

Short-Term Administration of Alibernet Red Wine Extract Failed To Affect Blood Pressure and To Improve Endothelial Function in Young Normotensive and Spontaneously Hypertensive Rats

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Received December 7, 2012

Accepted February 12, 2013

On-line July 17, 2013

Summary

As wine polyphenols were shown to possess many positive effects in mammals, including improvement of vascular function, this study investigated the effect of the Slovak Alibernet red wine extract (AWE) on blood pressure and vascular function in young normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Six weeks old, male, WKY and SHR were treated with AWE for three weeks at the dose of 24.2 mg/kg/day. Blood pressure (BP), determined by tail-cuff plethysmography, was significantly elevated in SHR vs. WKY and AWE failed to affect it. Lipid peroxidation was evaluated by determination of thiobarbituric acid-reactive substances. Vascular function was assessed in rings of the femoral artery using Mulvany-Halpern's myograph. Maximal endothelium-dependent acetylcholine (ACh)-induced relaxation was reduced in control SHR vs. WKY rats by approximately 9.3 %, which was associated with a significant decrease of its NO-independent component. AWE failed to affect maximal ACh-induced relaxation, both its NO-dependent and independent components, compared to controls of the same genotype. AWE however reduced lipid peroxidation in the left ventricle of both WKY and SHR and in the liver of SHR. In conclusion, three-week administration of AWE failed to reduce BP and to improve endothelial function in the femoral arteries of both genotypes investigated.

Key words

Antioxidant • Blood pressure • Endothelial function • Nitric oxide
• Red wine polyphenols

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Introduction

Natural polyphenols are a wide group of substances produced in secondary metabolism of plants. Depending on the chemical structure, they are divided into several subgroups (Ramassamy 2006), such as phenolic acids, non-flavonoid polyphenols and flavonoids. The positive influence of natural polyphenols on human health was suggested about twenty years ago as a possible explanation of the French paradox (Hertog *et al.* 1995). Nowadays, there is epidemiological evidence that moderate consumption of food and drinks rich in natural polyphenols, including red wine, is beneficial to human health (Pilšáková *et al.* 2010).

Several studies reported that consumption of red wine was associated with lower incidence of cardiovascular diseases (Carollo *et al.* 2007, Costanzo *et al.* 2011). Additionally, treatment with either red wine (RW) or other dietary sources, as e.g. purple grape juice, were found to reduce the susceptibility of LDL to oxidation and to improve endothelium-dependent vasodilatation in patients with coronary heart disease (Stein *et al.* 1999). As to blood pressure, dealcoholized

red wine reduced both systolic and diastolic blood pressure (BP) in subjects at high cardiovascular risk (Chiva-Blanch *et al.* 2012). Hypertensive subjects who were moderate wine drinkers had a significantly lower risk of death from all causes than had abstainers (Renaud *et al.* 2004).

The mechanisms involved in beneficial effects of natural polyphenols include reduction of platelet aggregation (Demrow *et al.* 1995), increased HDL concentration (Bornhoeft *et al.* 2012), reduced LDL oxidation and concentration (Nigdikar *et al.* 1998, Agouni *et al.* 2009, Bornhoeft *et al.* 2012), and improvement of antioxidant defense system (Scola *et al.* 2010, Lionetto *et al.* 2011). Besides antioxidant properties, which play a significant role in cardioprotection, red wine polyphenols affect vascular function *via* modulation of nitric oxide (NO) bioavailability. Improved vascular function was shown in several experimental models of human diseases after administration of either red wine or its constituents. Red wine compounds (RWC) prevented metabolic and cardiovascular alterations in obese rats (Agouni *et al.* 2009), improved flow-mediated dilatation and plasma NO level in hypercholesterolemic rabbits (Zou *et al.* 2003), and restored endothelial function in deoxycorticosterone acetate-salt hypertensive rats (Jiménez *et al.* 2007).

In our previous experiments, red wine polyphenolic compounds were shown to prevent excessive increase of BP in rats exposed to simultaneous administration of L-NAME, a well known NO synthase inhibitor (Pechánová *et al.* 2004). In rats with fully developed NO-deficient hypertension, administration of RWC (after cessation of L-NAME treatment) produced a greater readiness to blood pressure decrease than did spontaneous recovery and was associated with improvement of endothelial function and NO production (Bernátová *et al.* 2002). Additionally, estrogen receptor alpha has been identified as a key target of RWC on the endothelium (Chalopin *et al.* 2010).

