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Egg white protein hydrolysate reduces blood pressure, improves vascular relaxation and modifies aortic angiotensin II receptors expression in spontaneously hypertensive rats

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ABSTRACT

Angiotensin converting enzyme (ACE) inhibitory peptides from egg have demonstrated reduction of blood pressure (BP) *in vivo*. In this study we evaluated the effects of egg white hydrolysate (EWH) prepared by thermolysin and pepsin in spontaneously hypertensive rats (SHRs). Fourteen to sixteen week old SHRs were implanted with telemetry devices. After recovery, the rats were divided randomly into three groups: untreated, EWH low dose (250 mg/kg BW), and EWH high dose (1000 mg/kg BW) for 12 days. BP showed a significant reduction in the EWH high dose group compared to untreated controls. BP reduction was associated with enhanced *ex vivo* vasodilation reduced oxidative/nitrosative stress, reduced angiotensin converting enzyme and angiotensin II type 1 receptor expression, while enhanced angiotensin II type 2 receptor expression. Circulating level of angiotensin II was unaffected. Thus, EWH exerted anti-hypertensive effects in SHRs through multiple mechanisms of vascular relaxation and RAS modulation.

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Abbreviations: ACE, angiotensin converting enzyme; Ang II, angiotensin II; ANOVA, analysis of variance; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; AUC, area under the curve; BP, blood pressure; bpm, beats per minute; BW, body weight; DBP, diastolic blood pressure; EWH, egg white hydrolysate; HR, heart rate; L-NAME, N-nitro-L-arginine methyl ester; MAP, mean arterial blood pressure; MCh, methacholine; NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat; SNP, sodium nitroprusside

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1. Introduction

Hypertension, the persistent elevation of blood pressure (BP) over 140/90 mm Hg, is associated with an increased risk of cardiovascular diseases and is a growing health problem worldwide (Chockalingam, 2008; Danaei et al., 2013). A number of underlying mechanisms, including hyperactivity of the renin–angiotensin system (RAS), inflammation, oxidative stress and impaired nitric oxide generation, contribute to the pathogenesis of hypertension. While a number of pharmaceutical antihypertensives are currently available for clinical usage, many are known to have significant side-effects in a disease that requires lifelong therapy (Khanna, Lefkowitz, & White, 2008). Moreover, some cases of hypertension are not adequately controlled by commonly used pharmaceutical agents (Viera, 2012). Hence, there has been a growing interest in developing novel therapies for hypertension from natural sources.

Food derived proteins and peptides are a major source of bioactive compounds with potential therapeutic applications (Aluko, 2015; Balti et al., 2012; Girgih, Alashi, He, Malomo, & Aluko, 2014; Saiga-Egusa, Iwai, Hayakawa, Takahata, & Morimatsu, 2009). Egg is an inexpensive and nutritious source of many proteins which can be used to generate novel bioactive peptides. Our previous research has identified three peptides IRW (Ile-Arg-Trp), IQW (Ile-Gln-Trp) and LKP (Leu-Lys-Pro) from ovotransferrin hydrolysate (prepared by thermolysin and pepsin) with anti-inflammatory, antioxidant and angiotensin converting enzyme (ACE) inhibitor properties (Majumder et al., 2013, 2015; Majumder & Wu, 2011). Indeed, these peptides showed potent BP lowering effects in spontaneously hypertensive rats (SHRs), a well-known animal model of hypertension (Dornas & Silva, 2011). The isolation of specific proteins like ovotransferrin from egg white as well as using individual bioactive peptides derived from this isolated protein is expensive. Hence, there is a greater feasibility associated with using enzymatic hydrolysates of whole egg white (instead of the constituent proteins or peptides) for therapeutic applications. It is not known if egg white hydrolysate (EWH), as a source of ovotransferrin with *in vitro* ACE-inhibitory activity, would also exert antihypertensive effects *in vivo*. Given this background, we fed EWH (generated by thermolysin and pepsin) orally to SHRs to evaluate its potential as an anti-hypertensive treatment and examine the mechanisms of action focusing on various aspects of the RAS.

