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**Red wine extract decreases pro-inflammatory markers nuclear factor-κB and inducible NOS in experimental metabolic syndrome.**

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Short running title: Red wine extract and metabolic syndrome.

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**Abstract**

We aimed to analyse the effects of alcohol free Alibernet red wine extract (AWE) on nitric oxide synthase (NOS) activity and pro-inflammatory markers like nuclear factor-ᴋB (NFᴋB) and inducible NOS (iNOS) protein expressions in experimental metabolic syndrome. Young 6-week-old male Wistar Kyoto (WKY) and obese, spontaneously hypertensive rats (SHR/N-cp) were divided into control groups and groups treated with AWE (24.2 mg/kg/day) for 3 weeks (n=6 in each group). Total NOS activity and endothelial NOS (eNOS), iNOS and NFᴋB (p65) protein expressions were determined in the heart left ventricle and aorta by Western blot and immunohistochemical analysis. All investigated parameter were significantly increased in the aorta of SHR/N-cp rats. Pro-inflammatory markers like NFᴋB and iNOS were increased in the left ventricle as well. AWE treatment did not affect total NOS activity and eNOS expression in the aorta, however it was able to decrease NFᴋB and iNOS protein expressions in both left ventricle and aorta. In conclusion, in the cardiovascular system, Alibernet red wine extract decreased NFκB and iNOS protein expressions elevated as a consequence of developed metabolic syndrome. This effect may represent one of the protective, anti-inflammatory properties of Alibernet red wine polyphenols on the cardiovascular risk factors related to the metabolic syndrome.

**Key words:** red wine polyphenols, hypertension, obesity, metabolic syndrome, heart, aorta, nitric oxide, eNOS, iNOS, NFᴋB

**Introduction**

The metabolic syndrome represents a cluster of obesity, dyslipidaemia, hypertension and insulin resistance/hyperglycaemia and belongs to the candidate leading factors of the human morbidity and mortality on cardiovascular diseases in near future. Chronic inflammation participates as a critical mechanism in its pathogenesis (for review see Belin de Chantemele and Stepp[1]). Studies identifying and analysing the compounds that may have an effect in the prevention and early treatment of metabolic syndrome complications appear to have a great benefit to the patients.

Spontaneously hypertensive corpulent rats (SHR/N-cp) represent an effective model of metabolic syndrome that may provide sufficient information in the research concerning the pharmacological and non-pharmacological interventions of this chronic disease [2.] The model is characterized by the similar complications, macro and microvascular included, as in humans [2-5].

A number of publications have demonstrated that the moderate red wine consumption had protective effect on the cardiovascular system [6-8]. Various natural compounds rich of polyphenols, included red wine, red grape berry, wild blueberry and others, have shown their protective effects in the cardiovascular disease and metabolic syndrome (for review see Tangney and Rasmussen, 2013 [9]). Leibowitz et al. [10] have documented the positive role of cultured cells derived from red grape berry in fructose treated Sprague-Dawley rats. Such treatment was able to decrease blood pressure, plasma triglyceride and insulin levels and increase eNOS signaling impaired due to the  fructose treatment [10]. Downregulation of inducible nitric oxide synthase and COX2 expression have been demonstrated in obese Zucker rat after wild blueberry diet leading to restoration of impaired vasoactivity in these

animals [11]. The polyphenols isolated from red wine have shown their strong anti-oxidant and anti-inflammatory effect in different animal and human studies [6, 12-14].

The presented study investigated the effects of alcohol-free Alibernet red wine extract (AWE) in the experimental rat model of metabolic syndrome. Particularly, total NOS activity, eNOS, iNOS and NFκB expressions were analysed in the heart left ventricle and aorta.

## Methods

### Experimental protocol

The alcohol-free AWE was prepared from the red wine obtained from the Slovak State Institute of Viniculture (Modra, Slovakia) as described and characterized previously [15]. The concentration of total phenols in AWE is 24172 mg/L (in GAE mg/L).

