-PCR, plasma angiotensin-converting enzyme activity by fluorimetry and kidney histopathology were also analysed. Both HEW1 and HEW2 treatments did not affect systolic blood pressure and basal cardiac function; however, they improve the endothelial dysfunction occurring in resistance arteries from ZFR. Additionally, HEW2 reduced oxidative stress that would contribute to the increased nitric oxide bioavailability, therefore contributing to the protective effect of this treatment.

In conclusion, our data confirms that these egg white hydrolysates, by its antioxidant properties, could be an interesting non-pharmacological tool for cardiovascular diseases in metabolic pathologies such as MS.

Keywords: Egg white proteins, enzymatic hydrolysis, cardiovascular diseases, metabolic syndrome, Zucker rats.

1. Introduction

Obesity, caused or potentiated by the modern lifestyle, is a fast growing problem that is reaching epidemic proportions worldwide, and is associated with an increased risk of premature death (Kopelman, 2000; Briones et al., 2014). This pathology has been related with development of cardiovascular, lipid and glucose metabolism alterations. These disturbances are clustered to form the so-called metabolic syndrome (MS) that plays a key role in the onset of hypertension and vascular alterations (Agouni et al., 2009) and, consequently, the development of cardiovascular long-term complications.

A growing body of evidence indicates that obesity is accompanied by a state of chronic inflammation associated to oxidative stress (Hajjar and Gotto, 2013). In addition, in obesity models and patients it has been described the presence of endothelial dysfunction, characterized, among others, by alterations of endothelium-dependent vasodilator responses. Interestingly, the presence of endothelial dysfunction is associated with poor prognosis in patients with cardiovascular diseases (Virdis et al., 2013). One of the underlying mechanisms to the endothelial dysfunction is the enhanced production of reactive oxygen species (ROS) leading to reduction of nitric oxide (NO) bioavailability (Virdis et al., 2013).

The current therapeutic tools used for treatment the cardiovascular complications in MS are lifestyle interventions, pharmacotherapy and surgery. Concerning pharmacotherapy, the requirement to combine different drugs leads to side effects that affect the patient's quality of life (Atkinson, 2008). Therefore, a dietary intervention has been suggested to be the safest and most cost-effective option for concomitant use in those alterations present in MS or to

delay the onset of the pharmacotherapy in these diseases (Sae-tan et al., 2011; Poulose et al., 2005).

The development of nutritionally enhanced food products designed to suit specific health concerns is of particular interest because many bioactive compounds from foods may be potentially useful to decrease some MS-related complications (Yuliana et al., 2011; Li et al., 2005). Particularly, some bioactive peptides, obtained from food proteins during gastrointestinal digestion or food processing, have shown promising future in the prevention or treatment of lifestyle related diseases; these bioactive peptides include those with antihypertensive, antioxidant, immunomodulating, hypocholesterolemic or antimicrobial properties (Moreno-Fernández et al., 2018; Garcés-Rimón et al., 2018; Garcés-Rimón et al., 2016; Miguel et al., 2004; Moughan et al., 2014; Zhang et al., 2017). Until now, most of the studies performed with bioactive peptides have evaluated their biological properties separately; however, some of them have shown to possess more than one physiologically significant property (Garcés-Rimón et al., 2015; Lafarga et al., 2016). Then, the multifunctional properties displayed by these peptides can increase their impact towards the improvement of more than one target disease or multiple symptoms of a disease, such occur in MS (Udenigwe and Aluko, 2012).

In the present study, we analyzed the potential beneficial effects as dietary treatment of two different egg white protein hydrolysates obtained after enzymatic hydrolysis. They were selected by their multifunctional *in vitro* properties [angiotensin converting enzyme (ACE) inhibition, antioxidant, hypocholesterolemic and dipeptidyl peptidase IV (DPP-IV) inhibitory activity] and characterized by the degree of proteolysis expressed as the percentage of hydrolysis of ovalbumin (Garcés-Rimón et al., 2016). These hydrolysates were tested in an experimental model of MS, with special emphasis on the effects on development and progression of hypertension as well as cardiovascular complications. For this purpose, we

investigated the effects of the egg white protein hydrolysates on blood pressure as well as cardiac and vascular function and renal histology in Zucker fatty rats (ZFR). Besides, the contribution of oxidative stress to the observed effects was also evaluated.

2. Methods

2.1. Preparation of egg white hydrolysates

Pasteurized egg white was hydrolyzed with two different food grade enzymes Enzyme/Substrate (egg white) ratio of 2:100 (w/v) to obtain two protein hydrolysates, namely HEW1 and HEW2. HEW1 was obtained by using pepsin from pork stomach (E.C. 3.4.23.1. BC PEPSIN 1:3000 Biocatalysts, United Kingdom). The egg white was acidified with concentrated food grade HCl (37%) to pH 2. The samples were incubated at 37°C under constant stirring in a thermostatic water bath for 8 hours. Inactivation of pepsin was achieved by increasing the pH to 7.0 with food grade NaOH (5 N).

HEW2 was obtained by using aminopeptidase from *Rhizopus oryzae* (E.C. 3.4.11.1, PEPTIDASE 433P, Biocatalysts, United Kingdom). The egg white was adjusted to pH 7.0 with food grade NaOH (5 N). The hydrolysis was carried out at 50°C under constant stirring in a thermostatic water bath for 24 hours. Hydrolysis was stopped by heating the samples at 95°C for 15 minutes in a water bath, followed by cooling to room temperature.

Both hydrolysates were centrifuged for 15 min at 4500 g, and the supernatants were stored at -20°C until use.

2.2. Animal models

Animal care and experimental procedures conformed to the current Spanish laws (RD 53/2013) and are conformed to the Directive 2010/63/EU of the European Parliament for animal experiments. The experiments were approved by the Ethic Committee at Universidad Rey Juan Carlos (URJC).

For experiments, we used ZFR, a widely and well-characterized animal model of MS, which present many of the human MS features such as insulin resistance, dyslipidemia, hyperinsulinemia and hypertension (Aleixandre and Miguel, 2009). Thirty male 8-week old ZFR and ten male 8-week old Zucker lean rats (ZLR), purchased from Charles River Laboratories (Charles River Laboratories, Barcelona, Spain), were used in this study. During treatment, animals were maintained at a room temperature of 23ºC, with 12 h light/dark cycles, and were fed *ad libitum* with a solid standard diet (A04 Panlab, Barcelona, Spain). The ZFR were randomly divided into 3 groups of 10 animals receiving from 8^{th} to 20^{th} week: a) tap water (control), b) 750 mg/Kg/day HEW1 in drinking water (HEW1 group), c) 750 mg/Kg/day HEW2 in drinking water (HEW2 group). The ZLR were in turn fed with the standard diet and tap water until the 20^{th} week of life. During treatment, the body weight of the animals was weekly recorded up to the 20^{th} week of life. Daily intake of drinking fluids and freely accessible feed was also estimated weekly in the animals from the different groups throughout the experimental period.

At the end of the treatment, rats were deeply anaesthetized with an intraperitoneal injection of equitesin (2.1 g chloral hydrate, 1.06 g MgSO4, 0.46 g pentobarbital, 21.4 ml propylene glycol and 5.7 ml ethanol in 20 ml of H_2O) at a dosage of 0.3 ml/Kg body weight. Then, a catheter coupled to a pressure transducer was inserted into the right carotid artery of the animals for direct measurements of systolic (SBP) and diastolic (DBP) blood pressure and heart rate (HR) using a PowerLab/4e system (PanLab S.L., Barcelona, Spain). Recording of these cardiovascular parameters lasted for 15 min. Thereafter, rats were sacrificed by decapitation and blood, heart, aorta, mesenteric arcade and kidneys were collected. Blood was collected to obtain plasma which was divided into aliquots and kept frozen at -80ºC until analysis of ACE activity. Kidneys were processed to perform histopathological analysis.

