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Egg White Hydrolysate: A new putative agent to prevent vascular dysfunction in rats following long-term exposure to aluminum

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ABSTRACT

Aluminum (Al) is toxic for humans and animals. Here, we have tested the potential for Egg White Hydrolysate (EWH) to protect against cardiovascular changes in rats exposed to both high and low dietary levels of Al. Indeed, EWH has been previously shown to improve cardio metabolic dysfunctions induced by chronic exposure to heavy metals. Male Wistar rats received orally: Group 1) Low aluminum level ($AlCl_3$ at a dose of 8.3 mg/kg b.w. during 60 days) with or without EWH treatment (1 g/kg/day); Group 2) High aluminum level ($AlCl_3$ at a dose of 100 mg/kg b.w. during 42 days) with or without EWH treatment. After Al treatment, rats co-treated with EWH did not show vascular dysfunction or increased blood pressure as was observed in non EWH-cotreated animals. Indeed, co-treatment with EWH prevented the following effects observed in both aorta and mesenteric arteries: the increased vascular responses to phenylephrine (Phe), the decreased ACh-induced relaxation, the reduction on endothelial modulation of vasoconstrictor responses and the nitric oxide bioavailability, as well as the increased reactive oxygen species production from NAD(P)H oxidase. Altogether, our results suggest that EWH could be used as a protective agent against the harmful vascular effects after long term exposure to Al.

1. Introduction

The burgeoning human exposure to aluminum (Al) brings several consequences for health (Exley, 2013). The neurotoxicity of Al and its involvement with neurodegenerative and neuromuscular diseases is well established (Crépeaux et al., 2017; Gherardi et al., 2016; Mirza et al., 2016). Recently, evidences suggest Al as a new variable influencing cardiovascular risk and highlight its possible role in the development of cardiovascular diseases (Costello et al., 2014; Lind et al., 2012; Neophytou et al., 2016; Subrahmanyam et al., 2016). Al possess ability to be accumulated in human arteries (Bhattacharjee, 2013; Minami et al., 2001). It seems does not accumulate uniformly within arteries showing a gradient of deposition within the arterial walls from the aorta to the posterior cerebral artery that supplies blood to the hippocampus and, highest affinity for human brain endothelial cells (Bhattacharjee et al. 2013). The blood level of Al was related to

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atherosclerotic plaques formation in elderly (Lind et al., 2012) and, its circulating presence was appointed as one of the risk factors of high arterial stiffness in healthy population (Subrahmanyam et al., 2016).

More recently, a retrospective cohort study revealed that a low serum Al ≥6 ng/mL is independently associated with mortality in patients on chronic hemodialysis (Tsai et al. 2018). Moreover, considering the pro-oxidant and proinflammatory effects of Al (Exley, 2004; Ruipérez et al., 2012), it is plausible to postulate its role as a cardiovascular risk agent.

Experimental studies support the cardiac sensitive to toxic effects of Al. Rats exposure to Al at 0.1, 50, and 200 mg/kg for 120 days show cardiac abnormalities with dose-dependent Al bioaccumulation, structural and ultrastructural cardiac abnormalities and diffuse inflammation (Novaes et al., 2018). Al exposure up to 256 mg Al/kg b.w. over 120 days raised blood pressure in rats, which was related to the increased oxidative stress and osmotic fragility of erythrocyte membranes (Zhang et al., 2016). At vascular level, our group has recently demonstrated that acute or sub-chronic exposures to Al promote opposite effects. Thus, 1-h of Al exposure reduces vascular reactivity (Schmidt et al., 2016) while rats exposure to Al for 60 days show vascular dysfunction with impairment of both relaxation and contraction (Martinez et al., 2017a). However, there are still many doubts regarding the cardiovascular consequences of human exposure to Al. We have observed that Al exposure at a level which might be considered equivalent to normal dietary intake was sufficient to promote vascular dysfunction in rats, and, surprisingly, these effects were almost the same when we treated rats at a higher (super-dietary level) dose of Al (Martinez et al., 2017a).

Considering the imminent increase of human body burden of Al and its consequences, the study of alternatives to prevent or reduce these following effects is a priority. In this respect, our group has studied the effect of several bioactive peptides derived from Egg White Hydrolysate (EWH) after pepsin hydrolysis for 8 h (Miguel et al., 2004). These released peptides seem to have potent anti-inflammatory, antioxidant and/or antihypertensive properties (Garcés-Rimón et al., 2016; Garcés-Rimón et al., 2016a; Miguel et al., 2006), which could be valuable to prevent or reduce the after effects of Al exposure. Previously, we have observed that the intake of EWH improves glucose metabolism abnormalities related with metabolic syndrome in obese Zucker rats and in a diet-induced obesity experimental model (Garcés-Rimón et al., 2018; Moreno-Fernández, 2018), prevents complications associated with heavy metals exposure (Rizzetti et al. 2016, 2017) as well as, the cognitive impairments after long-term Al exposure (Martinez et al. 2019). Specifically, on cardiovascular protection, EWH has demonstrated angiotensin converting enzyme (ACE) inhibition, antioxidant, vasodilator properties (Dávalos et al., 2004; Manso et al., 2008; Miguel et al., 2008; Miguel et al. 2007; Miguel et al., 2007a; Miguel et al. 2006; Miguel et al. 2004) and, antihypertensive effects after short (Miguel et al., 2005) or long-term oral administration to spontaneously hypertensive rats (Miguel et al., 2006).

Herein, we have investigated the proposed cardio protective effect of EWH against vascular dysfunction in rats exposed to both a low and high level of dietary Al as well as the possible underlying mechanisms.

2. Materials and methods

2.1. Preparation of EWH

The bioactive peptides were obtained by pepsin hydrolysis of crude egg white for 8 h, as previously described (Garcés-Rimón et al., 2016). Briefly, commercial pasteurized egg white was hydrolyzed with BC Pepsin 1:3000 (E.C. 3.4.23.1; from pork stomach, E:S: 2:100 w:w, pH 2.0, 38 °C) (Biocatalysts, Cardiff, United Kingdom) and the enzyme was inactivated by increasing the pH to 7.0 with 5 N NaOH. The hydrolysate was centrifuged at 2500 g for 15 min and the supernatants were frozen and lyophilized. EWH was characterized in previous work and some peptide sequences (RADHPFL, IVF, YAEERYPIL, FRADHPFL, RDILNQ, YRGGLEPINF or ESIINF) were identified (Miguel et al., 2004). Later, reverse-phase liquid chromatography–mass spectrometry (RP-HPLC-MS/MS) analyses confirmed the presence of these active sequences mentioned above in EWH with pepsin for 8 h (Garcés-Rimón et al., 2016). The consistency of the every hydrolysis process is checked by high performance liquid chromatography.

2.2. Animals

Male *Wistar* rats (90 days-old, 360 ± 11.2 g) were obtained from the Charles River Animal Laboratory, Barcelona, Spain. Animals were housed under standard conditions (constant room temperature, humidity, and 12:12 h light-dark) with water and food available *ad libitum*. All experimental protocols were performed in accordance with the guidelines stated by the Brazilian Societies of Experimental Biology and the European and Spanish legislation on care and use of experimental animals (EU Directive, 2010/63/EU for animal experiments; R.D. 53/2013). The experimental protocol was approved by the Ethics Committees on Animal Use at both Universidade Federal do Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014) and Universidad Rey Juan Carlos, Madrid, Spain (Process Number: 39/2012).

Male *Wistar* rats were randomly distributed into two main groups according to their Al exposure and received orally and once a day: Group 1) Low Al level - rats were divided into 4 subgroups (n = 8/each) (1a-d) and received for 60 days: a) Untreated - ultrapure water as the daily drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) $AlCl_3$ – Al at a dose of 8.3 mg/kg b.w. in the daily drinking water, representing human Al exposure by diet (Martinez et al., 2017); c) Hydrolysate - ultrapure water as the daily drinking water and EWH at 1 g/kg/day by gavage (Miguel et al., 2006); d)Hydrolysate-Aluminum - Al at 8.3 mg/kg b.w.; and Group 2) High Al level - rats were divided into 4 subgroups (n = 8/each) (2a-d) and received for 42 days: a) Untreated – ultrapure water by oral gavage; b) $AlCl_3$ – Al at 100 mg/kg b.w. by gavage, representing a high level of human exposure to Al (Prakash & Kumar, 2009); c) Hydrolysate - ultrapure water and EWH at 1 g/kg/day both by gavage; d) Hydrolysate-Aluminum - Al at 100 mg/kg b.w. In the Group 1 – Low Al level, rats were exposed to Al in their drinking water for 60 days to better represent the dietary human Al exposure (Martinez et al., 2017). In the Group 2 - High Al level, animals were treated for 42 days by oral gavage and received tap water as drinking water, this model was described to induce Al neurotoxicity representing a high level of human exposure to Al (Prakash & Kumar, 2009).

$AlCl_3$.6 H_2O solution was prepared in ultrapure water being the concentration of each stock solution of 0.034 M (Group 1; 8.3 mg/kg/b.w.) and 0.331 M (Group 2; 100 mg/kg/b.w.). All reagents and salts were obtained from Sigma-Aldrich and Merck (Darmstadt, Germany).

2.3. Systolic blood pressure

Indirect systolic blood pressure (SBP) was measured weekly prior the treatment and during all the treatment period, using non-invasive tail-cuff plethysmography (AD Instruments Pty Ltd, Bella Vista, NSW, Australia).

2.4. Vascular reactivity experiments

Rat body weights, food and water or Al intakes for groups that have received Al in the drinking water were measured once a week. At the end of the treatments, animals were anaesthetized with a combination of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, *ip*) and euthanized. Thereafter, the thoracic aorta and the third-order mesenteric resistance arteries (MRA) were carefully dissected out and cleaned of fat and connective tissues. For vascular reactivity experiments, the

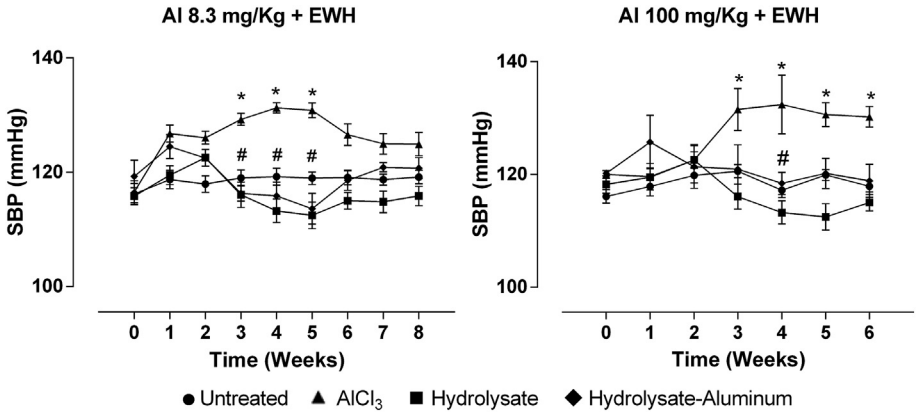

Fig. 1. Effects of EWH on non-invasive systolic blood pressure. Values of blood pressure (mmHg) after $AlCl_3$ exposure, co-treated or not with EWH. Data are expressed as mean ± SEM, n = 8, *P < 0.05 compared with their corresponding controls (Untreated rats), #P < 0.05 compared with $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni).