Male 6-week-old normotensive WKY and SHR/N-cp were divided into the control groups and groups treated with AWE in drinking water (24.2 mg/kg/day) for 3 weeks (6 animals in each). During the experiment, water consumption of each animal was controlled to ensure that each animal received the complete amount of AWE. All animals were housed at a temperature of 22–24°C and were fed with a regular pellet diet ad libitum. The spontaneously hypertensive rats with metabolic syndrome were obtained from Charles River US laboratories. All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, and conform to the European Convention on Animal Protection and Guidelines on Research. After 3 weeks of treatment, blood pressure (BP) was measured, animals were sacrificed and the samples of the left ventricle and the aorta were removed.

**Blood pressure and plasma glucose**

Blood pressure (BP) was measured by non-invasive tail-cuff-plethysmography as previously described [14,15]. Plasma glucose concentration was determined using commercial kit Glucose C II test Wako (Pure Chemical Industries, Japan).

**Total nitric oxide synthase activity and expression analysis**

Total nitric oxide synthase activity was determined in crude homogenates of left ventricle and aorta tissue by measuring the formation of L-citrulline [4,5-$^3$H] from L-arginine [4,5-$^3$H] (MP Biochemicals, California, USA) as previously described by Bredt and Snyder [16] with minor modification [17].

For Western blot analysis, samples of the left ventricle and aorta were used and probed with polyclonal rabbit anti-eNOS, anti-iNOS and anti-NF B - (p65) subunit antibodies (Santa Cruz Biotechnology, Inc., USA) as described elsewhere [18]. Bound antibody was detected using a secondary peroxidase-conjugated anti-rabbit antibody, the bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, UK) and analyzed densitometrically using Photo-Capt V.99 software.

**Immunohistochemical analysis**

Neutral buffered formalin solution fixed samples were used for immunohistochemical analysis of iNOS, eNOS and NFκB expression. The tissue samples were standardly processed, embedded in paraffin and sectioned; 4μm thick slices were deparaffinised and rehydrated in phosphate buffered saline solution (10 mM, pH 7.2). The slides were subsequently incubated 90 minutes at room temperature with the primary rabbit polyclonal antibody against iNOS (Santa Cruz Biotechnology, USA, sc-651) diluted 1:50, eNOS (Santa Cruz Biotechnology, USA, sc-8311) diluted 1:50 and NF B (p65) (Labvision, Thermo

scientific, USA, RB-1638) diluted 1:100 in DAKO REAL antibody diluent (Dako, Glostrup, Denmark). The slides were immunostained using anti-mouse/anti-rabbit immuno-peroxidase polymer (EnVision FLEX/HRP, Dako, Glostrup, Denmark) for 30 minutes at room temperature, according to the manufacturer's instructions. For visualisation, the diaminobenzidine substrate-chromogen solution was used (DAB, Dako, Glostrup, Denmark) for 5 minutes. Finally, the slides were counterstained with haematoxylin. As negative control, the slices of heart and aorta were subjected to the same procedure without staining with the primary antibody. The positivity was evaluated by optical microscope (Leica microsystems, Wetzlar, Germany).

**Statistics**

One-way analysis of variance and the Duncan test were used for statistical analysis. Data are presented as the means $\pm$ SEM. Values were considered to differ significantly if the probability value was less than 0.05 ($P < 0.05$).

**Results**

**Blood pressure and plasma glucose level**

At the end of experiment, both BP and plasma glucose level of SHR/N-cp rats were significantly higher than that of control WKY rats. AWE treatment did not change BP and plasma glucose level of WKY rats, while the treatment decreased these parameters of SHR/N-cp rats significantly (p<0.05) (Tab. 1).

**Total NOS activity**

Total NOS activity in the left ventricle was not changed significantly in any group. However, it was increased in the aorta of SHR/N-cp compared to WKY rats (p<0.05). AWE treatment did not change total NOS activity significantly (Tab. 2).