Heart, aorta and mesenteric arcade were used to evaluate the cardiovascular function as explained below.

2.3. Basal cardiac function

Hearts were rapidly removed, mounted and function evaluated using Langendorff set-up, as described (González et al., 2011). Following an equilibration period of 20 min, the basal cardiac variables coronary perfusion pressure (CPP), left ventricular developed pressure (LVDP), end diastolic pressure (EDP) and heart hate (HR) were recorded in isolated hearts for 30 min. After that, hearts and left ventricle were weighted to respectively calculate the cardiac mass index (CMI) and the left ventricular mass index (LVMI). Data obtained were compared among the different groups.

2.4. Vascular reactivity

Thoracic aorta were carefully dissected out and cleaned of connective and perivascular adipose tissues, taking care to avoid endothelium disruption. For reactivity experiments, aorta was divided into cylindrical segments of 3-4 mm in length and immediately mounted in an organ bath containing 5 ml of Krebs-Henseleit solution (KHS, in mM: NaCl 118; KCl 4.75; $NaHCO_3$ 25; $CaCl_2$ 2.54; KH_2PO4 1.19; $MgSO_4$ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at a resting tension of 2 g at 37°C, according to Lopez-Miranda et al (2004). Aortic rings were initially exposed twice to 75 mM KCl to check their functional integrity. Next, endothelial integrity was tested using acetylcholine (ACh, 10 μM) in segments previously precontracted with phenylephrine (Phe) at a concentration that produce close to 50% of the contraction induced by 75 mM KCl. A relaxation equal to or greater than 80% was considered demonstrative of the functional integrity of the endothelium. After a 45-min washout period, concentration-response curves to ACh (0.1 nM-300 μM) were determined in Phe-precontracted segments. Finally, and after other washout period, concentration-response curves to Phe (0.1 nM-300 μM) were

performed. A single concentration-response curve of each vasoactive agent was performed in each segment.

Third order mesenteric resistance arteries (MRA), 2 mm in length, were mounted in a small vessel dual chamber myograph for isometric tension measurement, as described (Hernanz et al., 2012). Segments contractility was tested by an initial exposure to a high K^+ solution (120 mM K^+, which was identical to KHS except that NaCl was replaced by KCl on an equimolar basis). The presence of endothelium was determined by the ability of ACh (10 μM) to relax arteries precontracted with Phe at a concentration that produce approximately 50% of the contraction induced by high K^+ solution in each case. Thereafter, a cumulative concentration-response curve to Phe (0.1-30 μM) was performed. Finally, concentration-response curves to ACh were determined in Phe-precontracted segments. The effect of N-nitro-L-arginine methyl ester (L-NAME) was analysed by their addition 30 min before the Phe and ACh concentration-response curves; due to the contractile effect induced by high ACh concentrations in MRA from ZFR, ACh from 1nM to 0.3 μM was used.

Vasoconstrictor responses were expressed as a percentage of the tone generated by high K^+ solution. Vasodilator responses were expressed as a percentage of the previous tone in each case. To compare the effect of L-NAME on the response to Phe and ACh in segments from different groups, results were expressed as differences of area under the concentration-response curves (dAUC) in control and experimental situations. AUCs were calculated from the individual concentration-response curve plots using a computer program (GraphPad Prism Software, San Diego, CA, USA); the differences were expressed as a percentage of the AUC of the corresponding control situation.

2.5. mRNA levels determination by qRT-PCR assay

The mRNA levels of the NADPH oxidase subunit NOX-1 were determined in mesenteric arteries as described previously (Hernanz et al., 2012). Total RNA was obtained by using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). A total of 1 μg of DNAse I treated RNA was reverse-transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a 20 μl reaction. PCR was performed in duplicate for each sample using 0.5 μl of cDNA as template, 1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 20x of Taqman Gene Expression Assays (Applied Biosystems, Rn00586652_m1). For quantification, quantitative RT-PCR was carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, from the CAT of Universidad Rey Juan Carlos) using the following conditions: 2 min 50°C, 10 min 95°C and 40 cycles: 15 s 95°C, 1 min 60°C. As a normalizing internal control we amplified β2 microglobulin (Rn00560865_m1). To calculate the relative index of gene expression, we used the $2^{-\Delta\Delta Ct}$ method, using samples from ZLR as control.

2.6. *In situ* detection of vascular superoxide anion production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate in mesenteric segments the $O_2^{\bullet-}$ production *in situ*, as previously described (Hernanz et al., 2012). Fourteen-micrometre frozen cross-sections were equilibrated in a Krebs-HEPES buffer (in mM: 130 NaCl, 5.6 KCl, 2 $CaCl_2$, 0.24 $MgCl_2$, 8.3 HEPES, 11 glucose, pH 7.4). Fresh buffer containing DHE (2 μM, 30 min, 37ºC) was topically applied onto each tissue section, cover-slipped and incubated for 30 min in a light-protected humidified chamber at 37ºC. Images were captured by a fluorescent laser scanning confocal microscope (Zeiss LSM 510, GmbH, Jena, Germany, objective x20, Ex 546 and Em 610 nm), using the same imaging settings in each case. The fluorescence intensity values were measured using the Metamorph image analysis software (Molecular Devices Corp., Downingtown, PA, USA). Samples from

ZLR or untreated ZFR were used as controls, and variations of fluorescence were calculated relative to control values.

2.7. Kidney histopathological analysis

Kidneys were obtained at the end of the treatment, fixed in buffered 10% formalin and embedded in paraffin. Each kidney was cut in sections of 5 µm and stained with hematoxylin-eosin. They were qualitatively studied under a Zeiss Axioskop 2 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) equipped with the image analysis software package AxioVision 4.6. A quantitative analysis of the glomerular area was also made in 2 to 4 slices of kidney per animal under a 20x objective.

2.8. Plasma ACE activity levels

ACE activity in plasma was measured by a fluorimetric method (Manso et al., 2008). For this, 3 µl of plasma were incubated with 40 µl borate buffer (pH=8.3) containing 5 mM of the ACE susbtrate hippuryl-histidil-leucine (HHL, Sigma-Aldrich, St. Louis, MO) at 37°C for 15 min. The reaction was stopped by adding 190 µl of NaCl 0.35 N. The generated product (His-Leu) was fluorimetrically measured at 37°C after 10 min incubation with 100 µl of 2% o-phthaldialdehyde in methanol. Fluorescence measurements were carried out in a Fluostar Optima plate reader (BMG Labtech, GmbH, Offenburg, Germany) with 350 nm excitation and 520 nm emission filters. A calibration curve with ACE from rabbit lung (Sigma-Aldrich) was included in each plate. Results were expressed as mU ACE / ml plasma.

2.9. Statistical analysis

The results are expressed as mean values ± S.E.M. for a minimum of 5 rats, and were analyzed by Student´s t-Test, one or two-way analysis of variance (ANOVA), followed by Bonferroni´s post hoc test, using the GraphPad Prism 4 software (San Diego, CA). Differences between means were considered to be significant when $P< 0.05$.

3. Results

3.1. Effect of chronic HEW administration on body weight gain, food and water intake and blood pressure

ZFR had a greater body weight gain when compared to ZLR (ZLR: 201.07 ± g, n= 10, untreated ZFR: 267.00 ± g. n= 10; $p<0.05$); at the end of the treatment there was no difference in the body weight gain between the three ZFR groups (HEW1: 271.60 ± g, n= 10; HEW2: 270.30 ± g, n= 10 ; $P> 0.05$). The water and food intake also did not differ between the groups during the treatment period (data not shown).