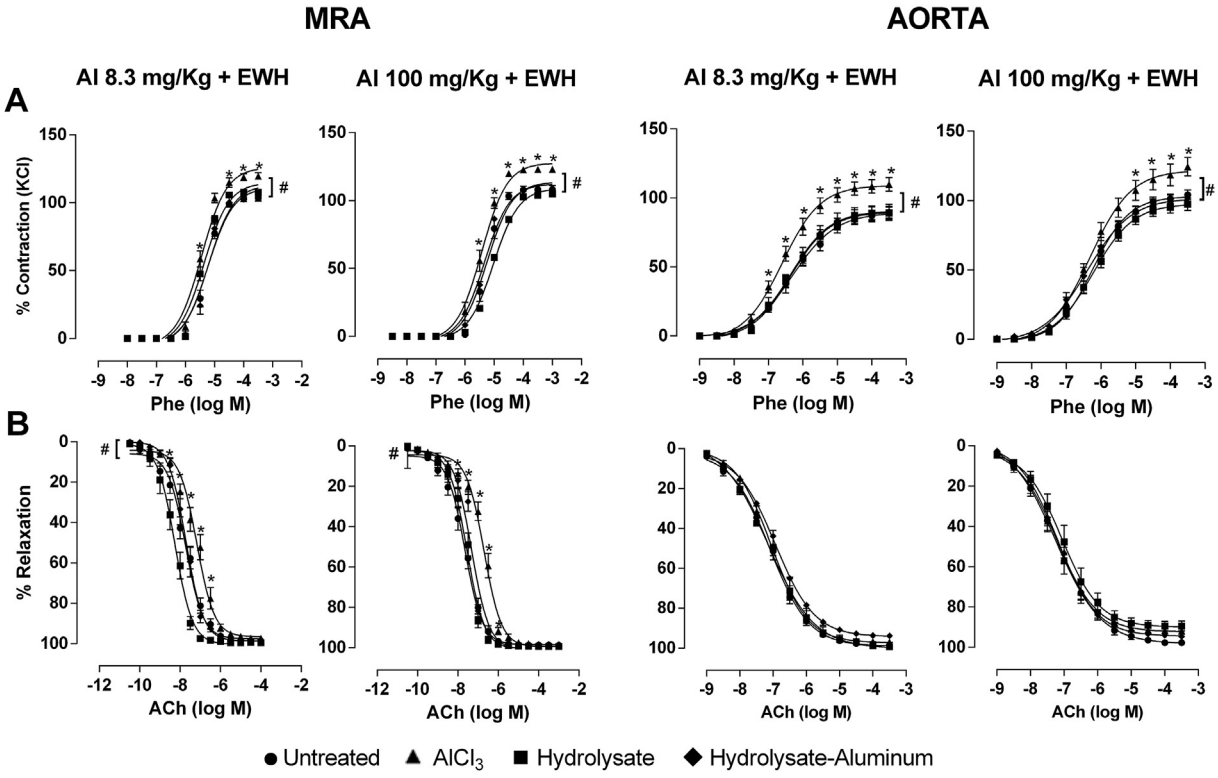

Fig. 2. Effects of EWH on vascular reactivity. Concentration–response curves to (A) phenylephrine and (B) acetylcholine in aorta and MRA segments. Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 compared with their corresponding Untreated groups, #P < 0.05 compared with $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni).

arteries were divided into cylindrical segments of 2 mm in length and placed into Krebs-Henseleit solution (in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 23; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 11 and EDTA 0.01), gassed with 95% O_2 and 5% CO_2 (pH 7.4). The remaining aorta and MRA segments were kept at −80 °C for further biochemical/biological assays.

Aortic segments were mounted in an isolated tissue chamber and maintained at a resting tension of 1.5 g at 37 °C. Isometric tension was recorded using an isometric force transducer (TSD125BX8, Biopac Systems, Inc, Santa Barbara, CA, USA) connected to an acquisition system (MP150WSW-SYS, Biopac Systems). MRA segments were mounted in a small-vessel dual chamber myograph (Multi Wire Myograph System, DMT620, ADInstruments, Australia) for measurement of isometric tension according to Wiggers et al. (2008). Segments were stretched to their optimal lumen diameter for active tension development. This was determined based on the internal circumference-to-wall tension ratio of the segments by setting their internal circumference, Lo, to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg.

After a 45-min equilibration period, aortic and MRA segments were respectively exposed twice to 75 and 120 mM KCl, first to check their functional integrity and again to assess the maximal tension developed. Afterwards, endothelial integrity was tested with acetylcholine (ACh, 10 μM) in segments that were previously contracted with phenylephrine (Phe) at a concentration that produced close to 50% of the contraction induced by KCl. After 60 min of washout, a single concentration response curve to Phe (0.01 nM–300 μM) was performed.

To evaluate the role of the endothelium in the vasoconstrictor responses to Phe, this vascular component was mechanically removed, and its absence was confirmed by the inability of ACh to induce relaxation greater than 10% of the previous contraction due to Phe. To evaluate the participation of nitric oxide (NO), potassium channels, ROS, prostanoids or AT1 receptors on Phe responses, the effects of the

Table 1
Effects of EWH on maximum response (R_{max}) and sensitivity (pD_2) in rats exposure to $AlCl_3$ for 60 days (8.3 mg/kg b.w. *per day* – Group 1), co-treated or not with EWH.

	Untreated		$AlCl_3$		Hydrolysate	Hydrolysate-Aluminum
	R_{max}	pD_2	R_{max}	pD_2	R_{max} pD_2	R_{max} pD_2
Aorta						
Control	85.6 ± 4.3	6.2 ± 0.1	112.1 ± 4.1$^{\#}$	6.9 ± 0.1	85.7 ± 6.5 6.8 ± 0.1	88.2 ± 7.4$^{\&}$ 6.2 ± 0.1
E−	111.6 ± 5.2*	6.1 ± 0.1*	108.1 ± 2.3	7.0 ± 0.1*	117.5 ± 5.1* 7.0 ± 0.1*	118.5 ± 4.1 7.0 ± 0.2*
L-NAME	128.5 ± 5.2*	6.8 ± 0.1*	125 ± 5.2	7.3 ± 0.1*	128.3 ± 3.6* 6.9 ± 0.2*	128.2 ± 2.5* 7.0 ± 0.1*
TEA	125.9 ± 4.1*	7.0 ± 0.0*	122.5 ± 5.2	7.2 ± 0.1*	117.1 ± 3.4* 7.0 ± 0.3*	119.1 ± 5.1 7.0 ± 0.2*
Apocynin	43.5 ± 5.1*	6.5 ± 0.1	45.6 ± 4.1*	6.1 ± 0.1	51.3 ± 2.3* 6.1 ± 0.1	49.3 ± 3.0* 5.9 ± 0.1*
SOD	88.2 ± 10.2	6.1 ± 0.1	88.9 ± 5.1*	5.9 ± 0.1*	75.1 ± 3.3 6.2 ± 0.1	80.6 ± 7.8 5.8 ± 0.1*
Indomethacin	54.1 ± 3.7*	6.3 ± 0.1	63.9 ± 4.2*	6.2 ± 0.1	55.9 ± 2.3* 6.1 ± 0.1	54.1 ± 2.4* 6.1 ± 0.18
NS 398	52.3 ± 5.7*	6.2 ± 0.1	54.1 ± 3.2*	6.0 ± 0.1	52.2 ± 3.3* 6.0 ± 0.1	51.8 ± 6.5* 6.2 ± 0.1*
MRA						
Control	110 ± 1.2	4.1 ± 0.1	124 ± 3.2$^{\#}$	5.0 ± 0.1$^{\#}$	110.1 ± 2.1 3.9 ± 0.2	112 ± 2.0$^{\&}$ 4.5 ± 0.3$^{\&}$
E−	122 ± 2*	5.3 ± 0.4	116.1 ± 2.9	4.0 ± 0.2	124.3 ± 2.5* 5.2 ± 0.4*	122.4 ± 1.8 5.3 ± 0.3
L-NAME	132.4 ± 2.7*	5.1 ± 0.1*	125.8 ± 3.1	4.9 ± 0.1	136.1 ± 4.2* 5.1 ± 0.6	132.4 ± 2.6 5.1 ± 0.1
Apocynin	115.2 ± 3.1	4.1 ± 0.1	113.5 ± 1.4*	4.2 ± 0.1*	118.1 ± 3.2 4.8 ± 0.2*	115.4 ± 2.2 4.1 ± 0.2
SOD	128.1 ± 1.5*	5.1 ± 0.2*	123 ± 3.6	4.7 ± 0.1	128 ± 4.8 4.9 ± 0.1*	129.4 ± 2.6 4.9 ± 0.1
Indomethacin	101.3 ± 2.1*	3.0 ± 0.2*	104.9 ± 2.1*	2.6 ± 0.4*	101.3 ± 4.6 3.1 ± 0.5	104.8 ± 4.2 3.9 ± 0.2
NS 398	124.2 ± 1.6*	4.6 ± 0.1	119.4 ± 2.3*	4.1 ± 0.1	125.7 ± 0.1* 4.6 ± 0.3	127.8 ± 2.3 4.2 ± 0.1

Parameters of maximal response (R_{max}) and sensitivity (pD_2) of the concentration-response curves to phenylephrine in both aorta and MRA before (control) and after endothelial damage (E−), L-NAME (100 μM), TEA (2 mM), apocynin (0.3 μM), SOD (150 U/ml), Indomethacin (1 μM) and NS 398 (1 μM) incubations. Results are expressed as mean ± SEM. R_{max}: maximal effect (expressed as a percentage of maximal response induced by KCl) and pD_2 expressed as a -log one-half R_{max}.*P < 0.05 compared to the corresponding control in each group, #P < 0.05 compared with the Untreated group, & P < 0.05 compared with the $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni).

Table 2
Effects of EWH on maximum response (R_{max}) and sensitivity (pD_2) in rats exposure to $AlCl_3$ for 42 days (100 mg/kg b.w. *per day* – Group 2), co-treated or not with EWH.