**Expression of inducible (iNOS) and endothelial NOS (eNOS)**

Significant increase of iNOS expression was determined in the left ventricle and aorta of SHR/N-cp rats (increase to $130.0 \pm 3.0\%$, p<0.05 and $124.0 \pm 3.6\%$, p<0.05, respectively) when compared to control WKY rats ($100.0 \pm 1.1\%$ and $100.0 \pm 1.0\%$, respectively). Parallel AWE administration prevented the increase of iNOS expression in both left ventricle and aorta tissues and restored it to the control levels ($95.0 \pm 2.8\%$ and $91.0 \pm 2.8\%$, respectively) (Fig. 1).

Similar increase of eNOS expression was described in the aorta of SHR/N-cp rats ($139.0 \pm 4.8\%$, p<0.05) as compared to control WKY rats ($100.0 \pm 1.3\%$). The AWE treatment did not, however, affect this increase. No change of eNOS expression was detected in the left ventricle in any group (Fig. 2).

**Expression of nuclear factor kappa B**

The expression of NFκB (p65) showed similar changes as expression of iNOS in all investigated groups (Fig. 3). Significant increase of NFκB (p65) expression was determined in the left ventricle and aorta of SHR/N-cp rats (increase to $125.0 \pm 3.7\%$, p<0.05 and $122.0 \pm 1.9\%$, p<0.05, respectively) when compared to control WKY rats. Parallel AWE administration prevented this increase and restored the expression to the control levels ($93.0 \pm 2.9\%$ and $95.0 \pm 1.0\%$ respectively).

**Histological analysis**

Both types of NOS and NFκB (p65) were present in myocardial cells of heart left ventricle and smooth muscle cells of aortal media, showing the irregular diffuse cytoplasmic granular positivity. Mild to moderate positivity of iNOS and NFκB well correlated with their protein expression analysed by Western-blot analysis (Fig. 4). eNOS showed strong positivity of the lining endothelial cells, mild to moderate positivity of cardiomyocytes and only weak positivity of smooth muscle cells of aortal media. No differences in the localization of NOS (neither isoform) and NFκB (p65) between the experimental groups were observed.

**Discussion**

Comparing to WKY, SHR/N-cp rats are characterized by increased body and heart weights as well as by impaired glucose metabolism [2], which was clearly confirmed by our study as well (Table 1). In SHR/N-cp rats, Alibernet red wine extract treatment was able to decrease both blood pressure and plasma glucose level significantly. Our study further clearly documented that in the cardiovascular system Alibernet red wine extract downregulated pro-inflammatory markers, like NFκB and iNOS elevated as a consequence of metabolic syndrome in rats.

It is well described, that the obesity in metabolic syndrome is accompanied by up-regulation of pro-inflammatory genes leading to oxidative stress and to the remodelling of extracellular matrix in tissues [19]. Inflammatory environment in affected arteries is partially responsible for the increased macrophage infiltration of the vascular wall and increased neointima proliferation and promotes the development of serious form of atherosclerotic vascular disease [20]. The fact that the inflammatory process in the vessel wall mediated by NFκB has negative effect on the vascular function has been well described [21, 22].

Recently it has been generally accepted that NFκB plays a pivotal role in the induction and maintenance of the inflammatory burden in the metabolic diseases, obesity and type 2

diabetes included [23]. This fact is in accordance with the finding of increased of NFκB expression in aorta and heart tissue of animals with metabolic syndrome in the present study. The NFκB is a key molecule of the intracellular transduction system that was found to be activated during the different stages of atherosclerosis. Inhibition of this pathway attenuates the atherosclerosis and its complications [24].

In our experimental conditions the increase of NFκB expression in the aorta of animals with metabolic syndrome was significantly prevented by AWE treatment and reached the level of NFκB expression found in controls. Hasegawa et al. [25] showed that the blockage of NFκB signalling in the endothelial cells decreases the obesity-induced macrophage infiltration of the vessel wall and prevents the insulin resistance onset. They proposed the NFκB signalling inhibition as the potential target for treating the metabolic syndrome [25]. Enhanced levels of NFκB kinase was shown to be partially responsible for the induction of insulin resistance and fat accumulation in peripheral tissues and it is considered as a novel therapeutic target for the treatment of insulin resistance associated with obesity also in other studies [26].