At 20th weeks of life, ZFR rats had significantly higher SBP than ZLR [ZLR: 91.69 ± 4.36 mmHg (n=9), untreated ZFR: 113.00 ± 3.33 mmHg (n=10); $p<0.05$], while values of DBP [ZLR: 62.12 ± 2.07 mmHg (n=9), untreated ZFR: 61.82 ± 3.11 mmHg (n=10); $p>0.05$] and HR [ZLR: 277.81 ± 8.94 beats/minute (n=9), untreated ZFR: 276.48 ±13.94 beats/minute (n=10); $p>0.05$] were similar. Neither the HEW1 nor the HEW2 treatments modified blood pressure or HR values in ZFR (data not shown).

3.2. Effect of chronic HEW administration on basal cardiac function

Basal cardiac function parameters in the different experimental groups are shown in Table 1. ZFR have similar left ventricular contractile (LVDP) and relaxant (EDP) function that ZLR. CPP was also similar between these two rat strains; however, ZFR showed a significantly lower HR than ZLR. HEW1 did not modify the basal cardiac function in ZFR; however, HEW2 corrected the bradycardia although it did not affect the coronary or the left ventricular function in ZFR. CMI and LVMI were significantly lower in ZFR than ZLR (Table 2). HEW1 did not modify these indexes, while HEW2 increased the CMI in ZFR.

3.3. Effect of chronic HEW administration on vascular function.

Aortic rings from ZFR had contractile response to Phe and endothelium-dependent vasodilator response to ACh that were similar to those observed in ZLR. Neither the HEW1

nor the HEW2 treatments modified aortic function in ZFR, resulting similar vascular responses to those obtained in Zucker fatty non-treated animals (data not shown).

Phe-induced contractile responses were slightly greater in MRA segments from ZFR than ZLR. This increase was reduced by both HEW1 and HEW2 treatments (Figure 1). The NOS inhibitor L-NAME leftward shifted the concentration-response curve to Phe in segments from both groups of rats; however, this effect was greater in ZLR than ZFR, as shown by the dAUC analysis (Figure 2). In MRA from ZFR treated with HEW2, but not in those from rats treated with HEW1, the effect of L-NAME was greater than the one observed in arteries from untreated ZFR rats (Figure 2).

ACh induced a concentration-dependent relaxation in MRA from ZLR pre-contracted with phenylephrine. However, in ZFR, ACh induced a biphasic response characterized by a relaxant response at concentrations of 1-300 nM followed by a contraction at higher concentrations. Treatment of ZRF with either HEW1 or HEW2 did not modify the profile, but improved the impaired ACh-induced relaxation (Figure 3).

The NOS synthase inhibitor L-NAME reduced the response to ACh in segments from both ZLR and ZFR; this effect was greater in ZLR, as shown by the dAUC analysis (Figure 4). In HEW1 and HEW2-treated rats, L-NAME also reduced the ACh-induced vasodilator response (Figure 4); the effect of L-NAME was similar in MRA from HEW1 treated and untreated ZFR; however, after HEW2 treatment this effect was higher than the one observed in arteries from untreated ZFR and similar to ZLR (Figure 4).

3.4. Effect of chronic HEW administration on oxidative stress and NOX-1 mRNA levels in MRA

The vascular superoxide anion production was greater in mesenteric segments from ZFR than ZLR segments, both in the media and in the adventitia layer (Figure 5). HEW2, but

not HEW1 treatment, reduced the greater superoxide anion production observed in ZFR in both layers (Figure 5).

The mRNA levels of the NADPH oxidase subunit NOX-1 were higher in ZFR than ZLR. HEW2 treatment decreased NOX-1 mRNA levels in ZFR compared to Zucker fatty non-treated animals, while HEW1 treatment did not modify those levels (Figure 6).

3.5. Effect of chronic HEW administration on plasma ACE activity levels

ZFR had significantly lower plasma ACE activity than ZLR (ZFR: 0.194 ± 0.017 mU / ml plasma, n= 10; ZLR: 0.234 ± 0.011 mU / ml plasma, n= 10; $p<0.05$). Neither the HEW1 nor the HEW2 treatments modified plasma ACE activity values, resulting similar values to those obtained in Zucker fatty non-treated animals (data not shown).

3.6. Effect of chronic HEW administration on kidney

Kidneys from ZLR showed no signs of glomerular or tubular pathology; in addition, no extravascular T or B-lymphocytes were present (Figure 7A, B). On the contrary, ZFR showed important changes in the kidney morphology. Thus, glomerular surface area was larger in ZFR compared with ZLR. Similarly, convoluted tubules appeared swollen and vacuolarized in certain areas (Figure 7C-F). This hypertrophy was observed no matter animals were fed with the hydrolysates or not (Figure 7G).

4. Discussion

The main finding of the present study is that egg white protein hydrolysates improve the vascular dysfunction associated to MS of resistance arteries. The reduction of oxidative stress elicited by HEW2, by increasing the NO bioavailability, would contribute to the protective effect of this treatment. Our results support the use of bioactive compounds from foods as dietetic supplementations in cardiometabolic disorders such as occur in the MS.

The existence of a relationship between complications associated to MS and cardiovascular diseases is widely known (Papakonstantinou et al., 2013), being hypertension

one of the most important risk factor for development of cardiovascular pathologies (Maraj et al., 2013). In the present study, SBP was slightly higher in 20 weeks-old ZFR than ZLR, in agreement to that found by other researchers; in fact, ZFR are considered as pre-hypertensive animals at young ages and hypertensive animals since 25-30 weeks of life (Oltman et al., 2006; Belobrajdic et al., 2011). Neither the HEW1 nor the HEW2 treatment did modify blood pressure in ZFR rats in spite of the important ACE inhibitory activity described *in vitro* for both hydrolysates (Garcés-Rimón et al., 2016) and the antioxidant properties observed for HEW2 *in vitro* (Garcés-Rimón et al., 2016) and *in vivo* (see below). Several studies, including some of our group, have shown the antihypertensive properties of food derived peptides (Contreras et al., 2009; Correa et al., 2011; Martínez-Maqueda et al., 2012; Fernandez-Musoles et al., 2013), including those derived from egg, in different hypertension models, without affecting blood pressure in normotensive animals (Miguel et al., 2005; 2007); this fact could explain that in the present study, the HEW treatments did not modify blood pressure values of ZFR, as at 20 weeks of life the animals did not reach important hypertensive values. Other food components have demonstrated antihypertensive effect in ZFR older than 25 weeks (Galisteo et al., 2005; Rivera et al., 2008); then, it is possible that the HEW used in the present study could reduce blood pressure in this rat strain at older age. However, it is important to note that the handling of 25-30 weeks-old ZFR is very difficult because at that age the obesity is very developed in these animals and their health become to get worse. In addition, the study with animals at those advanced ages could also be technically complicated and results difficult to interpret due to the physiological state of these animals. On the other hand, in the present study direct blood pressure evaluation was carried out in contrast to that employed in most investigations using indirect techniques, mainly tail cuff method. This indirect method requires a physical restraint and some degree of warming of the animal to ensure that the tail blood flow is sufficient for a measurement to be made.

Both restraint and warming constitute stressful factors that may affect blood pressure (Van Vliet et al., 2000).

Obesity is an important cardiovascular risk factor; then, a deep evaluation of the putative effect of egg hydrolysates on cardiovascular system was carried out. ZFR and ZLR have similar basal left ventricular function and coronary flow; however, HR was significantly lower in ZFR than in ZLR, suggesting that at 20 weeks of like, obese animals could be in a preliminary state of autonomic cardiac dysfunction (Brito et al., 2014). This alteration was appreciated in the isolated heart preparation but not in the *in vivo* HR measurement. The existence of compensatory systems that *in vivo* could mask the cardiac autonomic dysfunction would explain this difference. Our results are in accordance with other authors that describe lower HR values in obese Zucker rats in comparison with the control strain (Alonso-Galicia et al., 1996), although obese dogs and humans have higher HR values than their corresponding non-obese groups (Reisin et al., 1978; Rocchini et al., 1987; 1989). Treatment with both hydrolysates did not modify left ventricular function and coronary flow in ZFR. However, the HEW2, but not the HEW1 treatment, corrected the bradycardia observed in ZFR animals, suggesting a beneficial effect on cardiac autonomic dysfunction.