Regarding the positive effects of red wine, some studies related them to alcohol itself rather than to its non-alcoholic components (Hansen *et al.* 2005). Other studies failed to find antihypertensive effects of red wine or RWC (Retterstol *et al.* 2005, Andrade *et al.* 2009). It is important to mention that the polyphenolic content of red wine depends on many factors, including grape variety, provenance and process of vinification (Sato *et al.* 1996). Thus the effect of different red wines on the human and animal organism might be variable.

Despite extensive research, relatively little information is available on the vascular effects of red wine and RWC in spontaneously hypertensive rats, an experimental model of human essential hypertension (Pintérová *et al.* 2011). Previously we found elevated NO production in the aorta and left ventricle of SHR rats treated with alcohol-free extract from Alibernet red wine produced in the region of Modra, Slovak Republic (Kondrashov *et al.* 2012). The aim of the present study was to investigate the effect of the same alcohol-free extract from Alibernet red wine on blood pressure and vascular function in young normotensive and spontaneously hypertensive rats.

Methods

Animals

All rats used in the study (n=6 in each group) were born in our certified animal facility in order to maintain the same environmental background of all animals. All rats were housed at 22-24 °C on a 12:12-h dark-light cycle (lights on from 06.00 h till 18.00 h) and maintained on a standard pellet diet and tap water or non-alcoholic solution of Alibernet red wine extract (AWE) *ad libitum*. All procedures were used in accordance with the institutional guidelines and they were approved by the State Veterinary and Food Administration of the Slovak Republic.

At the beginning of the experiment, 6-week-old male normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were randomly divided into the control group and the group treated with AWE for 3 weeks. The animals were kept in groups of three per cage (35/55/20 cm). AWE was diluted in tap water in the final dose of 24.2 mg/kg/day (Kondrashov *et al.* 2012). AWE was administered to rats in the appropriate daily volume of water, assessed for each rat one week before onset of the experiment.

AWE was prepared from Alibernet red wine obtained from the Slovak State Institute of Viniculture, Modra, Slovak Republic. AWE was prepared in the evaporative condenser as described previously (Kondrashov *et al.* 2012). Wine was subjected to the process of dealcoholization and concentration, resulting in alcohol-free concentrated wine extract. The total phenolic content of AWE was 24172 mg/l of gallic acid equivalents (Kondrashov *et al.* 2012). After concentration, AWE was kept in the refrigerator at 4 °C.

Blood pressure and basic cardiovascular parameters

Blood pressure was determined non-invasively on the tail of the rats as described previously (Puzserova *et al.* 2013). Briefly, one week before experiment, the rats were handled and accustomed to the tail-cuff procedure. Blood pressure was further determined before experiment (basal) and at the 1st, 2nd and 3rd week of experiment. Body weight (BW) was recorded on the same days. At the end of experiment, the rats were killed by decapitation after a brief CO₂ anesthesia. Wet weight of the left heart ventricle (LVW) as well as the tibial length (TL) were determined.

Oxidative stress markers – TBARS

Thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxidation, were measured as described by Hu *et al.* (1989), with some modifications. To determine TBARS, 1 ml of tissue homogenate of the left ventricle and liver (10 %, w/v) was added to 2 ml of 7.5 % trichloroacetic acid and mixed. After centrifugation at 1 000g for 10 min, 1 ml of the supernatant was added to 0.5 ml of 0.7 % 2-thiobarbituric acid and heated in a water bath at 100 °C for 10 min. After cooling, the TBARS were measured at 532 nm. An extinction coefficient of 156 000 mol⁻¹ 1 cm⁻¹ was used for calculation of results.

Determination of vascular reactivity

Vascular function was determined in the femoral arteries as described previously (Puzserova *et al.* 2013). The segments of the arteries (approximately 1.28±0.03 mm long) were mounted as ring-shaped preparations in the Mulvany-Halpern's style small vessel wire myograph (Mulvany and Halpern 1977) chamber (Dual Wire Myograph System 410A, DMT A/S, Aarhus, Denmark).