We noticed the similar increase of NFκB expression also in the heart tissue of the animals with the metabolic syndrome. Although the role of NFκB expression in heart damage, unlike in aorta, is still controversial, several studies pointed its role in the process of hypoxia-induced cell injury and demonstrated the beneficial effect of its inhibition [27, 28], partially by attenuating myocardial inflammatory cascades during the ischemia [29, 30]. This may be interesting mainly due to the fact, that tissue hypoxia belongs to one of the crucial mechanisms of the heart injury in metabolic syndrome. It indicates that the inhibition of NFκB expression by AWE, as has been described in our experiment, may have beneficial effect on the heart tissue in this type of chronic damage. In this context, there is evidence that red wine polyphenols improved features of the metabolic syndrome in human studies as well [31, 32].

The increased NFκB expression was accompanied by increase in iNOS expression in both investigated tissues. Increased eNOS expression was determined in the aorta. Activation of NF B represents one of the mechanisms that have been suggested to be responsible for iNOS as well as eNOS upregulation [33-35]. Despite increased NOS expressions and elevated total NOS activity in the aorta of animals with metabolic syndrome, the increased reactive oxygen species formation in these animals may lead to decrease of NO bioavailability[15]. Indeed, increase of conjugated dienes, one of the markers of oxidative damage, has been demonstrated also in our model of metabolic syndrome (data not shown). This may be one of the factors participating in the pathogenesis of tissue injury in metabolic syndrome. The decreased bioavailability of NO supposed to be also responsible for impaired NO-mediated vasodilatation in diabetes and obesity [36, 37].

Thus, nitric oxide seems to have beneficial and protective effect in metabolic diseases. Barbato et al. [20] referred the significant reduction of neointimal proliferation and decrease of adhesion molecules ICAM-1 and P-selectin and receptors for oxidized low-density lipoproteins expression on the endothelial cells after experimental iNOS gene transfer in obese animals[20]. Such gene transfer, however, may produce overproduction of NO, which is not reachable under the physiological conditions and after prolongation could be even pro-inflammatory. In this context, higher NOS activity and increased eNOS expression in animals with metabolic syndrome may represent rather adaptive process to developing metabolic syndrome. In our experimental conditions AWE treatment did not affect this adaptive process. On the other hand, it decreased expression of pro-inflammatory iNOS protein. This may represent one of the beneficial effects of red wine extract.

In conclusion, our study provide direct evidence that polyphenolic compounds in Alibernet red wine extract decreased NFκB and iNOS expressions elevated due to the development of metabolic syndrome in rats. This may represent one of the protective effects of Alibernet red

wine polyphenols on the cardiovascular complications of risk factors related to metabolic syndrome.

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**Tab. 1.** Effect of AWE treatment on body weight, heart weight, blood pressure and plasma glucose in WKY and SHR/N-cp rats.

|  | Body weight (g) | Heart weight (g) | Blood pressure (mmHg) | Glucose (mg/dl) |
|---|---|---|---|---|
| WKY | 249 ± 5 | 809 ± 17 | 106±3 | 91±5 |
| WKY + AWE | 253 ± 7 | 841 ± 24 | 108±4 | 85±7 |
| SHR/N-cp | 315 ± 16* | 878 ± 20* | 174±2* | 165±9* |
| SHR/N-cp + AWE | 310 ± 16* | 845 ± 16 | 163±3*+ | 127±8*+ |

WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; * p<0.05 compared to WKY rats, + p<0.05 compared to the respective genotype

**Tab. 2.** Effect of AWE treatment on total NOS activity in the heart left ventricle and aorta of WKY and SHR/N-cp rats.