Cardiac and left ventricular mass indexes were smaller in the ZFR group than in the corresponding control rats. This could be explained by the increased cardiomyocyte apoptosis described in obesity (Lee et al., 2007). The HEW1 treatment did not modify the cardiac mass indexes in the obese animals; however, the chronic HEW2 treatment significantly increased both cardiac indexes in ZFR. These results suggest that the aminopeptidase egg white hydrolysate could protect against obesity-associated cardiac apoptosis, although further experiments are necessary to confirm this possibility.

Changes in vascular tone have been proposed as one of the most important mechanism implicated in the development of hypertension. It is known that vascular endothelium has a

pivotal role in the blood flow and in the maintenance of the vascular tone through an equilibrated release of vasoconstrictor and vasodilator mediators. Although vascular responses were not altered in aorta from obese rats, in mesenteric resistance arteries from ZFR the Phe-induced vasoconstrictor response was increased and the vasodilation elicited by ACh was reduced, confirming the endothelial dysfunction already described in patients and in obesity models (Campia et al., 2012). Several studies have shown beneficial effects of bioactive peptides derived from dietary proteins on endothelial dependent vascular responses. In this sense, we have previously reported endothelium-dependent vasodilator responses induced by bioactive peptides derived from egg proteins (Miguel et al., 2006; García-Redondo et al., 2010). In addition, several experimental and clinical studies have also found improvement of vasodilator function using bioactive peptides from milk proteins in different conditions (Sipola et al., 2002; Ballard et al., 2013). However, little is known about the vasodilator effect of these bioactive peptides on metabolic diseases. Only a report has shown that consumption of a hydrolysate of egg lysozyme with alcalase improves endothelial dysfunction in the aorta of diabetic Zucker rats (Wang et al., 2012). Herein we found that both HEW1 and HEW2 improved vascular dysfunction to Phe and ACh in MRAs, suggesting a protective role of these nutrients in the endothelial dysfunction related to obesity.

Endothelial dysfunction observed in resistance arteries from obese Zucker rats is associated with reduced NO (Villalba et al., 2009; Bouvet et al., 2007; Romanko and Stepp, 2005). Accordingly, the contribution of NO to both Phe- and ACh-induced responses was lower in mesenteric arteries from obese than from lean rats. There is evidence that elevated oxidative stress is a major mechanism contributing to the reduction of NO bioavailability in obesity. Thus, increased levels of reactive oxygen species have been found in resistance arteries from ZFR (Bouvet et al., 2007; Katakam et al., 2005). In addition, the expression of some subunits of the NAD(P)H oxidase, one of the main source of reactive oxygen species at

vascular level, is increased in vessels from ZFR (Bouvet et al., 2007). Furthermore, it has been suggested that treatment of ZFR with statins may improve vascular dysfunction by mechanisms involving the reduction of oxidative stress (Erdos et al., 2006; Shinozaki et al., 2007). In this study we observed elevated superoxide anion levels as well as increased expression of the NAD(P)H oxidase subunit NOX-1 in mesenteric arteries from ZFR; this increased oxidative stress might contribute to reduce the NO bioavailability and therefore to the endothelial dysfunction in this rat strain. Treatment with HEW2 reduced the NOX-1 expression and the superoxide anion production, which might contribute to explain the improvement of vascular dysfunction elicited by this treatment. These results agree with the *in vitro* antioxidant activity already described for HEW2 (Garcés-Rimón et al., 2016), although the precise mechanism by which this hydrolysate reduces oxidative stress in obese animals remains to be elucidated. On the contrary, treatment of ZFR with HEW1, despite the improvement of vascular function, did affect neither the involvement of NO in vascular responses nor the oxidative stress. Further experiments are necessary to ascertain the mechanisms underlying the improvement of the vascular function induced by the treatment with this pepsin hydrolysate.

It has been demonstrated increased activity of renin-angiotensin system, both systemically and within adipose tissue, in obesity (Briones et al., 2014). Surprisingly, in our study the plasma ACE activity was slightly reduced in ZFR as compared to that found in ZLR, although no differences were observed between ZFR and HEW-ZFR groups. This data suggested that the *in vivo* effects of both hydrolysates were unrelated to ACE inhibition, although an *in vitro* ACE inhibitory activity has been described for both hydrolysates (Garcés-Rimón et al., 2016).

Renal pathologies are closely related to the development of several cardiovascular diseases and, particularly, with hypertension (Cain and Khalil, 2002; ~~Maggio and Greenwood,~~

~~1982;~~ Huting, 1993; ~~Foley et al., 1998~~). Since ZFR have renal damage (Maggio and Greenwood, 1982), and this could be related to the increased blood pressure observed in these animals, we performed the kidney histological analysis to analyze ~~go deep~~ whether the consumption of egg hydrolysates produces any change in this tissue. Our results agree with those previously described showing glomerular hypertrophy and interstitial damage in ZFR. Some researchers have pointed out that obese Zucker rats suffer from renal damage from week 12 onwards ~~of life~~ (Lavaud et al., 2001); such alterations occur in the absence of hypertension, type 2 diabetes mellitus and inflammation and seem to be caused by an increase in oxidative stress (Poirier et al., 2000; 2001). However, cardiac disturbances were not found (Conti et al., 2004), despite the acute renal damage found in 36 weeks old animals. These results are in accordance with ours since, although we found renal differences between lean and obese rats, they did not affect cardiac function. The consumption of HEWs did not modify the renal damage, which agrees with the lack of antihypertensive effect observed for both hydrolysates.

In conclusion, both HEW1 and HEW2 treatments improved the endothelial dysfunction in resistance arteries from a MS model, which might improve the prognosis in obese patients that have a high cardiovascular risk. However, these bioactive peptides did not modify cardiovascular parameters, such as blood pressure. The reduction of oxidative stress elicited by HEW2, that may increase NO bioavailability, would contribute to the protective effect of this treatment. Further experiments are necessary to analyze the mechanisms underlying the improvement of the vascular function induced by HEW1. The different outcomes of the administration of both hydrolysates, produced with pepsin or aminopeptidase, pointed at their differential peptide composition as responsible for the activity. Our data confirms that these products could be an interesting no-pharmacological tool for cardiovascular diseases in some metabolic pathologies.

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Table 1: Baseline cardiac values of Left Ventricular Developed Pressure (LVDP, mm Hg), End Diastolic Pressure (EDP, mm Hg) and Coronary Perfusion Pressure (CPP, mm Hg) in Zucker Lean rats (ZLR) and in Zucker fatty rats non-treated (ZFR) or treated with egg HEW1 or HEW2 (ZFR-HEW1 and ZFR-HEW2). Data are expressed as the Mean ± SEM *P<0.05 vs ZLR, and # P<0.05 vs ZFR, using one-way ANOVA and Bonferroni post-test. n=9-10.

	ZLR	**ZFR**	**ZFR-HEW1**	**ZFR-HEW2**
LVDP (mmHg)	98.2±7.7	103.4±9.2	126.4±10.5	102.6±12.5
EDP (mmHg)	-5.2 ±13.2	-5.6±3.6	-7.4±3.6	-8.6±2.5
CPP (mmHg)	66.4±2.2	71.3±5.4	65.7±4.2	70.9±5.2
HR (beats/minute)	237.7±9.2	195.7±10.2*	185.6±12.3	231.9±8.0#

Table 2: Cardiac Mass Index (CMI, mg/g) and Left Ventricular Mass Index (LVMI mg/g) in Zucker Lean rats (ZLR) and in Zucker fatty rats non-treated (ZFR) or treated with egg HEW1 or HEW2 (ZFR-HEW1 and ZFR-HEW2). Data are expressed as the Mean ± SEM *P<0.05 vs ZLR, and # P<0.05 vs ZFR, using one-way ANOVA and Bonferroni post-test. n=9-10.