	Untreated		$AlCl_3$		Hydrolysate	Hydrolysate-Aluminum
	R_{max}	pD_2	R_{max}	pD_2	R_{max} pD_2	R_{max} pD_2
Aorta						
Control	88.5 ± 3.3	6.1 ± 0.1	112.1 ± 3.1$^{\#}$	6.6 ± 0.1	87.7 ± 4.2 6.2 ± 0.1	88.2 ± 2.2$^{\&}$ 6.3 ± 0.1
E−	111.5 ± 4.2*	6.5 ± 0.1*	108.8 ± 2.1	7.0 ± 0.1*	118.6 ± 3.5* 7.0 ± 0.1*	111.5 ± 4.1 6.5 ± 0.1*
L-NAME	129.9 ± 3.1*	6.8 ± 0.1*	125.8 ± 3.4	7.1 ± 0.1*	128.8 ± 2.5* 7.0 ± 0.2*	129.1 ± 1.5* 6.9 ± 0.1*
TEA	125.8 ± 3.2*	7.1 ± 0.1*	123.2 ± 3.1	7.0 ± 0.1*	119.9 ± 2.3* 7.1 ± 0.3*	122.1 ± 4.1 7.1 ± 0.2*
Apocynin	49.9 ± 3.4*	6.7 ± 0.1	45.1 ± 2.2*	6.2 ± 0.1	51.2 ± 2.5* 6.5 ± 0.1	48.3 ± 3.0* 6.9 ± 0.1*
SOD	82.1 ± 3.2	6.1 ± 0.1	88.6 ± 3.1*	6.0 ± 0.1*	78.9 ± 3.3 6.2 ± 0.1	81.9 ± 3.8 6.1 ± 0.1*
Indomethacin	54.1 ± 3.1*	6.3 ± 0.1	61.9 ± 4.6*	6.4 ± 0.1	57.9 ± 4.3* 6.0 ± 0.1	54.2 ± 3.4* 6.3 ± 0.1
NS 398	53.1 ± 3.9*	6.0 ± 0.1	56.1 ± 3.6*	6.4 ± 0.1	59.2 ± 4.4* 6.1 ± 0.1	52.8 ± 3.5* 6.1 ± 0.1*
MRA						
Control	110 ± 1.4	4.5 ± 0.1	123.5 ± 2.4$^{\#}$	5.0 ± 0.1$^{\#}$	110.1 ± 2.1 3.9 ± 0.2	110 ± 2.4$^{\&}$ 4.7 ± 0.1
E−	122 ± 2.1*	5.0 ± 0.2	118.1 ± 2.7	4.3 ± 0.1	124.2 ± 3.5* 5.2 ± 0.1*	122.4 ± 1.9 4.8 ± 0.2
L-NAME	129.4 ± 2.9*	5.2 ± 0.3*	127.9 ± 4.1	5.0 ± 0.3	132.9 ± 2.2* 5.1 ± 0.4	128.4 ± 2.1 5.1 ± 0.2
Apocynin	115.9 ± 4.9	4.0 ± 0.2	113.3 ± 2.2*	4.4 ± 0.1*	117.2 ± 2.2 4.4 ± 0.1*	115.4 ± 2.2 4.3 ± 0.1
SOD	128.1 ± 1.5*	5.3 ± 0.1*	125.1 ± 3.6	4.8 ± 0.3	127.2 ± 2.4 5.2 ± 0.1*	127.5 ± 2.4 4.9 ± 0.1
Indomethacin	103.9 ± 3.1*	3.4 ± 0.1*	104.3 ± 2.9*	3.0 ± 0.2*	105.1 ± 1.5 3.9 ± 0.3	104.7 ± 3.2 3.8 ± 0.3
NS 398	124.2 ± 3.9*	4.5 ± 0.1	117.9 ± 3.2*	4.6 ± 0.2	126.3 ± 0.2* 4.6 ± 0.1	123.8 ± 1.4 5.0 ± 0.2

Parameters of maximal response (R_{max}) and sensitivity (pD_2) of the concentration-response curves to phenylephrine in both aorta and MRA before (control) and after endothelial damage (E−), L-NAME (100 μM), TEA (2 mM), apocynin (0.3 μM), SOD (150 U/ml), Indomethacin (1 μM) and NS 398 (1 μM) incubations. Results are expressed as mean ± SEM. R_{max}: maximal effect (expressed as a percentage of maximal response induced by KCl) and pD_2 expressed as a -log one-half R_{max}.*P < 0.05 compared to the corresponding control in each group, #P < 0.05 compared with the Untreated group, & P < 0.05 compared with the $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni).

nonspecific NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME 100 μM), the potassium channels blocker tetraethylammonium (TEA, 2 mM), the NADPH oxidase nonselective inhibitor apocynin (0.3 μM), superoxide dismutase (SOD 150 U/ml), the nonselective COX inhibitor indomethacin (1 μM), and, the selective COX-2 inhibitor NS 398 (1 μM) were investigated by their addition 30 min before Phe in intact vessels.

To evaluate the endothelial dependent and independent relaxations, concentration-response curves with ACh (0.01 nM–300 μM) and sodium nitroprusside (SNP, 0.01 nM–300 μM), respectively, were performed in segments previously contracted with Phe.

2.5. Reactive oxygen species levels

Levels of reactive oxygen species in the aorta and MRA were determined by the spectrofluorometric method described by Loetchutinat et al. (2005), with some modifications (Martinez et al., 2017). The ROS levels were expressed as fluorescence units.

2.6. Lipid peroxidation

Lipid peroxidation was measured in vessels as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. (1979), with modifications (Martinez et al., 2017). The results

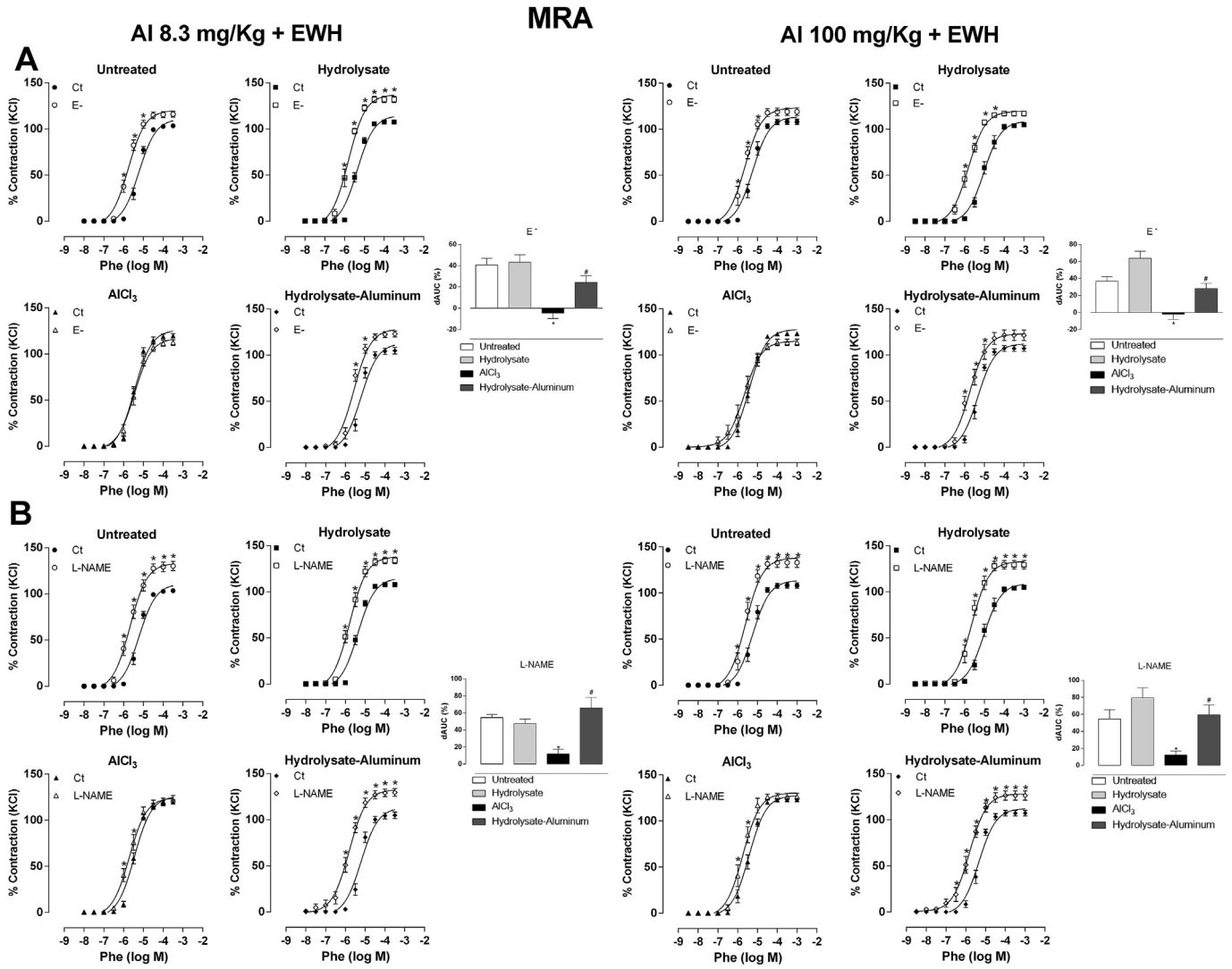

Fig. 3. Effects of EWH on NO-mediated vascular response in MRA. Effects of (A) endothelium removal (E−) and (B) L-NAME (100 μM) on the concentration-response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graph.

were expressed as nanomoles of MDA per mg of protein.

2.7. Ferric reducing/antioxidant power (FRAP) assay

The total antioxidant capacity in the aorta and MRA tissues were measured by FRAP assay (Benzie & Strain 1996), with modifications (Martinez et al., 2017). Results are presented with particular reference to Trolox equivalents.

2.8. In situ detection of vascular $O_2^{\bullet-}$ production

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate *in situ* superoxide radical anion ($O_2^{\bullet-}$) production in both aortic and mesenteric segments, as previously described (Briones et al., 2009). For quantification, five rings per animal were sampled for each experimental condition and, fluorescence was detected with a 568 nm long-pass filter (Zeiss Axioskop 2 microscope - Zeiss, Jena, Germany-Leica TCS SP2 equipped with a krypton/argon laser, × 20 objective, zoom 4x). The mean fluorescence densities in the target region were calculated using NIH Image J software version 1.46r (http://rsbweb.nih.gov/ij/), using the same imaging settings in each case.