| Total NOS activity (pkat/mg of protein) | Aorta | Heart left ventricle |
|---|---|---|
| WKY | 9.31 ± 0.55 | 5.18 ± 0.95 |
| WKY + AWE | 10.65 ± 0.75 | 5.28 ± 0.84 |
| SHR/N-cp | 28.03 ± 3.3 * | 4.08 ± 0.47 |
| SHR/N-cp + AWE | 30.15 ± 2.13 * | 5.19 ± 0.67 |

WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; * p<0.05 compared to WKY rats

**Figures Legends**

**Fig. 1. Inducible NOS expression** in the heart left ventricle and aorta tissue. WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; * p<0.05 compared to WKY rats

**Fig. 2. Endothelial NOS expression** in the heart left ventricle and aorta tissue. WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; * p<0.05 compared to WKY rats

**Fig. 3. NF|B expression** in the heart left ventricle and aorta tissue. WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; * p<0.05 compared to WKY rats

**Fig. 4. Immunohistochemical expression of NF|B and iNOS** in the heart left ventricle and aorta tissue. Significant increase of NFκB and iNOS expression in the heart left ventricle and aorta of rats with metabolic syndrome was prevented by AWE administration. WKY - normotensive Wistar Kyoto rats; SHR/N-cp - spontaneously hypertensive rats with metabolic syndrome; AWE - alcohol-free Alibernet red wine extract; Ao – aorta, LV – heart left ventricle
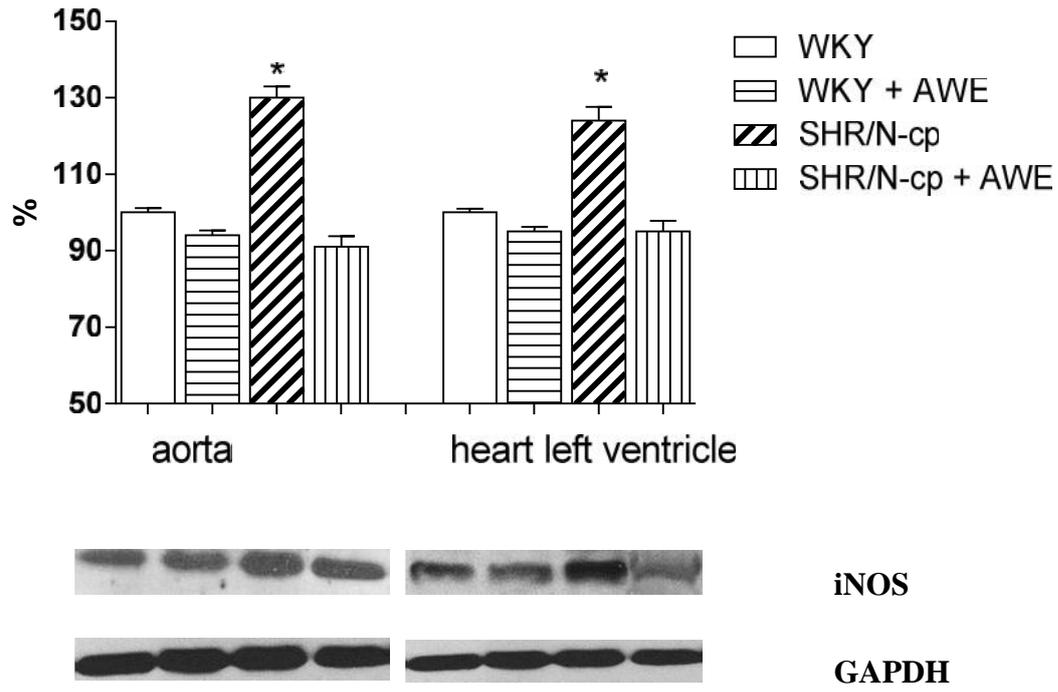
**Fig. 1.**

**Fig. 2.**

**Fig. 3.**

|  | WKY | WKY + AWE | SHR/N-cp | SHR/N-cp + AWE |
|---|---|---|---|---|

**NFkB**

Aorta



LV



**iNOS**

Aorta



LV