	ZLR	**ZFR**	**ZFR-HEW1**	**ZFR-HEW2**
CMI (mg/g)	3.63±0.16	2.72±0.09*	2.78±0.14	3.00±0.09#
LVMI (mg/g)	2.67±0.03	2.05±0.04*	2.01±0.09	2.09±0.06

Figure legends

Figure 1: Concentration-response curves to phenylephrine (Phe) in mesenteric resistance arteries from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). The results are the mean ± SEM. *P<0.05 using two-way ANOVA and Bonferroni post-test. n=7-9 animals (one ring per animal).

Figure 2: Effects of L-NAME (100 µM) on the concentration-response curves to phenylephrine (Phe) in mesenteric resistance arteries from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). The insert graph shows differences in the area under the concentration-response curve (dAUC) in L-NAME-treated and control segments. The results are the mean ± SEM. *P<0.05 vs. control segments using two-way ANOVA and Bonferroni post-test or vs. ZLR using Student's t-test; #P<0.05 vs. ZFR using Student's t-test. n=7-9 animals (one ring per animal).

Figure 3: Concentration-response curves to acetylcholine (ACh) in mesenteric resistance arteries from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). The results are the mean ± SEM. *P<0.05 using two-way ANOVA and Bonferroni post-test. n=7-9 animals (one ring per animal).

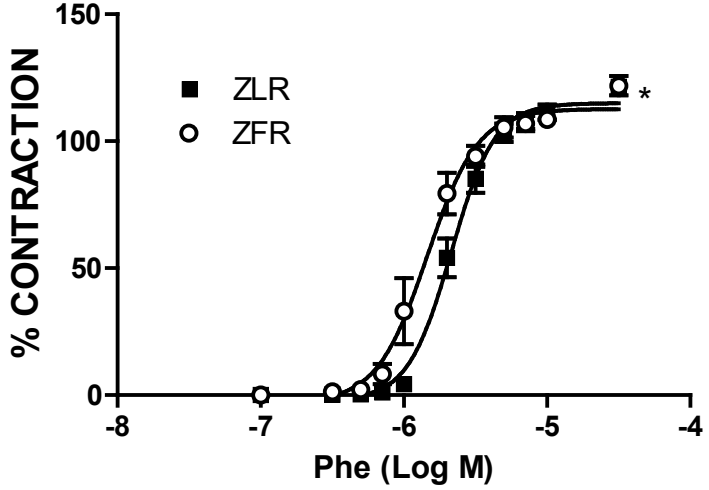
Figure 4: Effects of L-NAME (100 µM) on the concentration-response curves to acetylcholine (ACh) in mesenteric resistance arteries from ZLR and ZFR treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). The insert graph shows differences in the area under the concentration-response curve (dAUC) in L-NAME-treated and control segments. The results are the mean ± SEM. *P<0.05 vs. control segments using two-way ANOVA and

Bonferroni post-test or vs. ZLR using Student's t-test; #P<0.05 vs. ZFR using Student's t-test. n=7-9 animals (one ring per animal).

Figure 5: Quantification in the media and in the adventitia of vascular superoxide anion production in segments of mesenteric arteries from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). Vessels were labelled with the oxidative dye dihydroethidium and view by using a fluorescence confocal microscope (X20 objective). Image size 1024 x 1024 µm. *P <0.05 vs. ZLR, #P < 0.05 vs. ZFR by Student´s *t*-test. Data are expressed as mean ± SEM. n=6-7 animals (one ring per animal). The lower panels show representative fluorescent confocal photomicrographs.

Figure 6: Quantitative RT-PCR assessment of NOX-1 mRNA levels in mesenteric arteries from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) treated or not with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2). Data are expressed as mean ± SEM.*P < 0.05 vs. ZLR, #P < 0.05 vs. ZFR by Mann-Whitney nonparametric test. n=5-6 animals (one ring per animal).