2.9. Lucigenin chemiluminescence assay

A lucigenin-enhanced chemiluminescence assay was used to determine ROS NAD(P)H (NOX)-dependent production. Aortas were homogenized in lysis buffer (50 mM KH_2PO_4, 1 mM EGTA, and 150 mM sucrose, pH 7.4). The reaction was started by the addition of NADPH (0.1 mM) to the suspension containing the sample, lucigenin (5 μM), and assay phosphate buffer. Luminescence was measured in a plate luminometer (Auto-Lumat LB 953, Berthold Technologies, Bad Wildbad, Germany). The buffer blank was subtracted from each reading. Activity was expressed as relative light units per microgram of protein.

2.10. Quantitative real time PCR assay

The mRNA expression levels were determined by quantitative real-time PCR. Total RNA was obtained using TRIzol (Invitrogen Life Technologies). 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) in a 10-μl reaction. qRT-PCR was performed in duplicate for each sample using 0.5 μl cDNA as template, 1X iTaq™ Universal Probes Supermix (Biorad), and 20X Taqman Gene

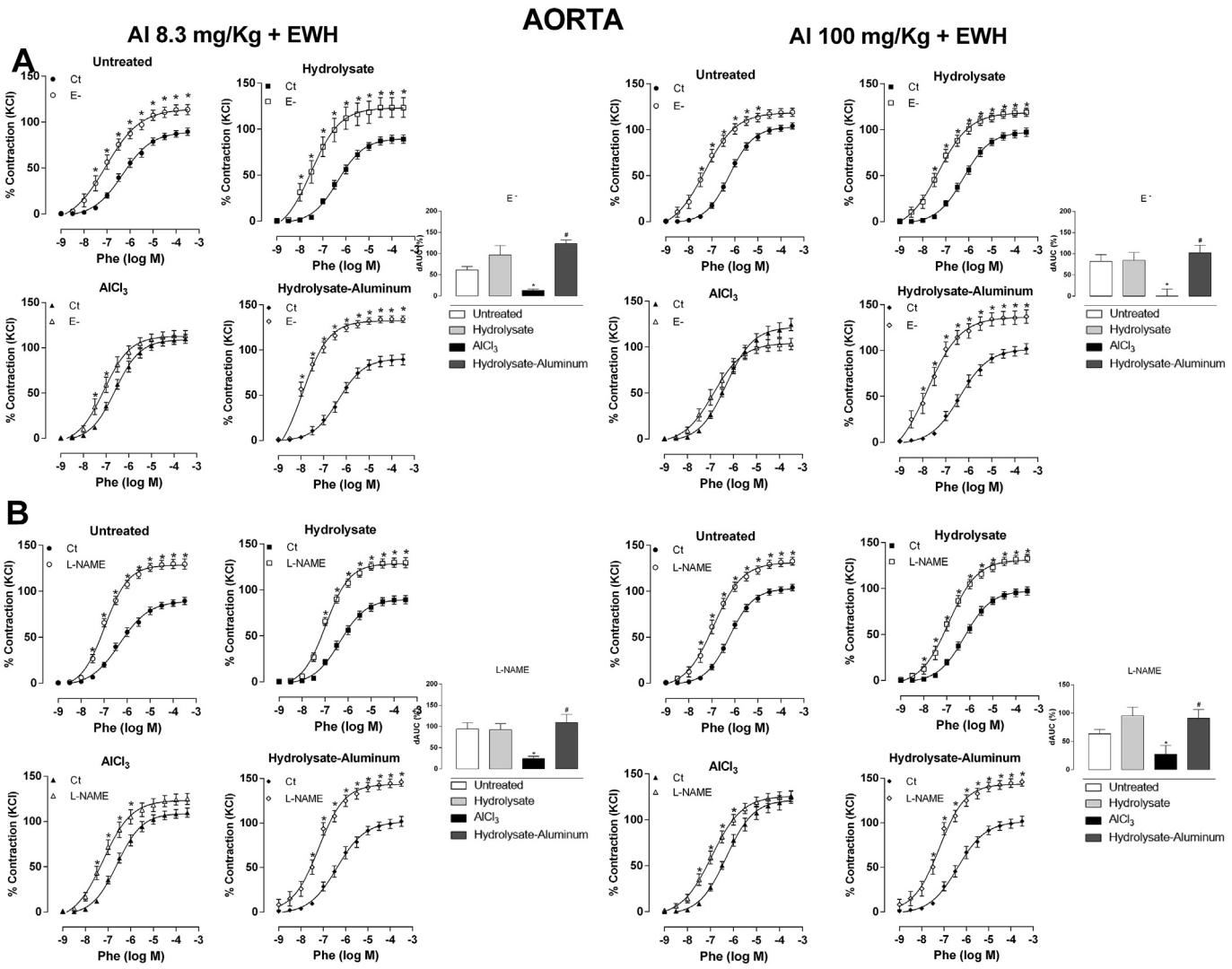

Fig. 4. Effects of EWH on NO-mediated vascular response in aorta. Effects of (A) endothelium removal (E−) and (B) L-NAME (100 μM) on the concentration-response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

Expression Assays (COX-2: Rn00568225_m1, NOX-1: Rn00586652_m1, SOD-1: Rn00566938_m1, TXA-2 synthase: Rn00562160_m1, Applied Biosystems) in a 10-μl reaction using the following conditions: 2 min at 50 °C and 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. qRT-PCR for eNOS (Fd: GAGAGTGAGCTGGTGTTTGG; Rv: GGT GAACATTTCCTGTGCTGT), NOX-2 (Fd: CCAGTGTGTCGGAATCTCCT; Rv: ATGTGCAATGGTGTGAATGG) and PGI-2 synthase (Fd: AGGATGC GCTGAAACGTGGAG; Rv: CCGAGGAAGAGGAAAGGATAG) were performed using the fluorescent dye SyBRGreen (iTaq FAST SyBRGreen Supermix with ROX, Bio-Rad, USA). For quantification, quantitative real-time PCR was carried out in a 7500 Fast (Applied Biosystems) adding a dissociation stage to the conditions already described above to show PCR product specificity. As a normalizing internal control, we amplified cyclophilin D (Rn01458749_g1). To calculate the relative index of gene expression, we employed the $2^{-\Delta\Delta Ct}$ method using untreated samples as a calibrator (Livak and Schmittgen, 2001). mRNA levels of the housekeeping gene were not modified by any of the treatments used.

2.11. Statistical analysis

Data are expressed as mean ± SEM. In the vascular reactivity experiments, vasoconstrictor responses in aorta and MRA were expressed as a percentage of the contraction induced by 75 mM and 120 mM KCl, respectively. Vasodilator responses were expressed as a percentage of the previous contraction to Phe. To compare the effect of L-NAME, TEA, apocynin, SOD, indomethacin and NS-398 -on the response to Phe in segments from each group, some 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; differences were expressed as the percentage of the AUC of the corresponding control situation. Results were analyzed using unpaired two-way ANOVA; when ANOVA showed a significant treatment effect, Bonferroni's post hoc test was used to compare individual means. Values of P < 0.05 were considered significant.

3. Results

3.1. Body weight, fluid and feed intakes

As we have previously observed (Martinez et al., 2019), body weight of rats, fluid (water or Al) and, feed intakes were not different between groups (data not shown).

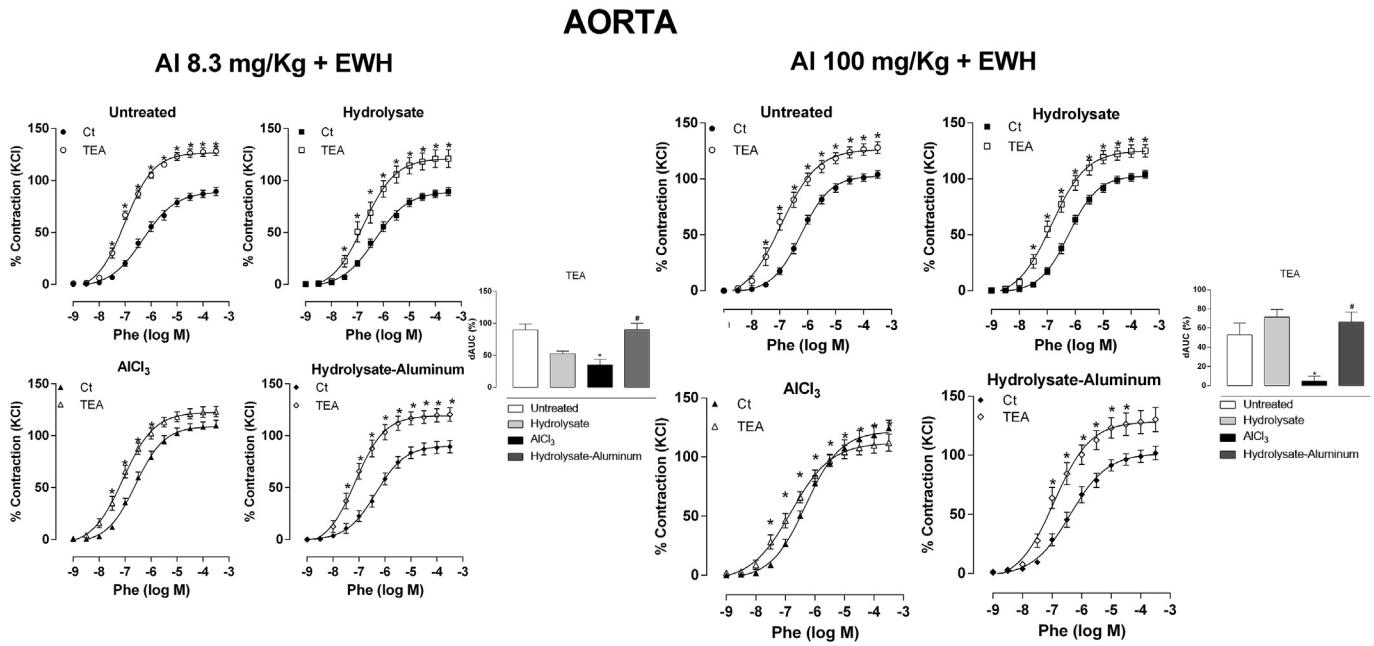

Fig. 5. Effects of EWH on K+ channels action on the vascular response in aorta. Effects of the K+ channels blocker TEA (2 mM) on the concentration-response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

3.2. Systolic blood pressure

Exposure to Al at both low and high level increased SBP after the 3rd week of treatment (Group 1 - Untreated: 119.2 ± 1.4 *vs* $AlCl_3$: 129.3 ± 1.1 mmHg; Group 2 - Untreated: 119.6 ± 1.4 *vs* $AlCl_3$: 131.3 ± 3.1 mmHg, n = 8, P < 0.05). The EWH treatment *per se* did not change blood pressure, however, prevented the increased blood pressure in rats exposed to both Al and EWH (Group 1 - Hydrolysate-Aluminum: 116.3 ± 1.3 mmHg, Hydrolysate: 116.1 ± 2.1 mmHg; Group 2 – Hydrolysate-Aluminum: 120.2 ± 4.2 mmHg, Hydrolysate: 116.3 ± 1.9 mmHg, n = 8) (Fig. 1A and B).