The experimental protocol consisted of the following steps: (1) Arteries were pre-constricted by serotonin (Ser, 1 µmol/l). When the contraction of the artery was steady, increasing concentrations of the endothelium-dependent vasodilator acetylcholine (ACh, from 0.001 to 10 µmol/l) were added in cumulative manner. (2) The same experiment was repeated after 25 min pre-incubation with the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 µmol/l) in the bath medium. (3) Finally, after Ser pre-constriction (1 µmol/l), the nitric oxide donor sodium nitroprusside (SNP, 0.001 to 10 µmol/l) was cumulatively added and the endothelium-independent relaxation response curve was recorded. The artery was four times

washed out with modified physiological salt solution (composition in mmol/l: NaCl 118.99, KCl 4.69, NaHCO₃ 25, MgSO₄·7H₂O 1.17, KH₂PO₄ 1.18, CaCl₂·2H₂O 2.5, Na₂EDTA 0.03 and glucose 5.5) and stabilized for 20 min after each step (Puzserova *et al.* 2013).

The extent of relaxation was expressed as the percentage of serotonin-induced precontraction. NO-dependent (i.e. L-NAME-sensitive) and NO-independent (i.e. L-NAME-resistant) components of ACh-induced relaxation were expressed as the area under the curve (AUC) in arbitrary units (au) calculated from individual dose-response curves. The NO-dependent component of endothelium-dependent relaxation was calculated as the difference of the AUC between ACh-induced relaxation before and after L-NAME pretreatment.

Resting wall tension (basal tension), which arises from the properties of the passive elements in the vascular wall, and normalized diameter were determined after the normalization procedure as described previously (Mulvany and Halpern 1977).

All the chemicals used in this study were purchased from Sigma-Aldrich (Germany). All drugs were dissolved in distilled water and concentrations are expressed as final concentration in the myograph chamber.

Statistical analysis

Data are presented as group mean values ± S.E.M. of the number (n) of observations. Results were analyzed by analysis of variance (ANOVA). One-way ANOVA was used where appropriate. In case of significant results pairwise comparison with Bonferroni adjustment was employed. Concentration response curves and blood pressure were compared using two-way ANOVA, followed by vertical contrast with Bonferroni adjustment. To assess depression present at high concentration of ACh-cumulative concentration response curves, the maximal response and the response at higher ACh concentration at a particular response curve were compared with Dunnet's test (see Fig. 3). Means were considered to differ significantly when p<0.05.

Results

Basic biometric and cardiovascular parameters

The respective basal blood pressure of control WKY, AWE-treated WKY (WKYA), control SHR, and AWE-treated SHR (SHRA) was 98±5 mm Hg,

97±5 mm Hg, 118±9 mm Hg ($p<0.05$ vs. WKY), and 119±2 mm Hg ($p<0.05$ vs. WKYA). AWE-treatment failed to alter BP vs. the respective control in either genotype investigated (Fig. 1).

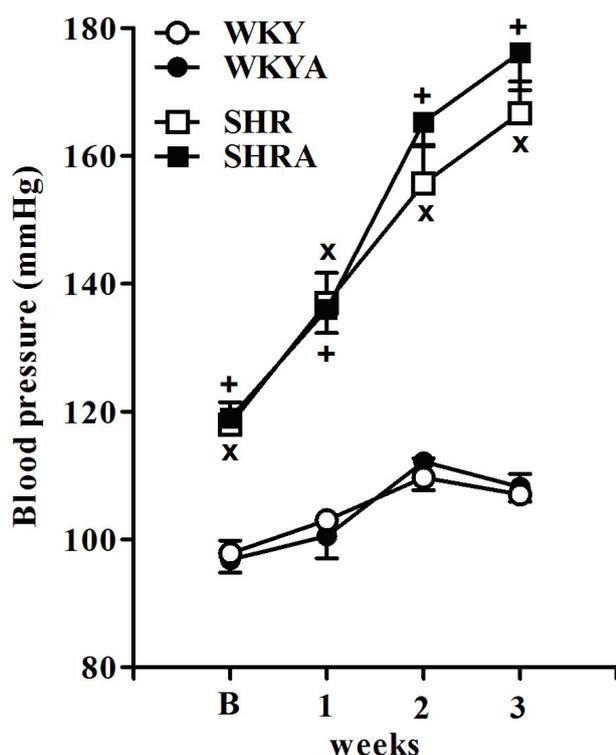
Final BW and TL were significantly lower in

both SHR groups vs. the respective WKY group (Table 1). Relative LVW was significantly increased in the SHR compared to WKY. AWE-treatment failed to affect these parameters, as compared to the control group of the same genotype (Table 1).