Figure 7: Representative images from Kidneys from Zucker Lean rats (ZLR) and Zucker fatty rats (ZFR) untreated or treated with HEW1 (ZFR-HEW1) or HEW2 (ZFR-HEW2) stained with hematoxylin/eosin. (A) Tubules and (B) glomerulus from ZLR. Tubules (C, arrows) and hypertrophied glomerulus (D, asterisk) from untreated ZFR (C). Hypertrophied tubular cells (arrows) of ZFR treated with HEW1 (E) or HEW2 (F). Bar, 100 □m. (G) Mean glomerular area ($\mu m^2 x 10^3$) of ZLR and ZFR untreated or treated with HEW1 and HEW2. * P<0.01 vs. ZLR. n=7-9 animals (one kidney section per animal).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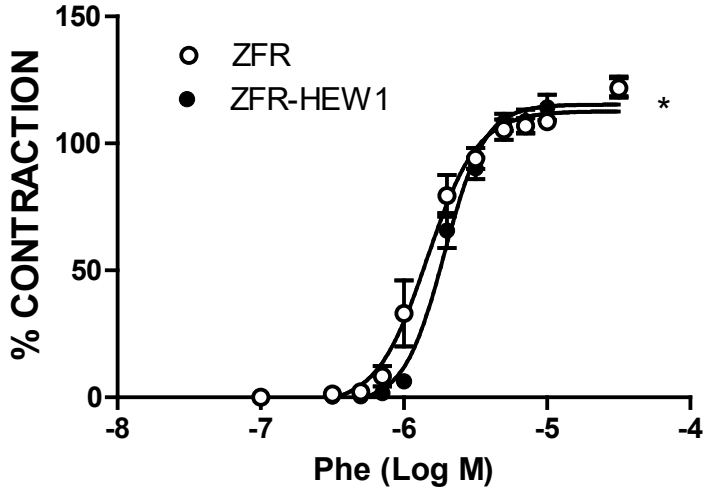
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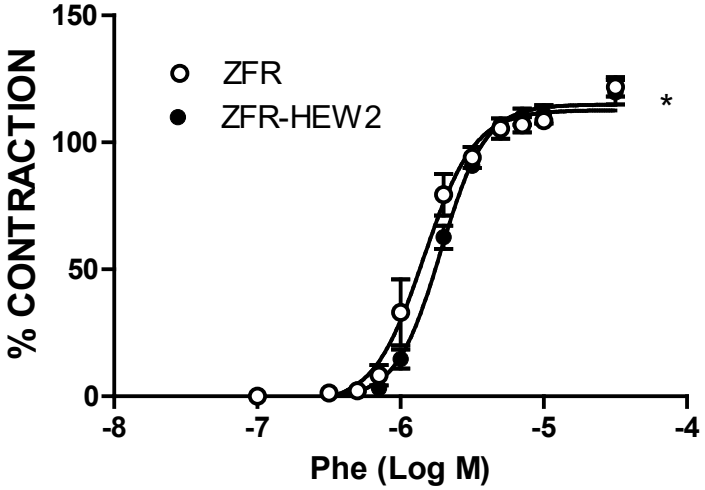
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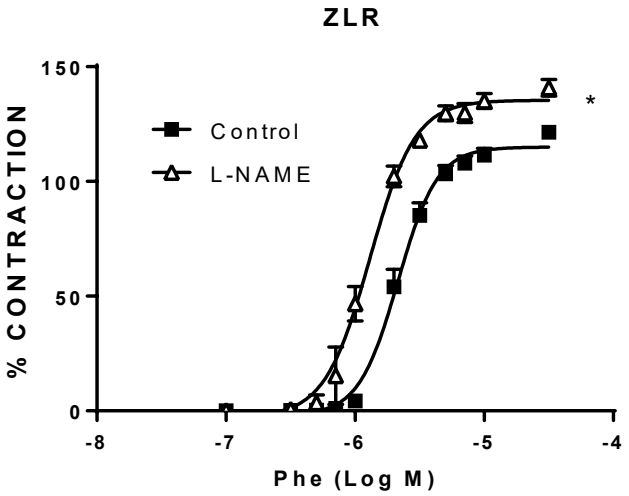