2. Materials and methods

2.1. Preparation of egg white hydrolysate

Hydrolysis of egg white was carried out as described previously (Majumder & Wu, 2011). Briefly, egg white slurry (5%, w/v) was first heated to 80 °C for 10 min and digested with thermoase PC10F (Amano Enzyme Inc. Nagoya, Japan) 0.1% w/w at pH 8 and 65 °C for 90 min. The pH was then adjusted to 2.5 for pepsin digestion. Pepsin (from porcine stomach, 10,000 units/mg, American Laboratories Inc. Omaha, NE, USA) was added at 1% w/w to the mixture at 55 °C and hydrolysis was carried out for another 180 min. After heat inactivation of enzymes, the hydrolysate was centrifuged and supernatant was collected and freeze dried for further experiments. The average protein content of the hydrolysate was 77.69%.

2.2. Ethics statement, animal model and experimental design

Rat experimental procedures were approved by the University of Alberta Animal Welfare Committee (Protocol # 611/09/10D) in accordance with the guidelines issued by the Canadian Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The feeding experiments were carried out using fourteen to sixteen week old male spontaneous hypertensive rats (SHRs) weighing 290.0 ± 10.5 g obtained from Charles River (Senneville, QC, Canada). Upon arrival, rats were acclimatized for one week at the University of Alberta animal facility with a 12:12 hour cycle of light:dark in a humidity and temperature controlled (60% RH, and 23 °C) environment and maintained on standard chow and water *ad libitum*. After one-week acclimation, SHRs were chronically implanted with DSI telemetry transmitters (PA-C40; Data Sciences International, Minneapolis, MN) as described previously (Majumder et al., 2013). After one week recovery following surgery, rats were randomly assigned into 3 groups (n = 6–7): untreated (control), EWH low dose (250 mg/kg BW) and EWH high dose (1000 mg/kg BW). EWH dosages were chosen based on the previous studies done in our lab (Jahandideh et al., 2014). Treatments were given orally once a day for 12 days after mixing with 20 mL of Ensure (Abbott Nutrition, QC, Canada). Ensure was used to enhance the palatability of the hydrolysate. The untreated group received the same volume of Ensure only (Jahandideh et al., 2014). BP was recorded on days 0 (baseline), 3, 6, 9, and 12 for 24 h (10 s of every 1 min) for all animals. On the morning of day 13, the animals were euthanized by exsanguination via excision of the heart under inhaled isoflurane anesthesia (isoflurane/oxygen; 1.0–2.5% mixture). Following sacrifice, blood was collected from the heart in EDTA coated tubes (BD Vacutainer, NJ, USA), and centrifuged (1000 *g* for 20 min at 4 °C) to obtain plasma. Tissues were removed immediately, rinsed with cold saline, weighed, flash frozen with liquid nitrogen and stored at –80 °C for further analysis. The mesenteric arteries were isolated immediately and used for *ex vivo* vascular function studies.

2.3. Data acquisition and signal processing

Chronic measurement of BP was done in a quiet room with minimal electrical interference as previously described (Jahandideh et al., 2014). Mean arterial blood pressure (MAP) was measured while systolic blood pressure (SBP) and diastolic blood pressure (DBP) were extracted from the observed signal. Heart rate (HR) was calculated between two consecutive points and expressed in beats per minute (bpm).

2.4. Vascular function studies

Second order branches of the mesenteric arteries were carefully excised from SHRs and used for *ex vivo* vascular function experiments. Isolation, mounting, normalization and validating

integrity of vessels were the same as described before (Jahandideh et al., 2014). Constrictor responses of vessels were determined by assessing responses to phenylephrine (PE, 10^{-8} to 10^{-4} M, Sigma Aldrich, Oakville, Canada). In two sets of vessels, the role of nitric oxide (NO) in endothelium-dependent relaxation was assessed via studying the methacholine (MCh, 10^{-10} to 10^{-4} M, Sigma) relaxation response of vessels in the presence or absence of the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 μM, Sigma). The contribution of vascular smooth muscle to relaxation was also assessed in a separate set of vessels through studying the relaxation response of vessels to sodium nitroprusside (SNP, 10^{-10} to 10^{-5} M, Sigma); which is an endothelium-independent relaxing agent. At the end of the experiment, the vessels were exposed to high potassium buffer to confirm their viability. Vessels with constriction less than 80% of their maximum constriction to PE were excluded from analysis.