Table 1. Basic biometric and vascular parameters of the femoral artery of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats.

| | WKY | | SHR | |
|----------------------|---------------------------|---------------------------|----------------------------|---------------------------|
| | Control (n=6) | AWE (n=6) | Control (n=6) | AWE (n=6) |
| Final BW (g) | 249 ± 5 | 253 ± 5 | 211 ± 5 ^x | 216 ± 4 ⁺ |
| TL (mm) | 36.2 ± 0.2 | 36.0 ± 0.6 | 31.4 ± 1.2 ^x | 33.1 ± 0.9 ⁺ |
| LVW/TL (mg/mm) | 12.1 ± 0.2 | 12.8 ± 0.8 | 14.1 ± 0.2 ^x | 14.4 ± 0.3 ⁺ |
| ND (μm) | 694 ± 17 | 683 ± 23 | 649 ± 22 | 612 ± 14 ⁺ |
| RWT (mN/mm) | 0.76 ± 0.10 | 0.74 ± 0.13 | 0.85 ± 0.11 | 0.59 ± 0.06 |
| Ser (mN/mm) | 7.06 ± 0.59 | 7.33 ± 0.49 | 6.64 ± 0.46 | 7.16 ± 0.36 |
| $E_{max ACh}$ (%) | 83.52 ± 2.15 | 75.95 ± 2.86 | 75.75 ± 2.38 ^x | 71.04 ± 4.66 |
| $E_{max L-NAME}$ (%) | 59.25 ± 2.86 [†] | 53.47 ± 6.53 [†] | 47.40 ± 4.26 ^{x†} | 40.31 ± 6.74 [†] |
| $E_{max SNP}$ (%) | 96.8 ± 0.7 | 97.7 ± 0.7 | 96.8 ± 1.3 | 94.9 ± 2.2 |

The final age of rats was 9 weeks. The concentration of serotonin was 1 μmol/l. AWE – Alibernet red wine extract; BW – body weight; $E_{max ACh}$ – maximal acetylcholine-induced relaxation; $E_{max L-NAME}$ – maximal acetylcholine-induced relaxation after L-NAME; $E_{max SNP}$ – maximal sodium nitroprusside-induced relaxation; L-NAME – N^G-nitro-L-arginine methyl ester; LVW – left heart ventricle weight; ND – normalized diameter of the femoral artery at 13.3 kPa; RWT – resting wall tension; Ser – vascular constriction induced by serotonin; TL – tibia length. Results are mean ± S.E.M. ^x $p<0.05$ compared to WKY control, ⁺ $p<0.05$ compared to AWE-treated WKY, [†] $p<0.05$ compared to the respective value before L-NAME



TBARS

AWE treatment led to significant reduction of TBARS in the left ventricle of both WKY and SHR. In the liver, the reduction of TBARS was seen only in SHR (Fig. 2).

Vascular function

There were no differences in the normalized internal diameter (ND) and resting wall tension of the femoral artery between control WKY and SHR. AWE reduced ND in SHR vs. WKYA (Table 1). AWE treatment failed to induce significant alterations either in serotonin-induced contraction or in maximal ACh-induced relaxation (E_{max}) before and after L-NAME ($E_{max L-NAME}$) administration.