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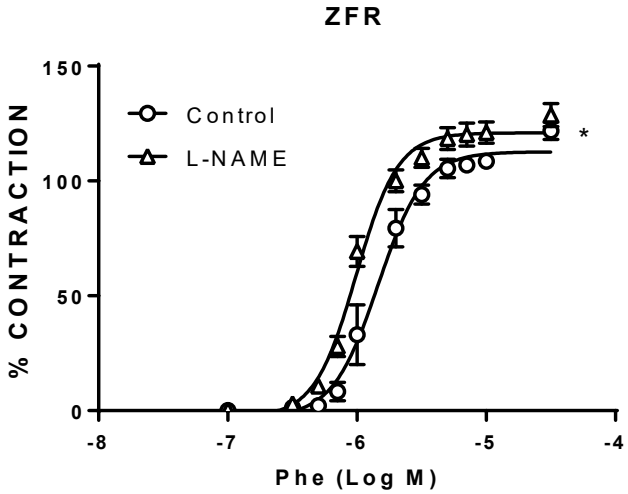
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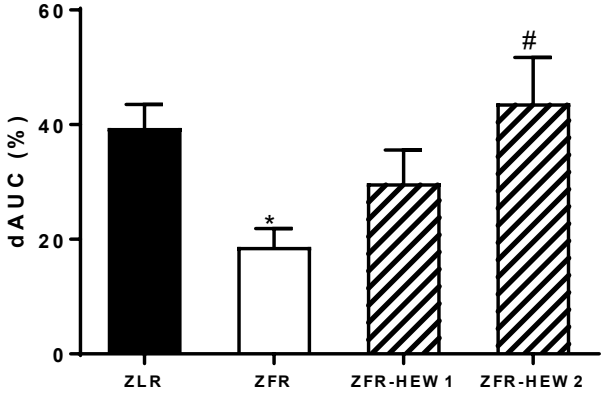
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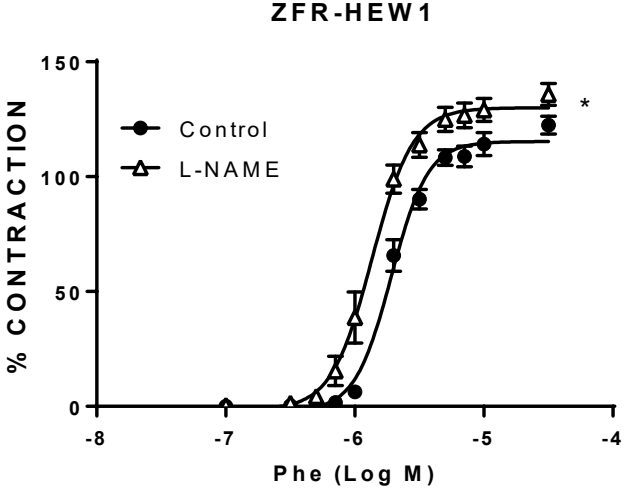
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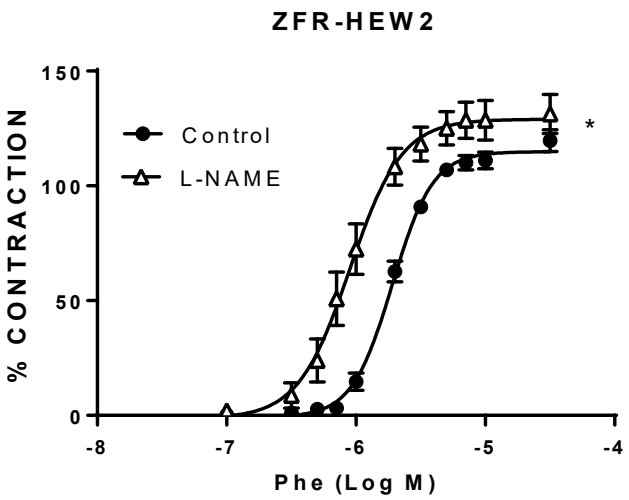
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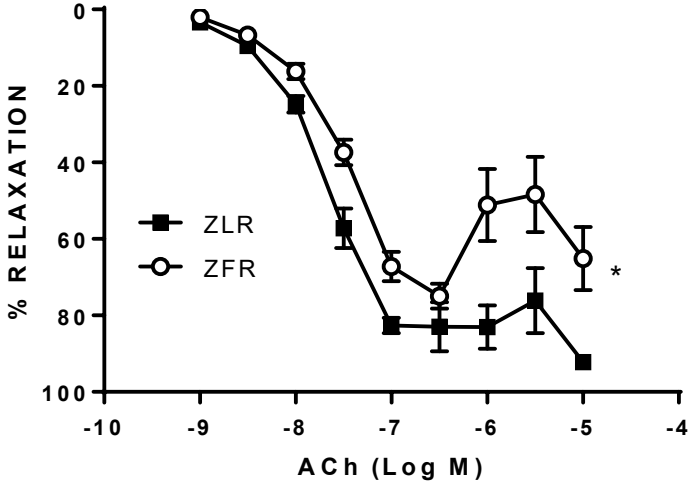
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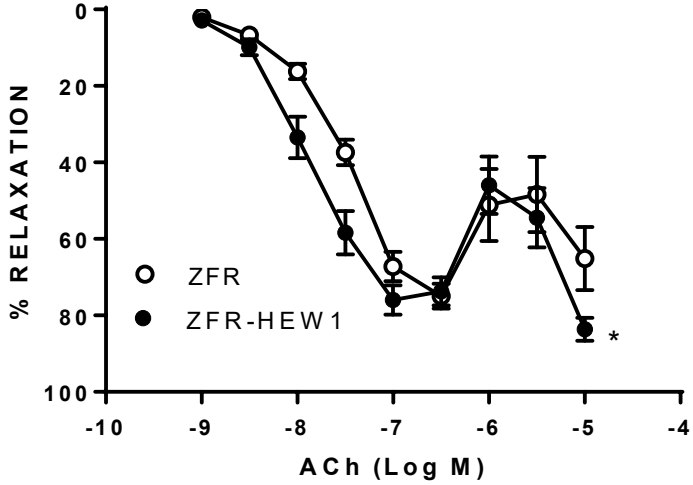
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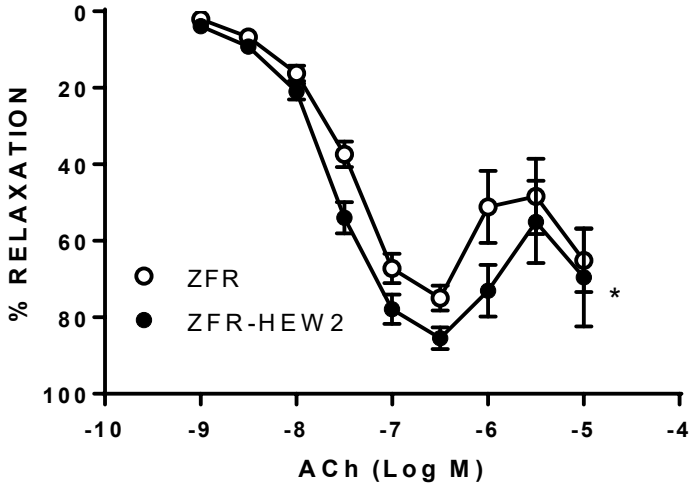
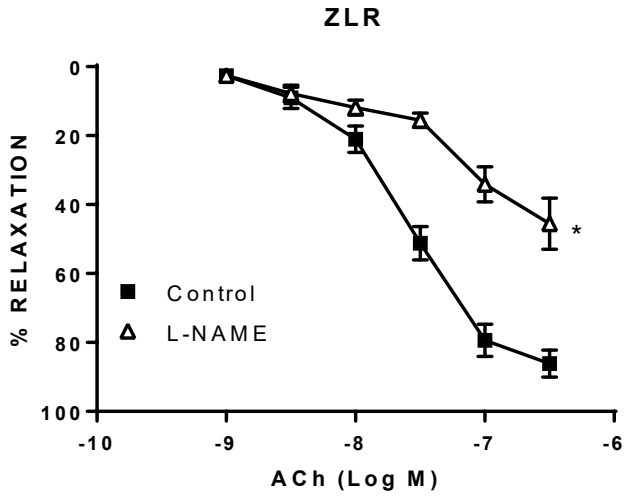
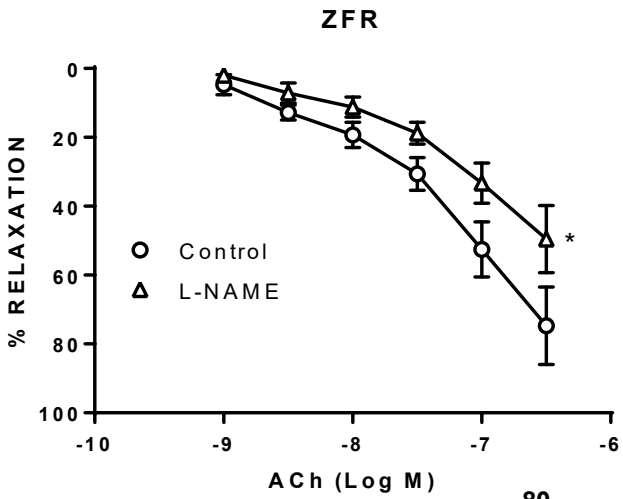
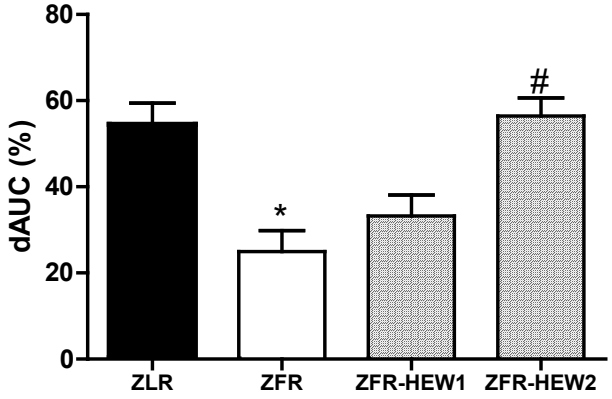
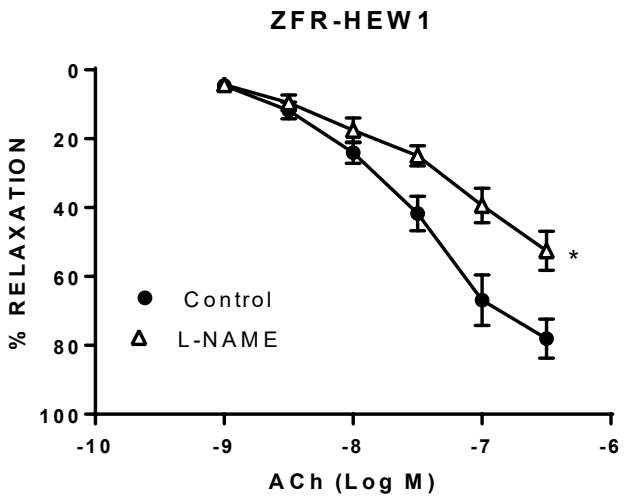
ZFR-HEW1
*