2.5. *Plasma analysis for circulating Ang II*

Plasma concentrations of angiotensin II (Ang II) were quantified by ELISA kit (Ang II ELISA, Cayman Chemical, Ann Arbor, MI, USA) based on the manufacturer's instructions.

2.6. *Immunofluorescence*

Aortic specimens were embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen immediately in liquid nitrogen for subsequent analysis. Ten micrometer tissue sections were prepared, fixed in cold acetone and incubated with blocking buffer (1% BSA in phosphate-buffer saline) for 1 hour. The sections were then immunostained overnight at 4 °C with rabbit polyclonal antibodies for nitrotyrosine (Dilution 1:200; Chemicon, Temecula, CA, USA), ACE, ACE2, angiotensin II type 1 (AT1R) and type 2 receptor (AT2R) (Dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation with a secondary antibody (dilution 1:500; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) was performed for 30 min in the dark at room temperature. For nitrotyrosine, glass cover-slips were mounted with a Vectashield H-1200 Mounting Kit, containing nuclear stain, DAPI (Vector Laboratories, Burlington, ON, Canada) on the corresponding slides and immediately visualized under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using SlideBook imaging software (Olympus) and presented at 100× magnification. For ACE, ACE2, AT1R and AT2R, glass cover-slips were mounted with PBS and scanned by Odyssey Sa system (Licor Biosciences) with 20 μm resolution. A control image with the secondary antibody alone was used to detect any nonspecific binding. The images were then quantified by subtracting the background fluorescence in the control image, so only the fluorescence from specific immunostaining was visible.

2.7. *Statistical analysis*

All data presented are mean ± SEM of 4–7 experiments, as indicated in the figure legends. MCh curves were fitted using nonlinear regression, and values for area under the curve (AUC) were compared. Statistical analysis was performed using GraphPad Prism software (version 6.0). For analyses of BP data, we used a two-way analysis of variance (ANOVA) with Tukey's post-hoc test. For all other studies, a t-test was used for comparison between 2 groups while a one-way ANOVA (with Dunnett's post-hoc test) was performed for comparison involving 3 or more groups. A p-value <0.05 was considered statistically significant.

3. Results and discussion

3.1. *EWH reduces BP in SHRs*

Baseline MAP in all experimental groups was above 160 mmHg; which is an indication of hypertension in SHRs. The pathogenesis of hypertension in SHRs is similar to human essential hypertension (Okamoto & Aoki, 1963). In this study, although both doses of EWH tended to reduce BP in SHRs over the experimental period compared to the untreated group, this reduction was only significant in the high dose treated group (1000 mg/kg BW) ($p < 0.001$, Fig. 1A–C). HR was not different between the groups (Fig. 1D). Hydrolysis of egg white breaks down the protein structure generating bioactive peptides and possibly free amino acids which may be critical for the antihypertensive effects. Hydrolysis of the egg white with thermolysin and pepsin generated a complex array of peptides which could potentially contribute to the observed antihypertensive effects. The hypotensive effects of egg-derived peptides have been reported in the literature (Duan et al., 2014; Jahandideh et al., 2014; Majumder et al., 2013; Matoba, Usui, Fujita, & Yoshikawa, 1999; Miguel, Lopez-Fandino, Ramos, & Aleixandre, 2005; Rawendra et al., 2013; Yu, Zhao, Liu, Lu, & Chen, 2011). Interestingly, the presence of tripeptides IRW, IQW, LKP with proven antihypertensive properties has also been confirmed in our EWH preparation through LC–MS/MS analysis (*data not shown*). Utilizing protein hydrolysates instead of single peptides has the advantage of reduced cost of processing and easier incorporation into food system as a natural component. Moreover, the available peptides in the whole protein hydrolysate may have other beneficial biological activities. Since the BP was only significantly changed in high dose EWH treated animals, all the subsequent experiments were carried out on this group compared to untreated group.