Fig. 1. Effect of AWE on blood pressure of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. AWE – Alibernet red wine extract; B – basal blood pressure; WKYA, SHRA – rats treated with Alibernet red wine extract. Results are mean ± S.E.M. of n=6 rats. ^x $p<0.05$ compared to control WKY group, ⁺ $p<0.05$ compared to AWE-treated WKY

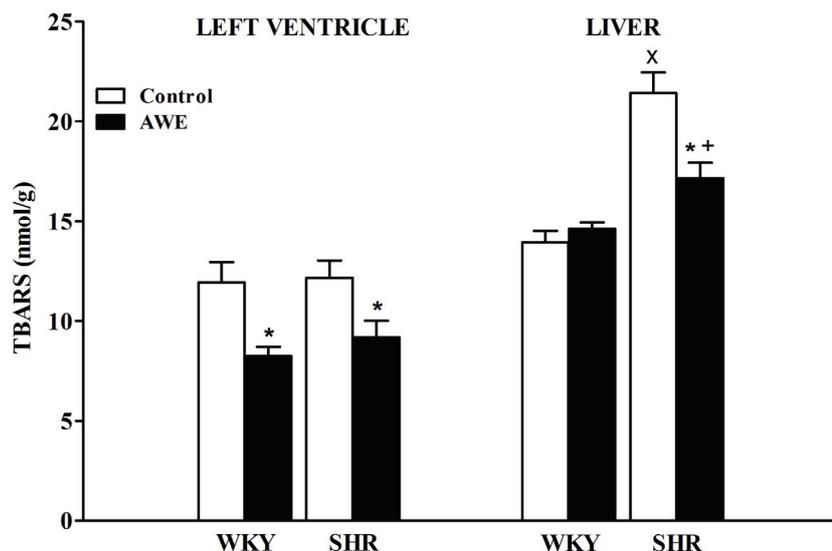


Fig. 2. Influence of AWE on oxidative status of Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. AWE – Alibernet red wine extract; TBARS – thiobarbituric acid-reactive substances. Values represent mean \pm S.E.M. of $n=6$ rats. * $p<0.05$ compared to the respective control group, ^X $p<0.05$ compared to WKY control, ** $p<0.05$ compared to AWE-treated WKY

Maximal endothelium-dependent relaxation (calculated from individual dose-response curves) of the femoral artery was reduced in control SHR vs. control WKY (Table 1). However, higher concentrations of ACh (greater than $0.3 \mu\text{mol/l}$) led to contractions in SHR, resulting in reduced relaxation as compared to lower ACh concentration (Fig. 3B, D). There was also a significant decrease of total relaxation response in SHR vs. WKY when calculated as area under the curve (313.49 ± 13.87 au vs. 359.15 ± 21.02 au, $p<0.05$).

Acute blockade of NO synthesis by L-NAME ($300 \mu\text{mol/l}$) reduced significantly vasorelaxation in all groups investigated compared to the respective value without L-NAME (Fig. 3C, D). AWE had no effect on endothelium-independent sodium nitroprusside-induced relaxation of the femoral artery in either genotype investigated (Fig. 3E, F).

The NO-dependent (i.e. L-NAME-sensitive) component of relaxation was similar in the control WKY and SHR group and AWE failed to affect it significantly, as compared to the control group of the same genotype (Fig. 4). However, the NO-dependent component of relaxation was increased significantly in AWE-treated SHR vs. AWE-treated WKY.

NO-independent (i.e. L-NAME-resistant) relaxation was reduced in the control SHR group vs. WKY and AWE did not improve it (Fig. 4).

Discussion

This study investigated the effect of a non-alcoholic extract from the Slovak Alibernet red wine on blood pressure and vascular function in young

normotensive and spontaneously hypertensive rats. Three-week treatment of rats with AWE had no effect on blood pressure and ACh-induced relaxation of rats, regardless their genotype. However, AWE reduced lipid peroxidation in the left ventricle of both WKY and SHR, as well as in the liver of SHR.

As mentioned above, red wine and its constituents may affect the cardiovascular system *via* several mechanisms. First of all, RWC possesses significant antioxidant properties resulting from both direct scavenging of free radicals and modulation of the antioxidant defense system. In humans, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were elevated in blood after repeated red wine consumption (Fernández-Pachón *et al.* 2009, Noguer *et al.* 2012). Resveratrol, an antioxidant found in grapes and wine, was shown to increase the gene expression of SOD and GPx and to suppress the expression of pro-oxidative NADPH oxidase in human endothelial cells (Spanier *et al.* 2009). In diabetic rats, RWC prevented the decrease of antioxidant enzymes (SOD, CAT and GPx) and lipid peroxidation in the heart and liver (Udayakumar *et al.* 2010). As mentioned above, we showed recently that AWE, as used in this study, increased SOD activity but not expression in the left heart ventricle in SHR, while no changes were observed in WKY (Kondrashov *et al.* 2012). Interestingly, in this study we found reduced lipid peroxidation in the left ventricle of both WKY and SHR and in the liver of SHR. Similarly, the antioxidant capacity of various red wines correlated with the reduction of lipid peroxidation in the liver, kidney and lung of Wistar rats, without affecting CAT, SOD and