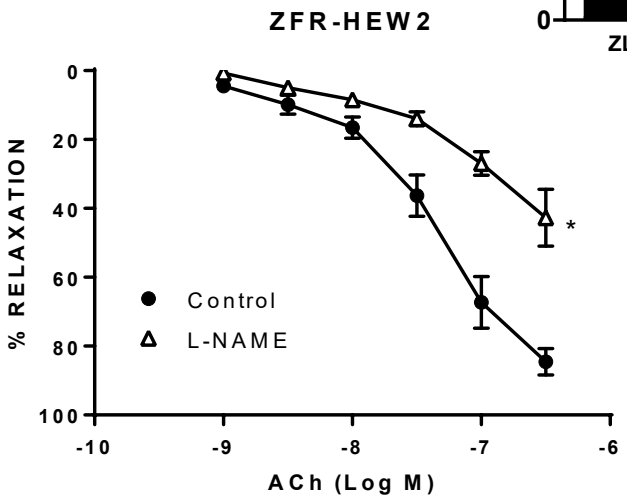

% RELAXATION
0
20
40
60
80
100
-10
-9
-8
-7
-6
-5
-4
ACh (Log M)
ZFR
ZFR-HEW2
*

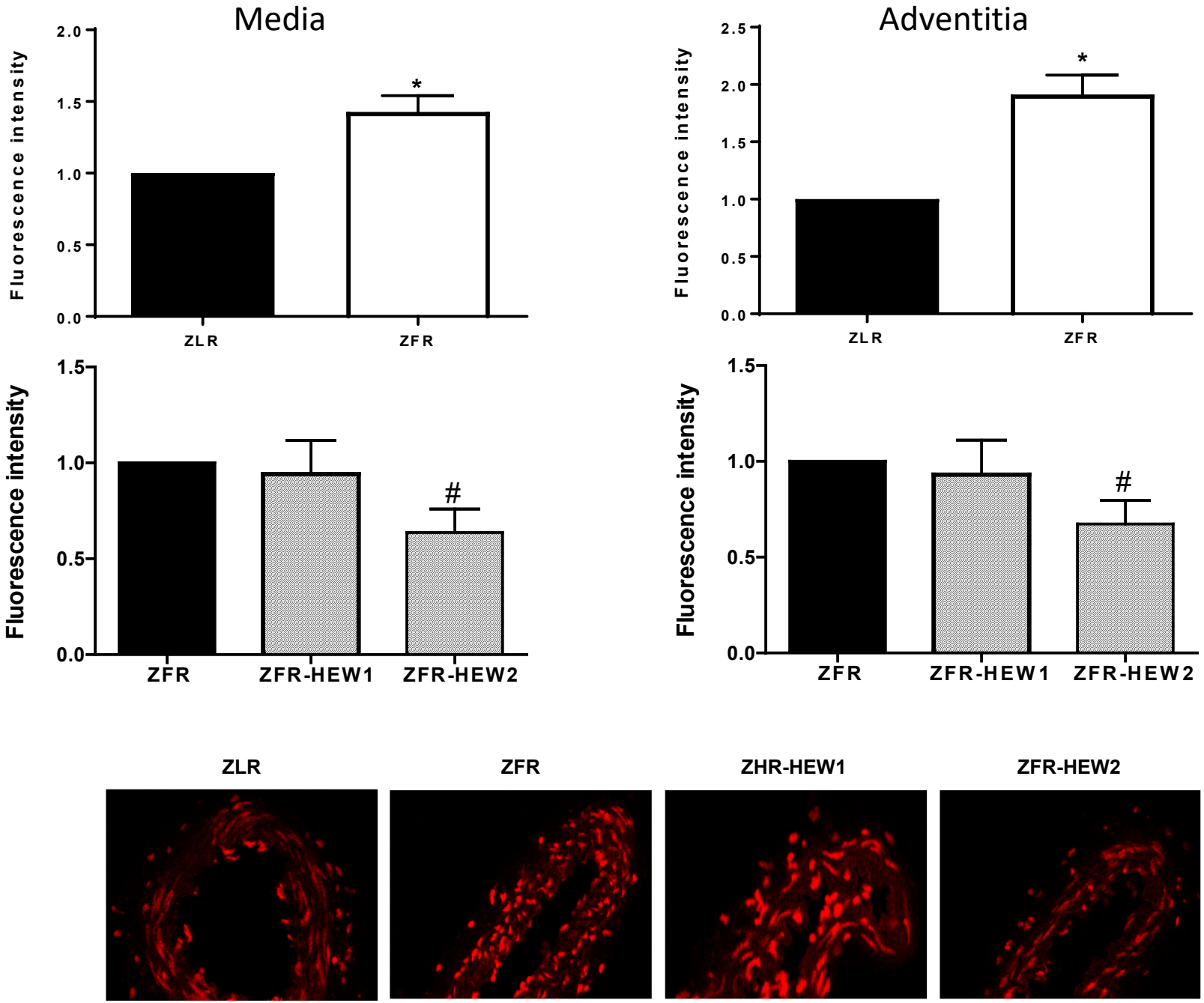

ZLR
% RELAXATION
0
20
40
60
80
100
-10
-9
-8
-7
-6
ACh (Log M)
Control
L-NAME
*

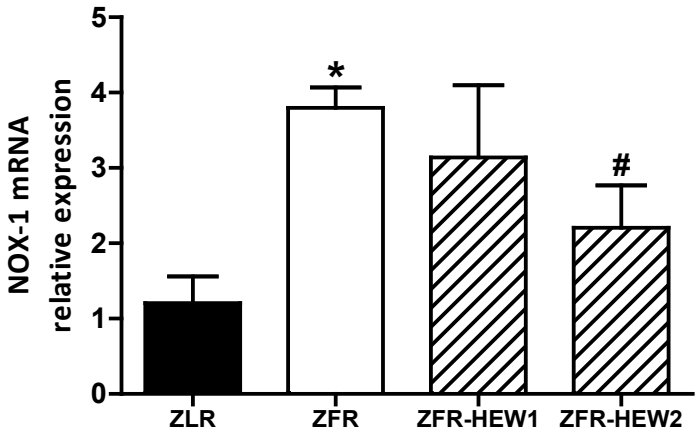

ZFR
% RELAXATION
0
20
40
60
80
100
-10
-9
-8
-7
-6
ACh (Log M)
Control
L-NAME
*

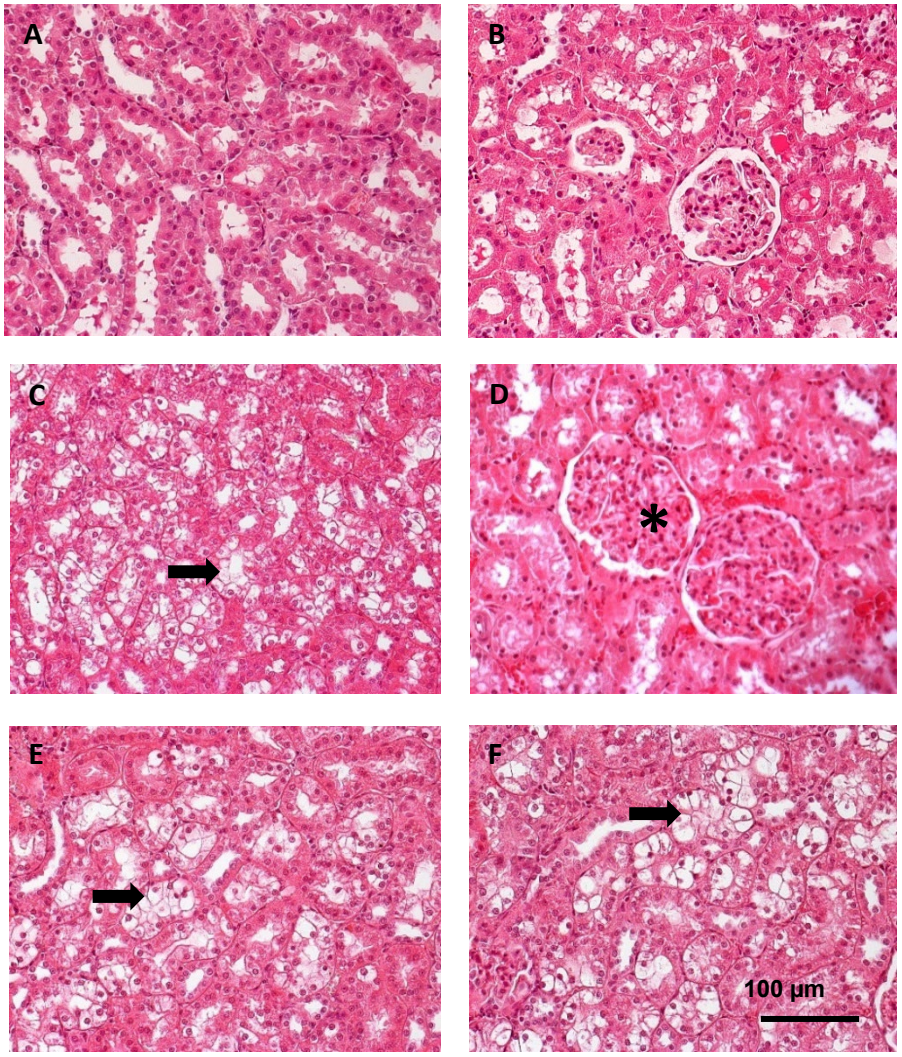

dAUC (%)
0
20
40
60
80
*
#
ZLR
ZFR
ZFR-HEW1
ZFR-HEW2

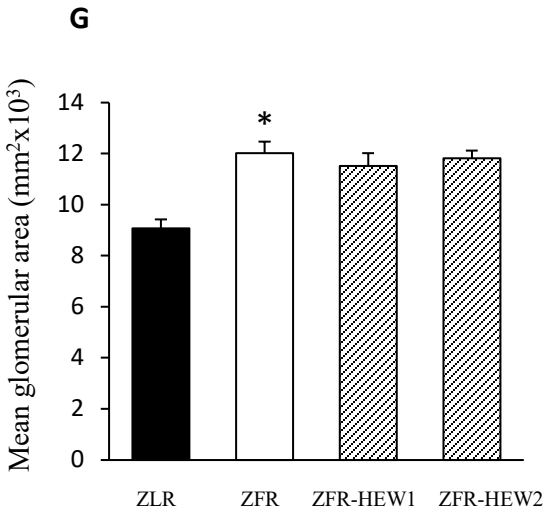

ZFR-HEW1
% RELAXATION
0
20
40
60
80
100
-10
-9
-8
-7
-6
ACh (Log M)
Control
L-NAME
*


ZFR-HEW2
% RELAXATION
0
20
40
60
80
100
-10
-9
-8
-7
-6
ACh (Log M)
Control
L-NAME
*


Media
Fluorescence intensity
2.0
1.5
1.0
0.5
0.0
*
ZLR
ZFR
Adventitia
Fluorescence intensity
2.5
2.0
1.5
1.0
0.5
0.0
*
ZLR
ZFR
Fluorescence intensity
1.5
1.0
0.5
0.0
#
ZFR
ZFR-HEW1
ZFR-HEW2
Fluorescence intensity
1.5
1.0
0.5
0.0
#
ZFR
ZFR-HEW1
ZFR-HEW2
ZLR
ZFR
ZHR-HEW1
ZFR-HEW2


NOX-1 mRNA
relative expression
5
4
3
2
1
0
*
#
ZLR
ZFR
ZFR-HEW1
ZFR-HEW2


A
B
C
D
E
F
100 µm


G
Mean glomerular area (mm²x10³)
14
12
10
8
6
4
2
0
*
ZLR
ZFR
ZFR-HEW1
ZFR-HEW2