3.2. *EWH improves vascular relaxation, reduces nitrosative stress and modifies Ang II receptors expression*

SHRs show impaired vasodilation, higher circulating levels of Ang II as well as increases in oxidative stress and inflammation compared to normal rats (Zicha & Kunes, 1999). The effect of EWH on vascular function in SHRs was evaluated as one of the possible mechanisms for its antihypertensive effects. Since mesenteric arteries contribute significantly to the alteration of BP through affecting systemic vascular resistance (Pannirselvam, Wiehler, Anderson, & Triggle, 2005), this vascular bed was studied to investigate the effects of the treatment on *ex vivo* vascular function. As evident in Fig. 2A, vasodilation to MCh was significantly enhanced by treatment with EWH compared to the untreated group ($p < 0.05$). Enhanced NO is

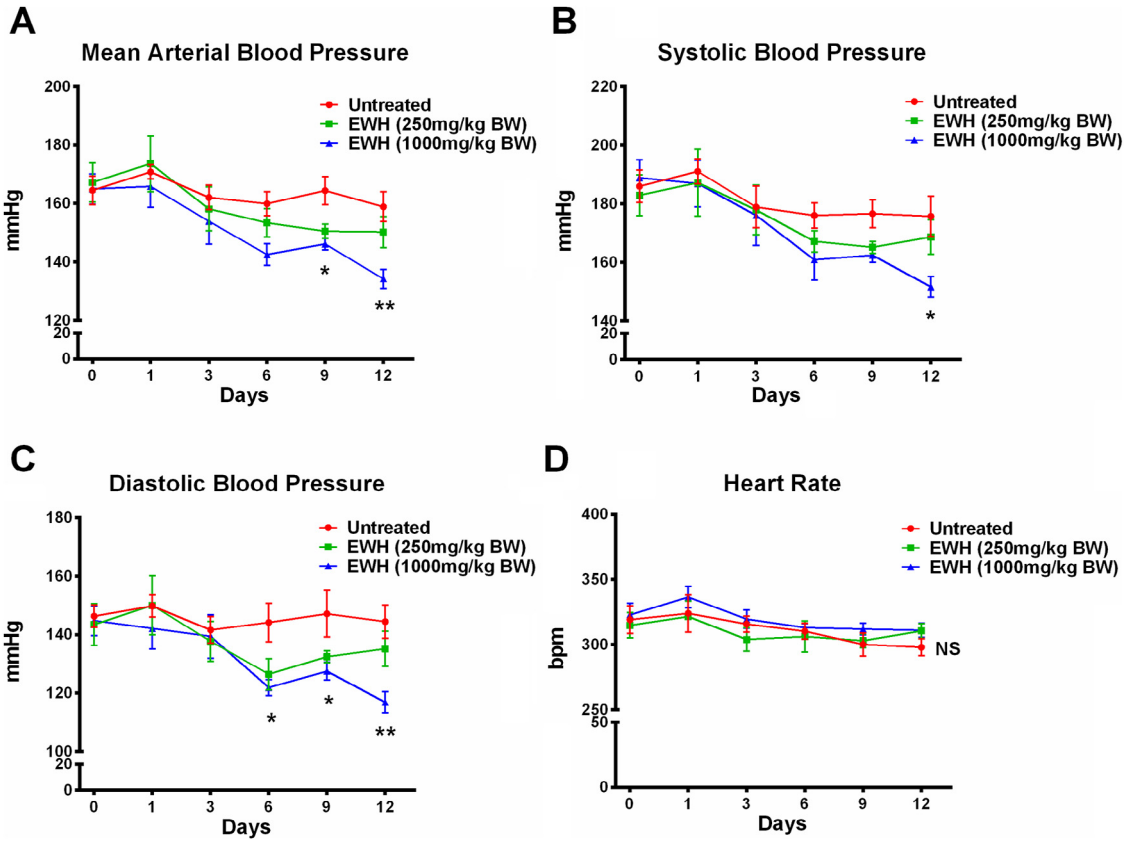

Fig. 1 – Egg white hydrolysate (EWH) reduces BP in SHRs. (A, B, and C) MAP, SBP, and DBP (mmHg) values for untreated or EWH treated (250 and 1000 mg/kg BW) SHRs over a period of 12 days. BP values for each represent the mean BP recorded over a 24 hr period. (D) HR (bpm) of SHRs in treatment groups over 12 days. Treatment with EWH (1000 mg/kg BW) significantly lowered MAP (A), SBP (B), and DBP (C) but not heart rate (D). Data represented as mean ± SEM from n = 6–7 animals per treatment group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared to untreated group. NS indicates not significant compared to the untreated group.