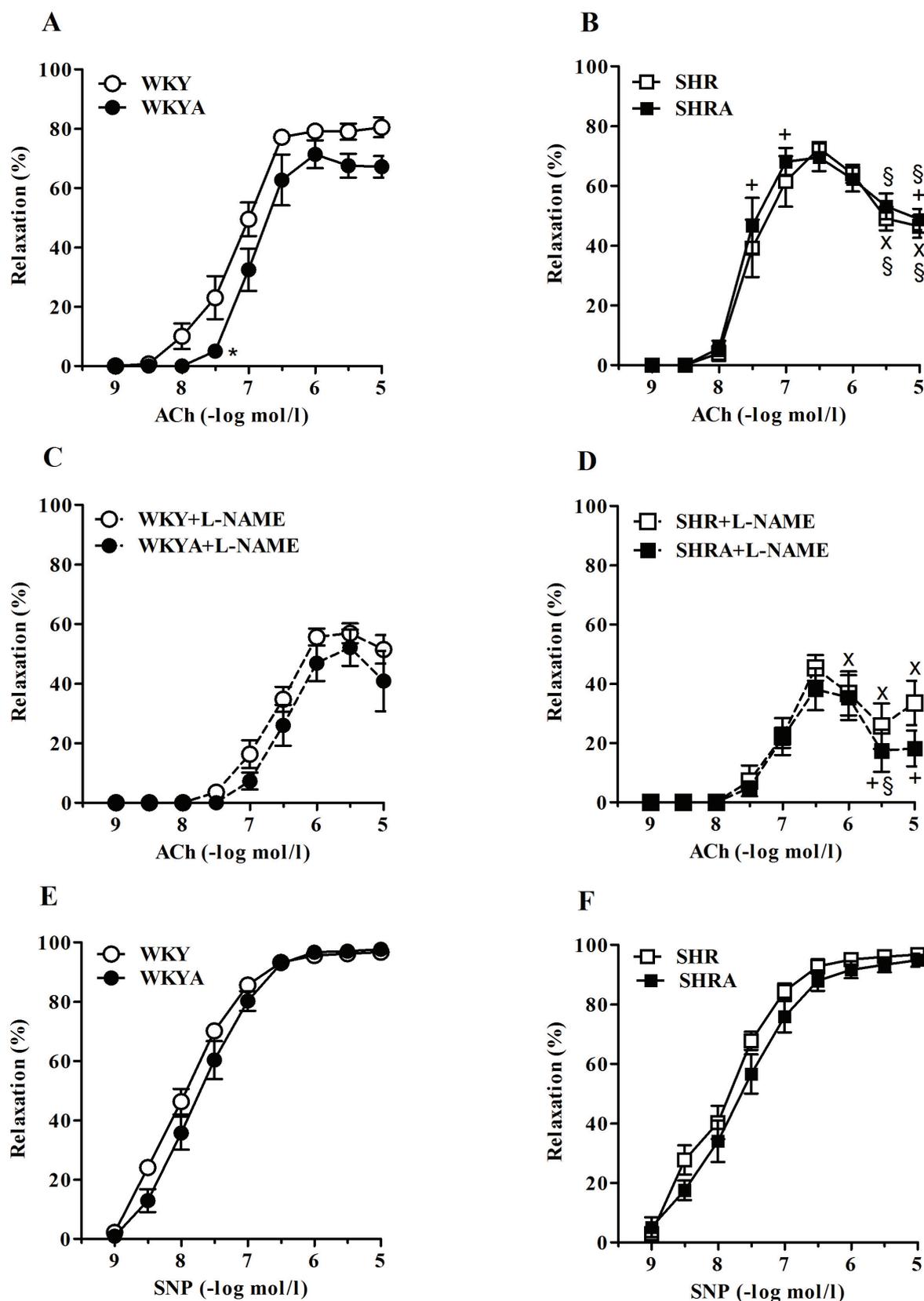


Fig. 3. Effect of AWE on relaxation of the femoral artery. **A, B** – ACh-induced relaxation, **C, D** – ACh-induced relaxation after inhibition of nitric oxide production with L-NAME, **E, F** – SNP-induced relaxation. ACh – acetylcholine; AWE – Alibernet red wine extract; L-NAME – N⁶-nitro-L-arginine methyl ester; SHR – spontaneously hypertensive rats; SHRA – AWE-treated SHR; SNP – sodium nitroprusside; WKY – Wistar-Kyoto rats; WKYA – AWE-treated WKY. Relaxation was determined in serotonin (1 μ mol/l)-precontracted artery segments. Values represent mean \pm S.E.M. of n=6 rats. *p<0.05 compared to respective control group, ^xp<0.05 compared to WKY control, ⁺p<0.05 compared to WKYA, [§]p<0.05 compared to maximal relaxation (ACh concentrations 0.3 μ mol/l)

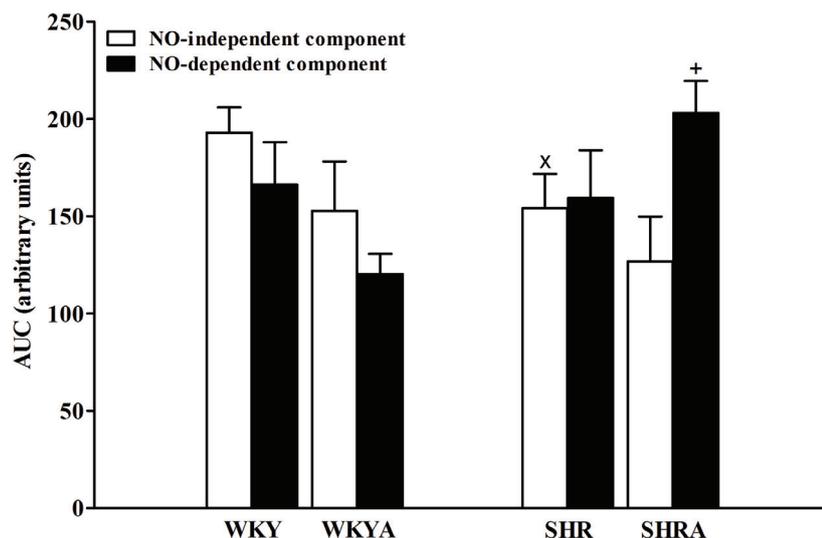


Fig. 4. Influence of AWE on NO-dependent and NO-independent components of acetylcholine-induced relaxations. AUC – area under the curve; AWE – Alibernet red wine extract; NO – nitric oxide; SHR – spontaneously hypertensive rats; SHRA – AWE-treated SHR; WKY – Wistar-Kyoto rats; WKYA – AWE-treated WKY. ^x $p < 0.05$ compared to WKY control, ⁺ $p < 0.05$ compared to WKYA. Values represent mean \pm S.E.M. of $n = 6$ rats.

GPx activities (Rodrigo *et al.* 2005). Abovementioned data suggest that the antioxidant effect of RWC and/or red wine in rats without pathologies might result from direct scavenging properties of RWC. However, in conditions of elevated oxidative stress, as observed in diabetes (Udayakumar *et al.* 2010) or in experimental hypertension (Rush *et al.* 2007), both direct scavenging and activation of the antioxidant enzyme cascade may be involved in reducing oxidative damage. Thus antioxidant effects of red wine or RWC seem to be tissue specific and related to the oxidative status of the organism.