3.3. Vascular reactivity in conductance and resistance arteries

The response to KCl either in aorta or MRA were not affected by any of the treatments (Group 1 – Aorta: 4.0 ± 0.2, MRA: 3.8 ± 0.4; Group 2 – Aorta: 4.0 ± 0.4, MRA: 4.0 ± 0.3 mN/mm, n = 15 to 20, P > 0.05). However, Al treatment at both low (Group 1) or high levels (Group 2) increased the vasoconstrictor responses to Phe in aorta and MRA while the endothelium-dependent responses induced by ACh was decreased only in MRA (Fig. 2A and B, Table 1 and 2). EWH treatment *per se* did not alter Phe and ACh responses (Fig. 2A and B, Table 1 and 2). Nevertheless, rats exposed to Al and co-treated with EWH showed Phe and ACh responses which were similar to those found in Untreated and EWH groups (Fig. 2A and B, Table 1 and 2). The vasodilator response induced by SNP were unaffected by any treatments (data not shown). These results suggest that EWH seems to prevent the increased vasoconstrictor responses in both conductance and resistance arteries as well as, the impaired endothelial function in resistance arteries after Al exposure.

3.4. Endothelial modulation of vasoconstrictor response

To investigate the role of EWH on endothelial modulation on vasoconstrictor responses after Al exposure, the effect of endothelium removal and incubation with the NOS inhibitor L-NAME (100 μM), were investigated. Both endothelium removal and NOS inhibitor addition left-ward shifted the concentration–response curves to Phe in aorta and MRA segments from all groups, this effect being smaller in preparations from rats treated with either 8.3 mg/kg (Group 1) or 100 mg/kg of Al(Group 2), as shown by the dAUC values (Figs. 3 and 4). The impairment on endothelial modulation seems to be prevented by EWH, since the NO participation on vasoconstrictor responses of rats exposed to Al and co-treated with EWH were similar to Untreated and EWH groups (Figs. 3 and 4). To go further in the understanding of the effects of EWH we have investigated the NO expression after Al exposure at human relevant dose level (8.3 mg/kg); EWH cotreatment did not prevent the decrease on eNOS gene expression in aorta (Untreated: 0.84 ± 0.1; Al: 0.61 ± 0.1; EWH: 0.75 ± 0.1; Al + EWH: 0.66 ± 0.1 relative expression, P < 0.05 Al *vs* Untreated). In order to verify the participation of K^+ channels on the vasoconstrictor responses, aortic segments were incubated with TEA, a K^+ channel blocker. TEA (2 mM) increased the Phe-induced contractile response in all groups, being this enhancement smaller in Al-treated rats; however aortic segments of rats exposed to Al cotreated with EWH showed contractile responses similar to those found in Untreated and EWH groups, as demonstrated by the dAUC values (Fig. 5). All these findings suggest that the impaired bioavailability of NO after Al exposure could be prevented by EWH administration; however, its effects seem not to be related to the improvement of eNOS gene expression.

3.5. ROS modulation of vasoconstrictor response

We have previously observed that Al increases oxidative stress impacting vascular function (Martinez et al., 2017). The nonselective NAD(P)H oxidase inhibitor apocynin (0.3 μM) and the ROS scavenger SOD (150 U/ml) both reduced the vascular response to Phe in MRA and aorta, being this effect greater in segments from Al-treated rats, as demonstrated by the dAUC values (Figs. 6 and 7). However, rats exposed to Al and co-treated with EWH showed Phe-induced responses similar to Untreated and EWH groups, as shown by the dAUC values (Figs. 6 and 7). Al exposure at both low (Group 1) and high (Group 2) doses increased vascular ROS and lipid peroxidation levels and, Al only at the lowest dose reduced the total antioxidant capacity in aorta; these alterations were prevented by EWH concomitant administration (Tables 3 and 4). Taking all together, these results suggest that the reduced

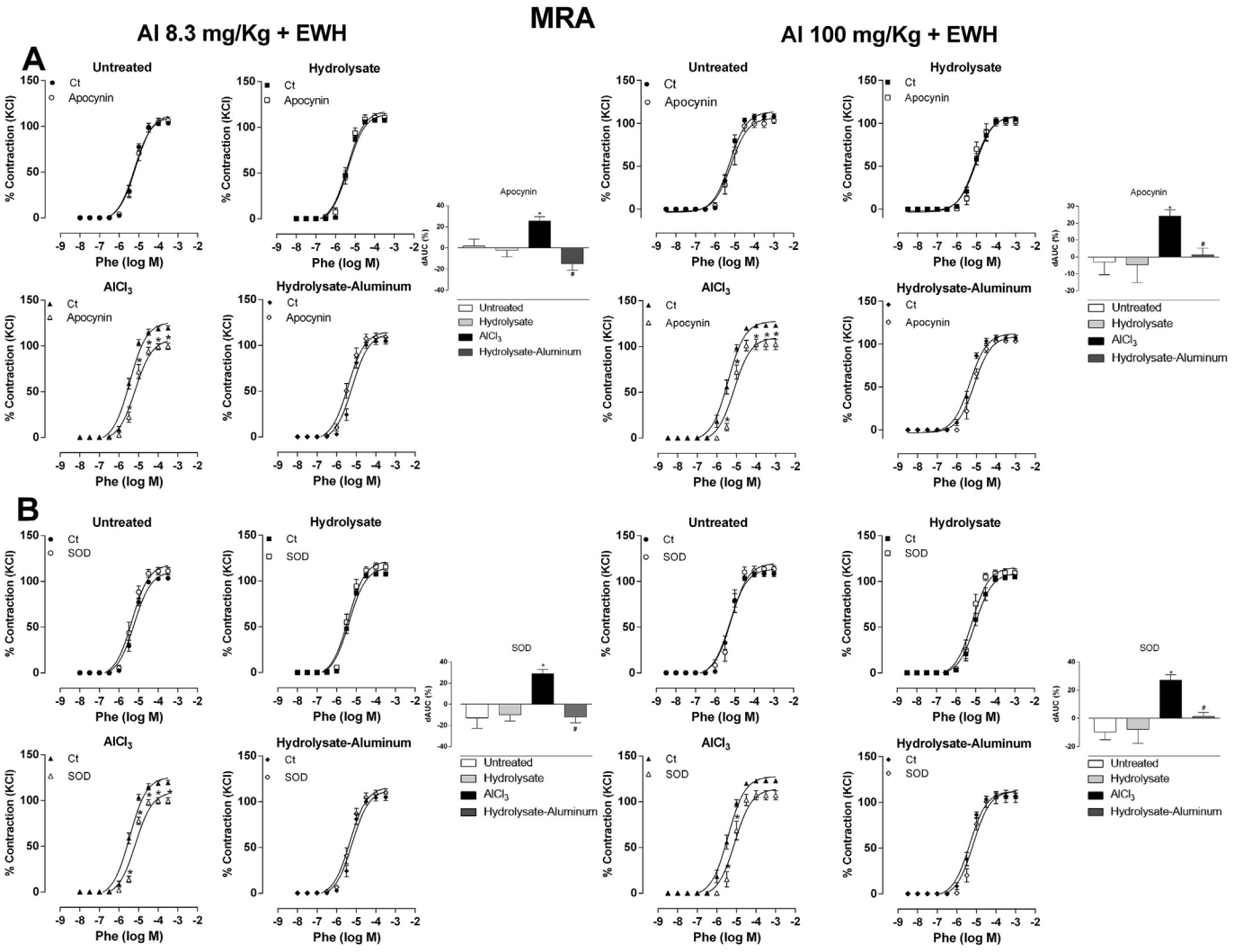

Fig. 6. Effects of EWH on ROS-mediated vascular response in MRA. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μM) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

bioavailability of NO could be related to the increase in oxidative stress observed after Al exposure and, the preventive effect of EWH could be related with its antioxidant action.

Therefore, to go further in the protective effect of EWH we investigated its possible role on NAD(P)H oxidase complex, important source of ROS at vascular bed (Martyn et al., 2006). Al exposure at human relevant dose (Group 1) increased the basal $O_2^{\bullet -}$ production in both conductance and resistance arteries and the EWH co-treatment prevented this enhancement (Fig. 8). Additionally, Al exposure increased the NAD(P)H oxidase activity and the NOX1 gene expression in aorta without affecting NOX2 mRNA levels (Fig. 9A,B,C); the co-treatment with EWH prevented the increase on NOX1 (Fig. 9B) but not the decrease of SOD1 gene expression induced by Al exposure (Fig. 9D).

3.6. Participation of COX-2-derived prostanoids in vasoconstrictor responses

To verify the participation of prostanoids on the increased response to Phe in Al-treated rats and the possible role of EWH in this pathway, we have used the non-selective COX inhibitor indomethacin and the selective COX-2 inhibitor NS 398. Indomethacin (1 μM) and NS 398 (1 μM) reduced the vasoconstrictor response to Phe in either aorta or MRA, being this reduction greater in segments from both Al-treated groups (Figs. 10 and 11). However, as observed by the dAUC values, this reduction was prevented by the co-treatment with EWH (Figs. 10 and 11), suggesting a preventive effect of EWH against the increased participation of contractile prostanoids from COX-2 on vessels of Al-treated rats.

Al exposure at the human relevant level (Group 1) increased the mRNA levels of COX-2 in aorta (Fig. 12A) while TXA-2 synthase and PGI_2 synthase remained unmodified; the COX-2 increase was not prevented by EWH treatment (Fig. 12A); however, EWH reduced mRNA levels of TXA-2 synthase while increased PGI_2 synthase in Al + EWH group (Fig. 12B and C). These results reinforce the participation of COX-2-derived prostanoids on the vascular impairment after Al exposure and, suggest a compensatory action of EWH modulating the delicate balance between vasodilators and vasoconstrictors in the regulation of vascular tone after Al exposure.