one of the possible endothelium-dependent mechanisms of vasodilation. Incubation of mesenteric arteries with L-NAME, a NOS inhibitor, reduced vasodilation in both untreated and EWH treated animals (Fig. 2B and C), suggesting a contribution of NO to relaxation in both groups. However, EWH further enhanced NO-dependent vasodilation compared to the untreated group as shown by a significant increase in the delta area under the curve (Δ AUC) of the MCh curves obtained for each of the two groups with and without NOS inhibition ($p < 0.05$, Fig. 2D). This increase in NO-dependent vasodilation may be due to the enhanced NO bioavailability through scavenging of free radicals or increased NO production in the vasculature. Vascular relaxation to SNP, an exogenous NO donor, was also significantly enhanced in EWH treated group compared to the untreated group ($p < 0.05$, Fig. 3); but to a lesser extent than MCh responses. This implies the involvement of either endothelium-independent mechanisms (in addition to endothelium-dependent ones) or enhanced NO bioavailability in EWH treated compared to untreated group. When the effect of EWH treatment on nitrosative stress in aortic sections of SHRs was further studied, we observed a significant decrease in aortic nitrotyrosine staining in EWH treated animals ($p < 0.01$, Fig. 4). This may explain the improved NO bioavailability and vasodilation effects observed in the vascular function experiments.

Ang II, the principal component of the RAS pathway, has diverse physiological actions regulating blood pressure and salt/water balance; elevated circulating Ang II level leads to high BP in SHR. Since EWH was initially identified as an *in vitro* ACE inhibitor, plasma Ang II levels were assessed in both EWH and untreated animals. Plasma Ang II levels were not significantly different between EWH and untreated groups ($p > 0.05$, Fig. 5), indicating that EWH may not work solely as an ACE inhibitor in reducing BP in SHRs. However, ACE expression in the EWH treated animals was significantly reduced compared to the untreated animals ($p < 0.05$, Fig. 6A). ACE2 is another contributor to Ang II plasma level; however feeding EWH did not affect the expression level of ACE2 ($p > 0.05$, Fig. 6B).

We further assessed the expression level of Ang II receptors. Ang II acts through two main receptors, angiotensin type 1 and type 2 receptors. Binding to AT1R receptor causes vasoconstriction in vascular smooth muscle cells and other potentially harmful consequences while binding to AT2R receptor induces opposing effects (Balakumar & Jagadeesh, 2014). Interestingly, EWH significantly reduced the expression of AT1R and enhanced concomitantly that of AT2R in SHRs compared to the untreated group ($p < 0.05$, Fig. 7A and B). Since the AT1R is responsible for the most known pathogenic effects of Ang II in the body, this finding offers a potentially novel way to