Another system involved in regulation of cardiovascular function that can be modified by RWC is the L-arginine/NO system. NO is well known to be involved in regulation of BP on both the central and peripheral level. In normotensive rats, reduction of NO led to hypertension, heart hypertrophy and fibrosis (Babál *et al.* 1997), reduced vasorelaxation and elevated vasoconstriction (Török and Kristek 2002). We observed that L-NAME-induced hypertension could be partially prevented by RWC by improving NO production (Pechánová *et al.* 2004). Activation of NO production or improvement of endothelium-dependent relaxation after red wine or RWC treatment was shown in the aorta of normotensive rats (Diebolt *et al.* 2001, Bernátová *et al.* 2002, Púzserová *et al.* 2006, Čačányiová *et al.* 2011) and mice (Chalopin *et al.* 2010), and in the porcine coronary artery (Ndiaye *et al.* 2005). In humans, red wine and RWC reduced BP and improved forearm endothelial function and NO production in young healthy individuals (Hamed *et al.* 2010). Similarly, a decrease of BP in association with elevation of plasma NO was seen after both red wine and RWC consumption in men at high

cardiovascular risk (Chiva-Blanch *et al.* 2012). On the other hand, Andrade *et al.* (2009) observed increased flow-mediated dilatation after short-term red wine consumption only in hypercholesterolemic but not in hypertensive or healthy subjects. Additionally, no significant effect of red wine on BP (as compared to basal value) was seen in the same study (Andrade *et al.* 2009). It is thus of interest that in SHR, the most frequently used experimental model of human essential hypertension, only a few studies have been conducted so far to determine the effect of RWC on BP and/or vascular function.

López-Sepúlveda *et al.* (2008) found that 5-week treatment of SHR adult females with RWC (40 mg/kg/day) reduced BP and improved endothelium-dependent relaxation of the aorta in association with reduction of superoxide. In our previous study, 8-week administration of RWC (20 mg/kg/day) to adult SHR males reduced BP and elevated vascular NO production in the aorta (Bernátová *et al.* 2007). Ten-week administration of a relatively high dosage of RWC (100 mg/kg/day) was found to improve endothelium-dependent dilatation of the cerebral arterioles, normalize wall stress and diameter, and alter the systemic oxidative state, yet it failed to reduce BP (Chan *et al.* 2008).

Differences in the effect of RWC on BP in the above mentioned studies, both human and experimental, may result from several factors. Of particular importance are the differences in the composition of red wine polyphenols and in the dose and duration of treatment applied in individual studies. Secondly, sex and age can play a significant role in modulation of BP and endothelial (dys)function both in humans (Nilsson *et al.*

2008, Kuneš *et al.* 2012) and in rats (Bernatova *et al.* 2009, Lišková *et al.* 2011, Kuneš *et al.* 2012).

Concerning the mechanism of ACh-induced relaxation, differences were found between normotensive and hypertensive rats. Several studies showed altered vascular acetylcholine-induced hyperpolarization and increased release of endothelium-derived constricting factors (EDCFs), including cyclooxygenase-generated prostaglandins, in the arteries of SHR at higher concentrations of ACh (Lüscher and Vanhoutte 1986, Fujii *et al.* 1993, Gündüz *et al.* 2011, Lišková *et al.* 2011). In the present study, we did not observe ACh-induced release of EDCFs in WKY rats. We however observed that endothelial dysfunction in the femoral artery of young SHR may have been due to the release of EDCFs and/or reduced hyperpolarization (i.e. reduced NO-independent component of relaxation) rather than to lack of NO. In addition, 3-week administration of AWE failed to affect BP and endothelium-dependent and endothelium-independent relaxation of the femoral artery in both young WKY and SHR males. However, elevated NO synthesis and increased SOD activity found in AWE-treated SHR (Kondrashov *et al.* 2012) resulted in an increase of the NO-dependent component of relaxation

(vs. AWE-treated WKY), presumably due to an increase in NO bioavailability. Furthermore, in WKY we did not see changes either in BP or in components of ACh-induced relaxation, in accordance with no alterations in their NO production and SOD activity (Kondrashov *et al.* 2012).

In conclusion, short-term administration of the Slovak Alibernet red wine extract reduced lipid peroxidation in the left ventricle and liver of SHR and in the left ventricle of WKY rats. In spite of improved oxidative status, AWE failed to modulate vascular function and blood pressure in both young normotensive and hypertensive rats.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

The authors thank Mrs. Jana Petova and Mrs. Maria Kovacsova for their technical assistance and for help with housing the animals. This study was partially supported by the Slovak Grant Agency for Science, grant No. 2/0084/10 and Slovak Research and Development Agency, grant No. APVV-0523-10 and APVV-0742-10.

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