4. Discussion

Our study supports recent evidence of Al-induced cardiovascular risk (Costello et al., 2014; Lind et al., 2012; Neophytou et al., 2016; Subrahmanyam et al., 2016). Thus, Al exposure at human dietary levels

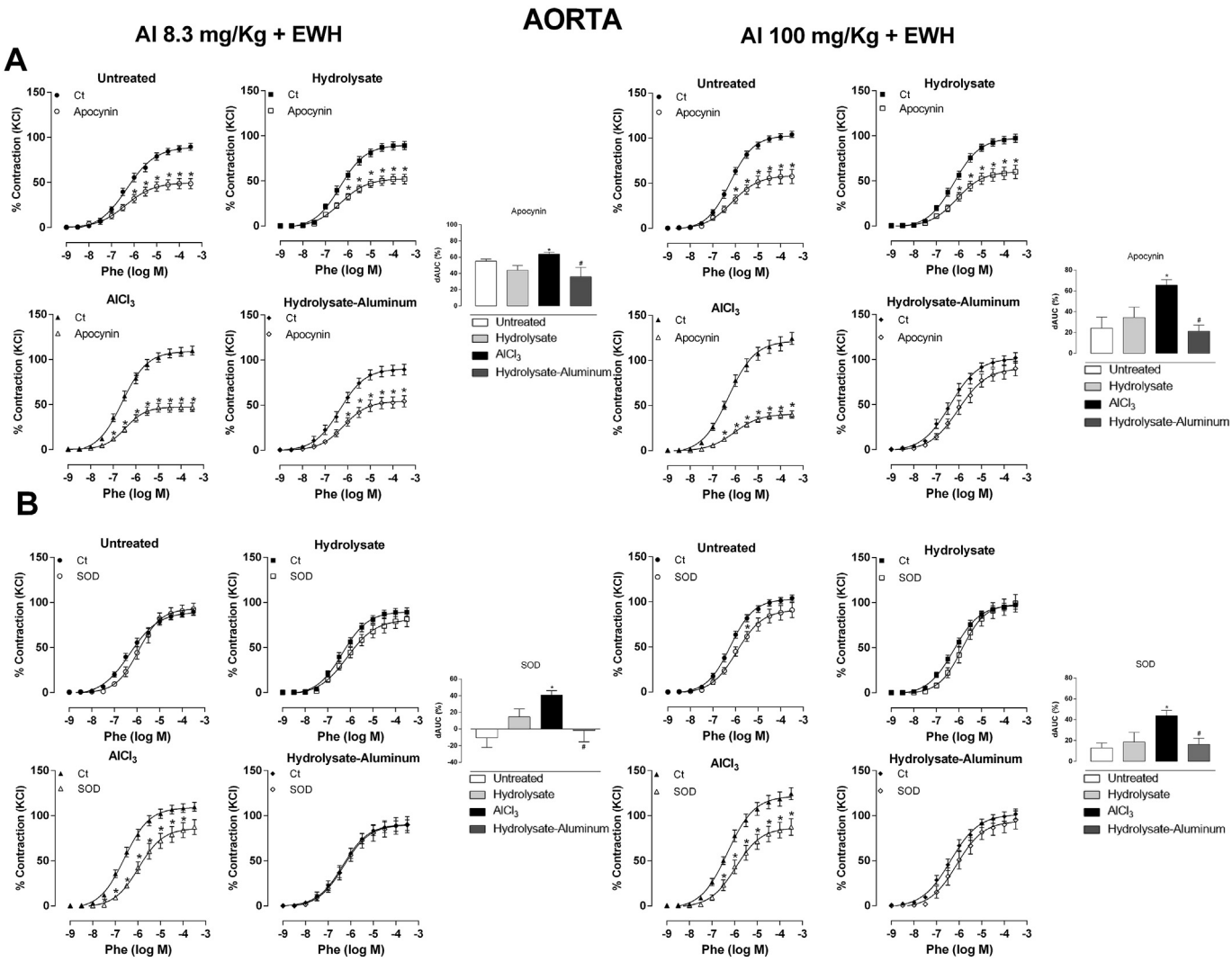

Fig. 7. Effects of EWH on ROS-mediated vascular response in aorta. Effects of (A) NOX oxidase inhibitor apocynin (0.3 μM) and (B) SOD (150 U/ml) on the concentration-response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

Table 3
Effects of EWH on biochemical parameters of oxidative stress in rats exposure to $AlCl_3$ for 60 days (8.3 mg/kg b.w. *per day* – Group 1), co-treated or not with EWH.

	Untreated		$AlCl_3$		Hydrolysate	Hydrolysate-Aluminum
	Aorta	MRA	Aorta	MRA	Aorta MRA	Aorta MRA
ROS	93.4 ± 7.9	111.2 ± 8.5	133.3 ± 12.2*	186.8 ± 21.8*	74.5 ± 4.9 50.2 ± 4.7	71.9 ± 8.1# 55.8 ± 5.8#
Lipid peroxidation	38.4 ± 2.3	7.1 ± 0.8	60.9 ± 5.5*	21.7 ± 3.0*	37.4 ± 3.5 10.4 ± 2.3	37.2 ± 3.1# 11.1 ± 1.7#
FRAP	64.1 ± 9.1	68.1 ± 14.8	26.7 ± 4.1*	59.2 ± 10.4	54.5 ± 5.4 61.7 ± 8.4	54.6 ± 5.4# 60.3 ± 7.9

Results are expressed as mean ± SEM, n = 8. *P < 0.05 compared to the Untreated group, #P < 0.05 compared with the $AlCl_3$ group. Two-Way ANOVA followed by Bonferroni. ROS, UF; Lipid peroxidation, nM MDA/mg protein; FRAP, mM.

Table 4
Effects of EWH on biochemical parameters of oxidative stress in rats exposure to $AlCl_3$ for 42 days (100 mg/kg b.w. *per day* – Group 2), co-treated or not with EWH.

	Untreated		$AlCl_3$		Hydrolysate	Hydrolysate-Aluminum
	Aorta	MRA	Aorta	MRA	Aorta MRA	Aorta MRA
ROS	269.4 ± 24.9	163.5 ± 12.9	404.1 ± 34.9*	244.9 ± 14.9*	190.1 ± 38.4 169.3 ± 19.4	171.3 ± 19.4# 214.4 ± 24.5
Lipid peroxidation	34.5 ± 2.9	11.4 ± 2.0	61.4 ± 8.9*	33.6 ± 1.7*	36.3 ± 4.1 18.8 ± 3.2	28.6 ± 2.1# 21.1 ± 5.6
FRAP	89.7 ± 2.9	78.7 ± 3.4	79.8 ± 4.3	90.3 ± 5.7	97.1 ± 8.1 84.2 ± 3.3	92.1 ± 5.6 75.7 ± 3.3

Results are expressed as mean ± SEM, n = 8. *P < 0.05 compared to the Untreated group, #P < 0.05 compared with the $AlCl_3$ group. Two-Way ANOVA followed by Bonferroni. ROS, UF; Lipid peroxidation, nM MDA/mg protein; FRAP, mM.

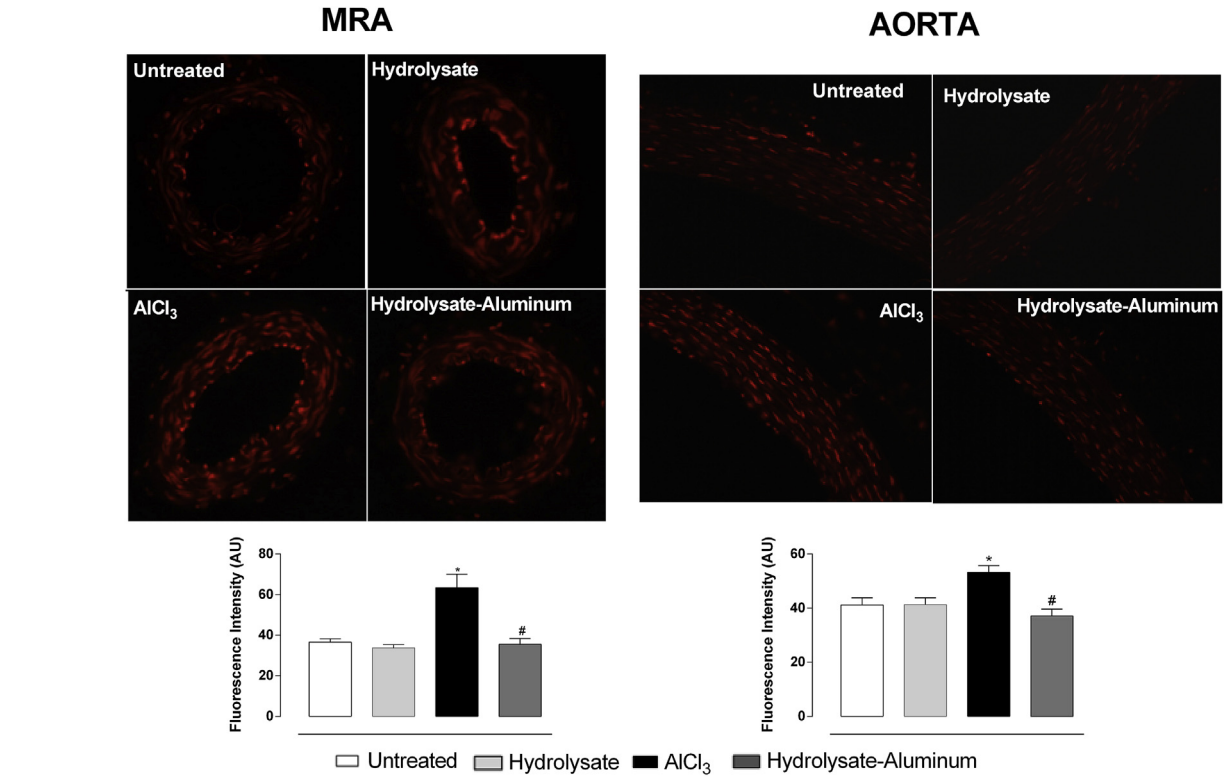

Fig. 8. Effects of EWH on vascular O_2^- production. Representative fluorescent photomicrographs of arterial sections labeled with the oxidative dye hydroethidine and vascular superoxide anion quantification in MRA and aorta. Data are expressed as mean ± SEM (n = 8). *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni). Au: Fluorescence intensity.