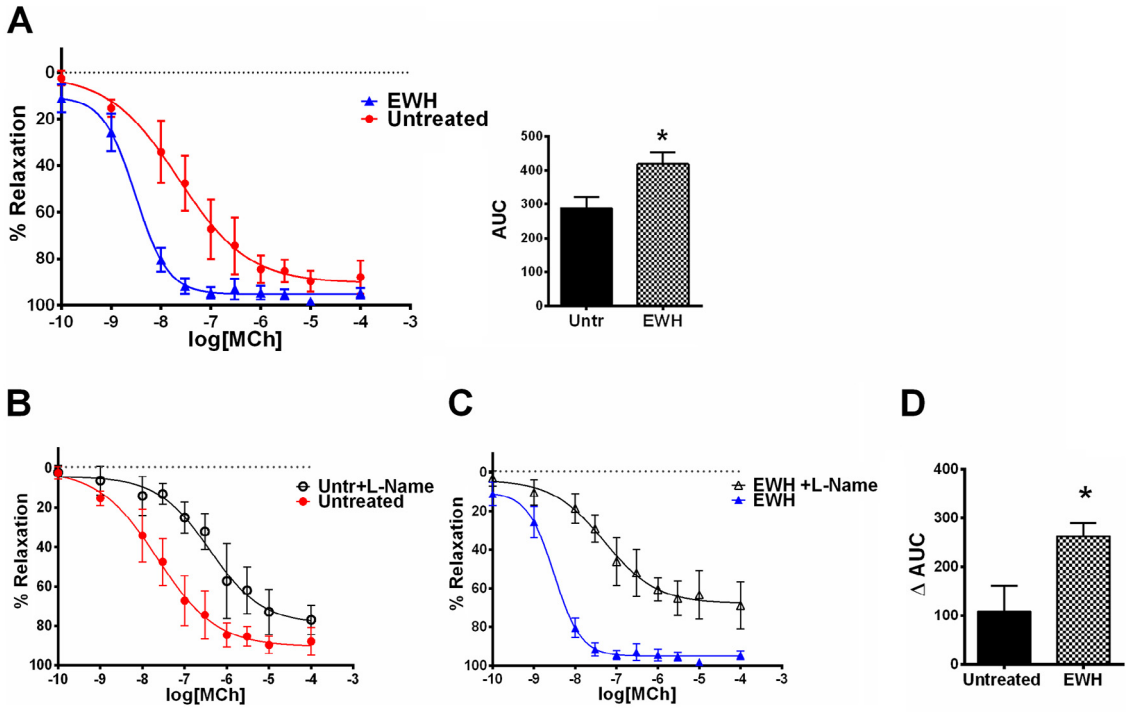

Fig. 2 – EWH treatment induces nitric oxide contribution to vasodilation in mesenteric arteries of SHRs. (A) EWH (1000 mg/kg BW) significantly increased vasorelaxation to MCh. Pre-incubation with L-NAME (100 μM) reduced vasorelaxation in untreated rats (B) as well as EWH treated rats (C). EWH further enhanced NO dependent vasorelaxation compared to untreated group (D). Data represented as mean ± SEM from n = 4–6 animals per treatment group. * indicates $p < 0.05$ compared to untreated group.

attenuate the effect of an overactive RAS pathway. This beneficial effect was further enhanced by increased AT2R expression, contributed collectively to improved vasodilation in vascular function experiments. Therefore, although rats in the EWH and untreated groups had almost the same levels of circulating Ang II, the antihypertensive effects observed upon EWH feeding were due mainly to different expression levels of Ang II receptors. It is possible that the EWH treatment increases the breakdown of receptor protein and/or reduces the protein synthesis of the receptor. To the best of our knowledge, this is the first study reporting the effects of a food compound on modulating AT1R and AT2R expression *in vivo*.

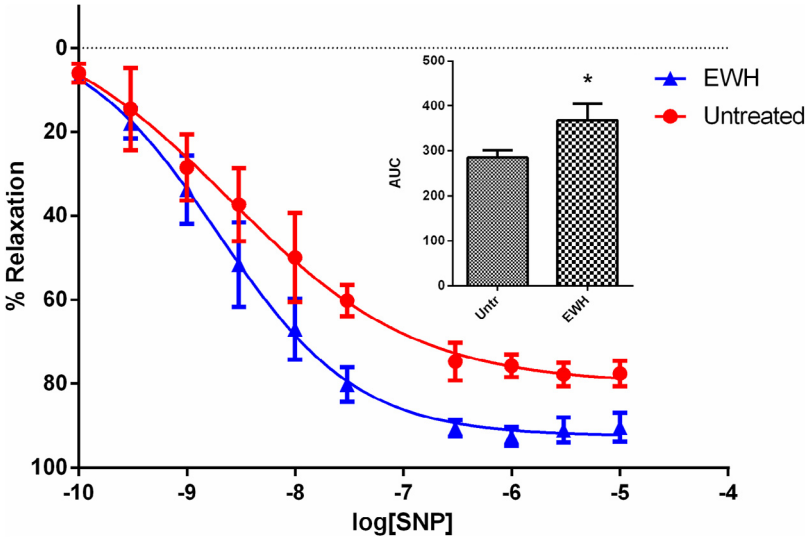

Fig. 3 – EWH treatment enhances vasodilation in the presence of exogenous nitric oxide in mesenteric arteries of SHRs. EWH (1000 mg/kg BW) significantly increased vasorelaxation to SNP in SHRs. Data represented as mean ± SEM from n = 4–7 animals per treatment group. * indicates $p < 0.05$ compared to untreated group.