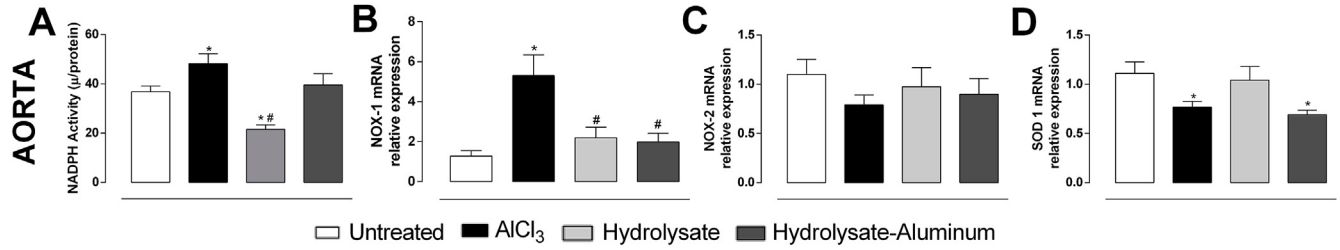

Fig. 9. Effects of EWH on NOX oxidase activity (A) and mRNA levels for NOX-1 (B), NOX-2 (C) and SOD 1 (D) in aortas. Data are expressed as mean ± SEM (n = 8). *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni).

produces an imbalance between vasoactive factors such as NO and COX-2 derived prostanoids and causes overproduction of ROS, thus leading to endothelial dysfunction and blood pressure increase. Here we have tested the effect of bioactive peptides derived from EWH against vascular dysfunction induced by Al. Dietary supplementation with EWH protects aorta and mesenteric arteries by restoring endothelial function after both low and high dietary Al exposures, probably by suppressing oxidative stress and decrease NOX1 gene expression as well as by changing the relative gene expressions of TXA-2 and PGI_2 synthases. Therefore, it is likely that the restored vascular homeostasis and NO availability is linked with the reduction of oxidative stress and normalization of vasoactive prostanoids, both being key effects of EWH treatment.

Al is everywhere; human exposure to Al can be reduced but not avoided. Humans are exposed to Al in myriad ways including diet, Al salts present in pharmaceuticals and cosmetics and, Al salts are used in about 60% of human and veterinary vaccines as adjuvants to potentiate the production of antibodies (Spickler and Roth, 2003). The effects of Al in the human body depend upon the achievement of a toxic threshold within specific compartments and these thresholds vary according to an individual's susceptibility and state of health (Gherardi et al., 2016). Recently, we have demonstrated that exposure of rats to Al at levels similar to that found in human diet resulted systemic toxic effects, suggesting that the average level of Al present in the human diet is sufficient to achieve this toxic threshold or burden (Martinez et al., 2017). By exposing rats to Al at a dose that "mimics" human exposure to this metal we identified the presence of cardiovascular risk markers such as vascular dysfunction and increased blood pressure and, surprisingly these effects were almost the same when we treated rats with a super dietary level of Al (100 mg/kg b.w.) (Martinez et al., 2017a). In the present study, the co-administration of EWH was able to reduce the vascular dysfunction in rats following long-term exposure to Al, highlighting the putative effect of a functional food ingredient which could be easily introduced in the human diet.

Nowadays, there is a search for "natural" and effective therapies to minimize the effects of environmental contaminants as well as to prevent diseases and epidemics of the 21st century. In this sense, food-derived compounds seem to be an effective remedy (Deng et al., 2012; García-Nino and Pedraza-Chaverri, 2014). EWH is produced after 8 h of hydrolysis digestion with pepsin releasing bioactive peptides with *in vitro* peroxyl radical-scavenging activity (574 µmol Trolox/g protein). The presence of amino acid sequences with aminoacids such as Pro, Lys

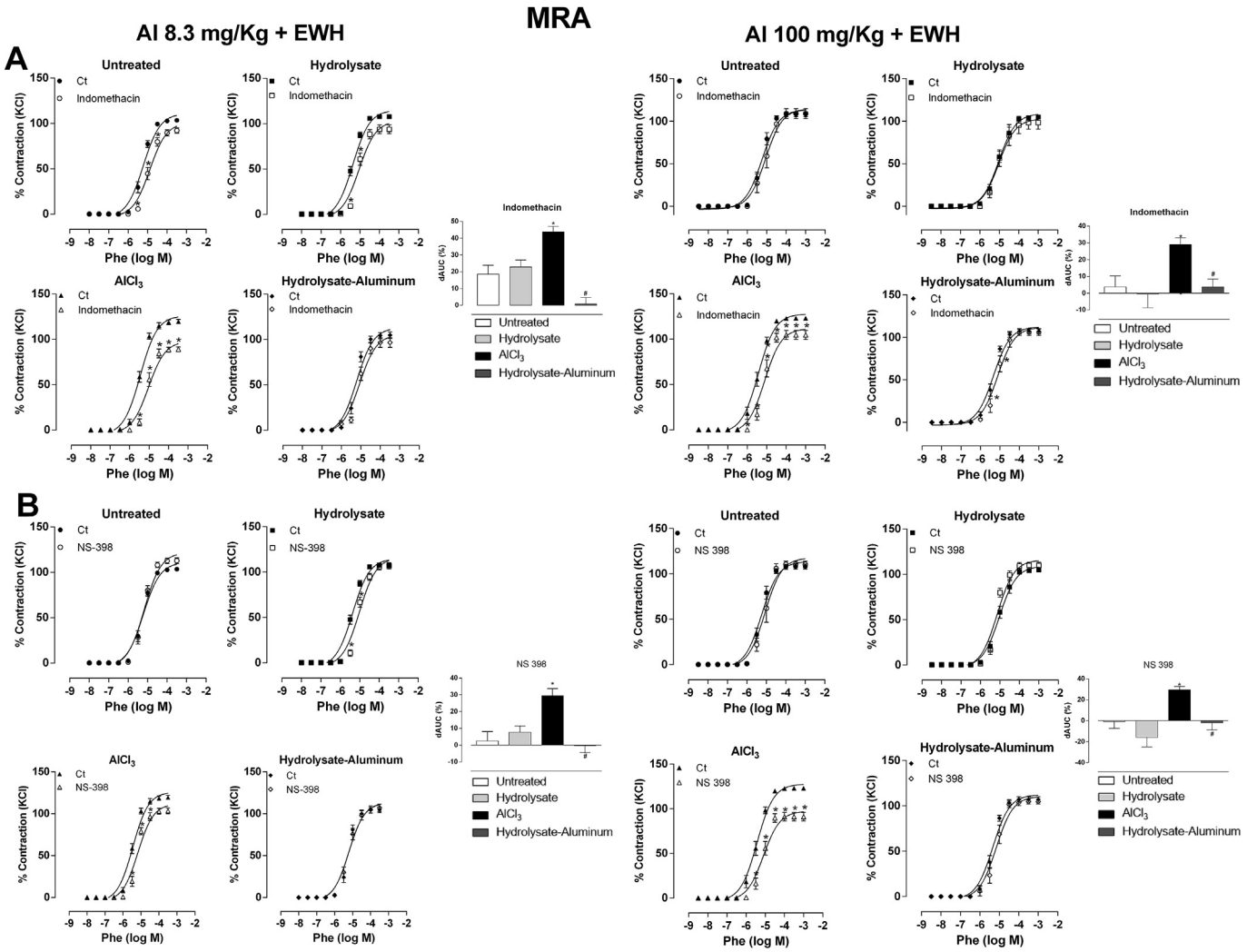

Fig. 10. Effects of EWH on COX-derived prostanoids role on vascular response in MRA. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μM) and (B) selective COX-2 inhibitor NS 398 (1 μM) on the concentration– response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

or Arg as a C-terminal residue confers ACE inhibitory activity, the presence of Arg or Tyr at the N-terminal position shows vascular-relaxing activity and, finally the presence of Tyr and Phe in C-terminal residue is related to scavenging free radicals and antioxidant properties (Miguel et al., 2004), therefore, considering these bioactive properties it is possible to postulate its role as a cardioprotective agent. Indeed, short and long-term administration of EWH showed antihypertensive effects in Spontaneous Hypertensive rats (SHR) (Miguel et al. 2005, 2006). EWH administration also decreased body and epididymal adipose tissue weights, reduced hepatic steatosis in Zucker rats (Garcés-Rimón et al., 2016) and improved glucose metabolism abnormalities in diet induced obesity experimental model (Garcés-Rimón et al., 2018). Regarding environmental contaminants, our group has reported the ability of EWH to prevent cardiovascular, reproductive and neurological complications related to mercury exposure (Rizzetti et al., 2017; Rizzetti et al., 2017a; Rizzetti et al. 2016); additionally, we have recently described that EHW improves the cognitive impairment observed after long-term Al exposure in rats (Martinez et al., 2019); all these effects have been attributed to its antioxidant and anti-inflammatory capacities. In the present study, rats treated with Al and co-treated with EWH did not develop vascular dysfunction or increased blood pressure, suggesting the efficacy of EWH to counteract the adverse effects after Al exposure at low and high levels.

Al has an important history as a neurotoxin (Exley 2013, 2017; Mirza et al., 2017) and, its toxicity is strongly related to oxidative stress in different organs (Novaes et al., 2018; Prakash and Kumar 2009). However, at cardiovascular system, only a few studies have devoted attention to the adverse effects of Al exposure. Isolated rat hearts exposed to Al at 100 μM showed a reduction of both coronary blood flow and isovolumetric systolic pressure (Gomes et al., 1994) while, 40 μM Al increased coronary flow and decreased heart rates (Korchazhkina et al., 1998). *In vivo*, Al exposure at 0.1, 50, and 200 mg/kg for 120 days promoted important pathological cardiac remodeling and degeneration (Novaes et al., 2018) as well as rats exposed to Al up to 256 mg/kg developed hypertension probably due to an increase in renin expression or erythrocyte membrane impairments (Ezomo et al., 2009; Zhang et al., 2016). The possible relationship between circulating Al levels and hypertension was also reported in humans (Granadillo et al., 1995; Jung et al., 2016).