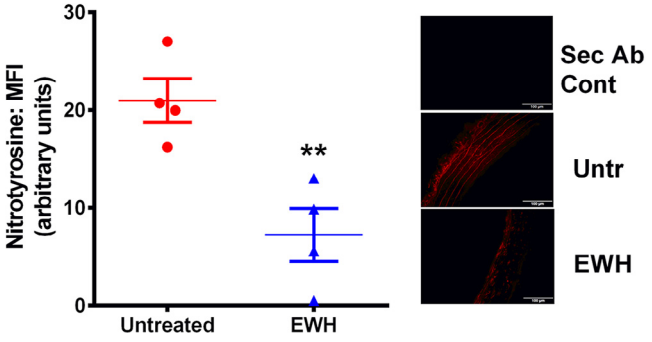

Fig. 4 – EWH treatment reduces tissue nitrotyrosine in SHRs. Immunostaining for nitrotyrosine in aortic sections of untreated and EWH (1000 mg/kg BW) treated SHRs. Data represented as mean ± SEM from n = 4 animals per treatment group. ** indicates $p < 0.01$ compared to untreated group.

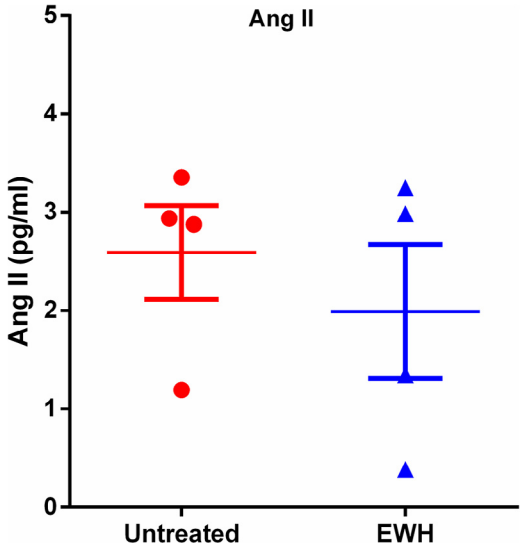

Fig. 5 – Effects of EWH on plasma circulatory Ang II. Ang II level was not affected after 12 days of treatment with EWH (1000 mg/kg BW). Data represented as mean ± SEM from n = 4 animals per treatment group.

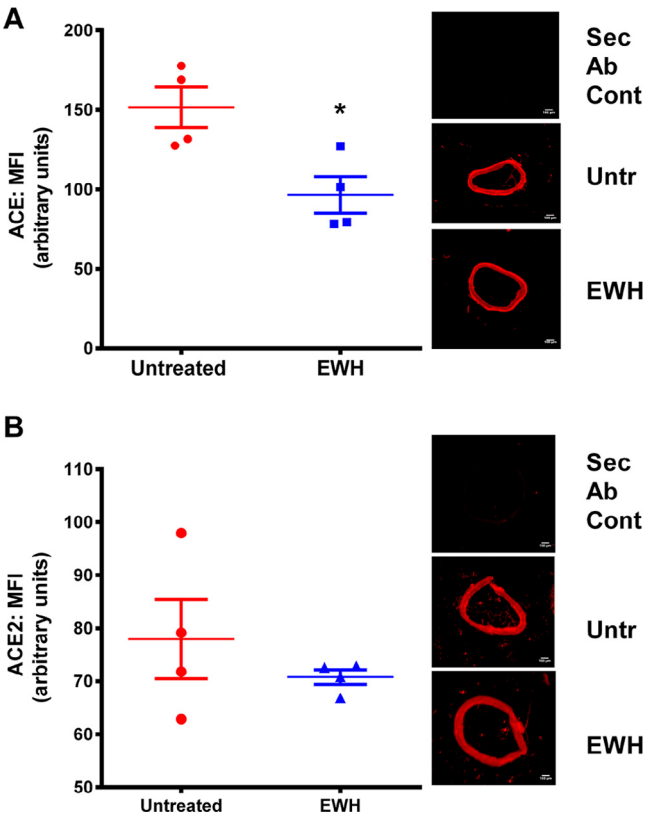

Fig. 6 – EWH reduces ACE but not ACE2 expression in aorta of SHRs. Immunostaining for ACE (A) and ACE2 (B) in aortic sections from untreated and EWH (1000 mg/kg BW) treated SHRs. Data represented as mean ± SEM from n = 4 animals per treatment group. * indicates $p < 0.05$ compared to untreated group.

Since BP is regulated by several organs in the body and is sex-specific, using only male SHRs and lack of normotensive control rats is one of the limitations of our study. The effects of EWH on sympathetic nervous system through binding to opioid receptors remain to be answered in future studies. It is also interesting to investigate the effects of EWH on BP post treatment to see if the effect on BP remains or not. As a well-known food allergy, the possible allergenicity of EWH especially in subjects already allergic to egg and egg products also needs to be evaluated in future studies.

4. Conclusion

The present study demonstrated the role of EWH in reducing BP in SHRs through several mechanisms. The reduction in BP was concomitant with an increased vasodilation, reduced nitrosative stress, reduced ACE and AT1R expression, as well as enhanced AT2R expression. While our findings are novel, further research is needed to ascertain the role of EWH on mechanisms involved in endothelial independent vasorelaxation to achieve a comprehensive understanding of underlying mechanisms. The findings from this study may establish the potential of egg derived bioactive peptides in the management of hypertension and associated complications.

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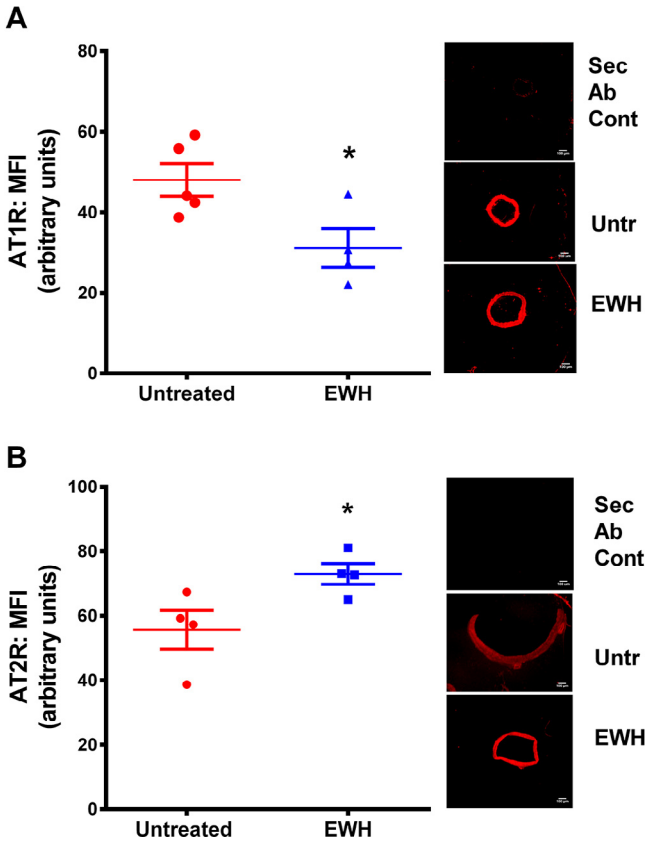

Fig. 7 – EWH reduces AT1R and increases AT2R expression in aorta of SHRs. Immunostaining for AT1R (A) and AT2R (B) in aortic sections from untreated and EWH (1000 mg/kg BW) treated SHRs. Data represented as mean ± SEM from n = 4–5 animals per treatment group. * indicates $p < 0.05$ compared to untreated group.

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