At the vascular bed, our group demonstrated that Al exposure for just 1 h is sufficient to compromise vasoconstrictor responses and to increase vascular ROS production (Schmidt et al., 2016). The cardiovascular risk of Al was further supported by our group by developing an experimental model of exposure to Al at an equivalent human dietary level. Thus, Al exposure for 60 or 42 days increased blood pressure and vascular reactivity, ROS and COX-2-derived prostanoids being

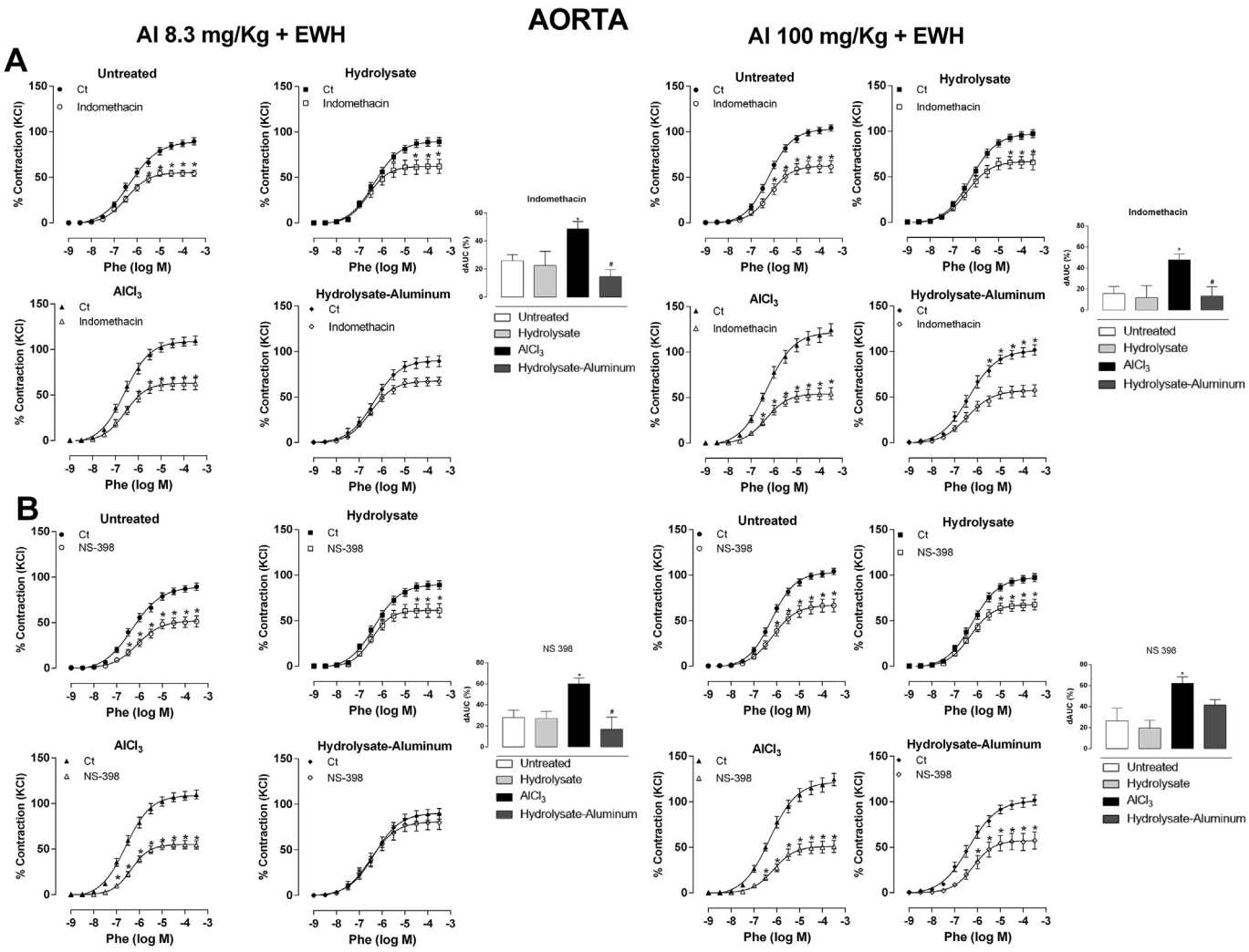

Fig. 11. Effects of EWH on COX-derived prostanoids role on vascular response in aorta. Effects of (A) cyclooxygenase inhibitor indomethacin (1 μM) and (B) selective COX-2 inhibitor NS 398 (1 μM) on the concentration– response curve to phenylephrine. The inset shows differences in the area under the concentration–response curves (dAUC). Data are expressed as mean ± SEM, n = 8 to 15, *P < 0.05 *vs* control curve, *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group (Two-Way ANOVA followed by Bonferroni) in dAUC graphs.

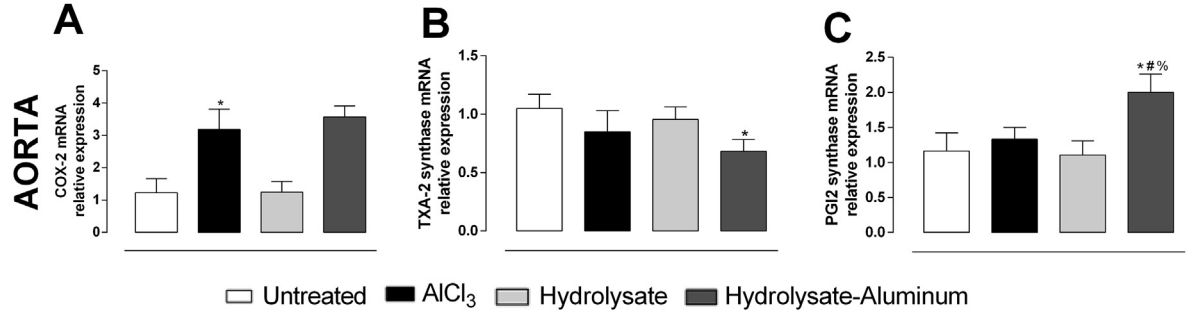

Fig. 12. Effects of EWH on COX-2 mRNA levels (A), TXA-2 synthase mRNA levels (B) and PGI2 synthase mRNA levels (C) in aortas. Data are expressed as mean ± SEM (n = 8). *P < 0.05 *vs* Untreated group, #P < 0.05 *vs* $AlCl_3$ group, & P < 0.05 *vs* Hydrolysate group (Two-Way ANOVA followed by Bonferroni).

important mediators of the vascular dysfunction after Al exposure (Martinez et al., 2017a). Results of the present study support our latest findings and suggest the potential of egg-derived peptides to minimize the adverse effects of Al on vascular function. Specifically, the co-treatment with EWH prevented the increased systolic blood pressure and endothelial dysfunction induced by Al exposure, likely by affecting the NO and prostanoid pathways as well as decrease prooxidant factors in both aorta and mesenteric arteries.

Al is known as a pro-oxidant agent due to formation of an Al superoxide radical cation (Exley, 2004; Ruipérez et al., 2012). At the vascular level, the oxidative stress can shift toward vasoconstrictor state and reduced vasodilation through several ways such as its effects on the NO pathway (Hernanz et al., 2014). In the presence of ROS, NO can rapidly react with $O_2^{\bullet-}$ promoting the formation of the oxidant $ONOO^-$, reducing the vasodilator effects of NO (Alvarez, 2008; Zou, 2007). In the present study, EWH did not prevent the reduction in eNOS gene

expression after Al exposure however decreased the NOX1 gene expression, impacting the main source of ROS at vascular level (Konior et al., 2014). Therefore, the increased vascular NO bioavailability induced by EWH could be related with a downregulation of prooxidant mechanisms.

The mammals isoforms NOX1, NOX2 and NOX4 are highly involved in the development of endothelial dysfunction observed in vascular pathologies such as hypertension and atherosclerosis (García-Redondo et al., 2016; Konior et al., 2014; Schramm et al., 2012). We have investigated the contribution of NADPH as a source of ROS after Al exposure, and, consequently as a target for EWH action. The *in vitro* exposure to apocynin and to the antioxidant SOD reduced the vasoconstrictor response to Phe more in segments from Al-treated rats, corroborating our last findings (Martinez et al., 2017a) and highlighting the involvement of the superoxide radical anion, probably from NADPH oxidase, on the increased vasoconstrictor response induced by Al. However, these effects were not observed in vessels from rats treated with Al and co-treated with EWH, therefore, it is suitable to speculate that the EWH could be acting in this pathway. The prevention of increased ROS, lipid peroxidation, basal $O_2^{\bullet-}$ production and mRNA levels of NOX-1 after Al exposure, reinforce the cardioprotective role of EWH even without direct effects on NADPH oxidase activity or by restoring the mRNA levels of cytosolic Cu/Zn SOD, decreased in Al-treated rats.

Previously, we have observed that Al induces a circuitous relationship between COX-2 products and ROS acting to induce vascular dysfunction and to increase blood pressure (Martinez et al., 2017a). Here, we have evaluated whether or not EWH could be acting on COX-2 pathway to prevent cardiovascular dysfunction induced by Al. In vessels from rats co-treated with Al and EWH, the respective unspecific and specific inhibitors of COX-2, indomethacin and NS 398, both promoted a reduction in the vasoconstrictor response to Phe, which was smaller than that observed in segments from Al-treated rats. As expected, Al induced expression of COX-2; however, EWH did not affect that expression. Nevertheless, interestingly, rats exposed to Al and co-treated with EWH showed reduced levels of TXA-$_2$ synthase while increased those of PGI$_2$ synthase; these results would suggest an effect of EWH in the synergistic actions of the two prostanoids, PGI$_2$ and TXA-$_2$. These COX-derived prostanoids show opposite effects and are critical for vascular homeostasis (Rahman, 2019). TXA-2 is involved in the critical regulation of the vascular wall by inducing platelet aggregation, smooth muscle contraction and is related to vascular changes in hypertension (Félétou et al., 2011; Nakahata, 2008), while PGI$_2$ is the most important cardioprotective prostanoid released by endothelium by mediating vasodilation and by inhibition of platelet aggregation (Rahman, 2019). In agreement, we have previously described that Al increases mRNA levels of TXA-2 R (Martinez et al., 2017a). Therefore, it is possible to postulate a compensatory role of EWH in the COX-prostanoids pathway activated in the presence of Al to maintain vascular homeostasis. Supporting our hypothesis, it seems that in the presence of NO, the blockade of PGI$_2$ production has no effect on vasodilatation (Verma et al., 2001); however, when NO is blocked, the residual dilation is due to increased PGI$_2$ synthesis (Beverelli et al., 1997), suggesting a compensatory role of PGI$_2$ when NO is reduced.

5. Conclusion

Taken as a whole, our data suggest that a functional food ingredient co-ingested with Al could be an alternative adjunctive agent in the prevention of Al-related cardiovascular complications. Al exposure at both low and high human equivalent dietary levels lead to an imbalance in vasoactive mediators with reduced NO bioavailability and overproduction of ROS, leading to endothelial dysfunction and increased blood pressure. Our data show that the co-treatment with EWH markedly prevents vascular dysfunction and the increased blood pressure induced by Al. We suggest that EWH likely prevents endothelial dysfunction by suppressing oxidative stress and affecting the NO and prostanoid pathways in both aorta and mesenteric artery. Although our results suggest a promising role for EWH to protect against Al-associated adverse effects, there remain many unresolved questions relating its mechanism of action that must be continuously addressed.

Author contributions

Conceived and designed the experiments: C.S.M., G.A.W., M.M., M.S., F.M.P. Performed the experiments: C.S.M., J.T.P., A.G.E., A.M. Analyzed the data: C.S.M., R.P., A.M., G.A.W. Wrote the manuscript: C.S.M. Contributed to interpretation of results and reviewing of the manuscript: C.S.M., G.A.W., M.M., C.E., M.S., M.J.A., F.M.P., D.V.V., R.P., A.M. All authors have read approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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