SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Beneficial effects of Allium ursinum in dyslipidaemia and in pulmonary arterial hypertension

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Beneficial effects of Allium ursinum in miscellaneous cardiovascular disorders

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The Examination takes place at the library of Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen
11 a.m., June 18, 2018

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
3 p.m., June 18, 2018

Table of Contents

1. Introduction and objectives

The main risk factors of cardiovascular diseases (CVD) and death caused by it, are hypertensive disorders, diabetes, dyslipidaemia, obesity, smoking, alcoholism, malnutrition and sedentary lifestyle.

Based on the most recent datas provided by the Hungarian Central Statistical Office (KSH), life expentancy at birth has increased from 68,2 to 72,3 in the last years between 2001 and 2015. Moreover, the decline in mortality caused by CVD has contributed significantly to the prolonged life expentency, in which preventive and healing medicine played a determinative role . The modern therapy of hypertensive disorders, diabetes, dyslipidaemia, developed coronary artery disease, congestive heart failure, arrythmias and cerebrovascular diseases, significantly reduced mortality.

Our constantly improving values are still far below the average of the European Union, where the life expentancy at birth is 77,9 years. Despite the fact that the mortality rate caused by ischemic heart diseases decreased in the greatest extent among the leading causes of death, it is still highly above the others in Hungary and Europe alike. Although it reflects the succes of intervention that mortality due to the coronary heart disease and stroke has decreased in the past decade. As a result, the prevalence of chronic heart failure has dramatically increased. Consequently, the treatment of chronic heart failure is a major public healt and economic burden in the industrialized countries. On the basis of previous thoughts it is important to emphasize that dyslipidemia is the most common influencing risk factor of cerebro- and carviovascular diseases. Dyslipidemia is characterized by the elevated LDL cholesterol, triglycerid and decreased HDL cholesterol level, which are risk factors for prematured and progressive atherosclerosis.

A number of studies have demonstrated that by only the arrangement of lipid status, may have pozitive influence on mortality caused by iscemic heart

and other vascular diseases, as primary and seondary prevention as well.

Finally, in relation to CVD, there should not be forgotten about the incurable pulmonary arterial hypertension (PAH), which is a rare, but progressive and malignant type of disorder. The development of PAH is often sneaking, wherefore the patient is often in an advanced stage after diagnosis. Due to the high mortality rate of affected patient within 5 years, we can not sufficiently highlight the need for development in PAH therapy as well.

Objectives

Eventhough that the Allium sativum and Allium ursinum related closely, the available scientific literatures about the latter are significantly modest. Although, the few *in vitro* and *in vivo* results of comparative studies are promising. Our team has find therefore worthy to investigate the effects of the wild garlic leaf lyophilisate in complex cardiovascular diseases models:

A) Ischemic/reperfusion injured hyperchlesterolaemic New Zealand white rabbits, held on/feed with cholesterol rich chow (HC model)
B) Monocrotaline-induced pulmonary arterial hypertensive Sprague Dawley rats with right ventricle dysfunction (PAH model)

We aimed in both studies to clarify the functional changes that may arise in molecular level as well.

2. Materials and methods

2.1. Animals

The animals received human care in compliance with “Principles of Laboratory Animal Care” by EU Directive 2010/63/EU for animal experiments. The duration of the adaptive feeding was 2 weeks. Animals were housed under a 12:12 h light-dark cycle in a specific pathogen-free environment and had free access to food and water. Allium ursinum lyophilisate-containing chow was produced in the laboratory of Dept. of Pharmaceutical Technology, University of Debrecen.

HC model

The experiments were carried out using adult male New Zealand rabbits with an average body weight of 2.5-3.0 kg. The rabbits were provided with laboratory rodent chow, or chow enriched with 2.0% cholesterol (Jurasko, Debrecen, Hungary), or cholesterol-supplemented chow containing 2% wild garlic leaf lyophilisate (WGLL) daily for 8 weeks ad libitum.

PAH modell

Male rats of the Sprague Dawley strain weighing 200–250 g were purchased from Charles River International Ltd. (Wilmington, MA, USA). Body weights were measured in all animals at the endpoint of the study (8 weeks). PAH was induced by a single subcutaneous injection of monocrotaline (MCT, 60 mg/kg, to the interscapular region), the specific toxic alkaloid of Crotalaria spectabilis (Sigma-Aldrich Co., St. Louis, MO, USA), whereas the control group recieved only the vehicle, Dimethyl sulfoxide buffer (DMSO buffer, Control group, n = 8). MCT-treated rats were then divided into 3 subgroups as follows: rats that received only MCT injection and developed PAH (PAH group, n = 8); rats that received MCT injection and were kept on standard chow enriched with 2% wild garlic leaf liophilizate (WGLL group, n = 8); and MCT-injected rats that were treated with 25 mg/kg sildenafil daily via oral (gavage technique) dosing (Sildenafil group, n = 8).

2.2. Sample lyophilization and Bioanalytics.

Deep-frozen Allium ursinum leaves (Toltelekgyar Ltd., Zalakomar, Hungary) were lyophilized for 24 hours in a Martin-Christ ALPHA 1-4 freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at an ambient pressure of 0.120 millibars (mb), with a condensor temperature of -50°C and shelf temperature of 35°C. The ratio of the frozen, leaf to fresh and desiccated plant material was 5:6:1. HPLC analysis was accomplished using a Waters 600 system (Waters Corporation, Milford, CT, USA), equipped with a 2998 photodiode array detector, on-line degasser, and

auto sampler, using a reversed phase Phenomenex Synergi 4 μm Hydro-RP 80Å (250 mm x 4.6 mm) column (Phenomenex, Torrance, CA, USA). Mass spectrometry (Matrix-assisted laser desorption/ionization–time of flight, MALDI-TOF MS) analyses of the compounds were performed in positive-ion mode using a Bruker Biflex III MALDI-TOF mass spectrometer equipped with delayed-ion extraction (Bruker Daltonics, Bremen, Germany).

2.3. Transthoracic Echocardiography (TTE)

Echocardiographic examination of animals was conducted under light anesthesia (ketamine 15 mg/kg, xylazine 3 mg/kg (i.m.) at the 8-week timepoint of the study. The chest of each animal was shaved, and the rabbit was positioned in a dorsal decubitus position. Echocardiographic measurements were performed using a Siemens Acuson 512 sonograph, with 7V3c probe at 7 MHz (*HC studyl*), and a Vivid E9 sonograph with i13L linear array probe at 14 MHz (*PAH study*). Complete 2-dimensional, M-mode (at papillary muscle levels), Doppler (PW), and tissue Doppler (TDI) echocardiograms were acquired and digitally stored for further analysis. Doppler imaging at the mitral and aortic valves was obtained from the apical 3- and 4-chamber views. ECG was continously monitored during echocardiographic examinations in all cases. Measurements included interventricular and left ventricular free-wall thickness in diastole and systole (IVSs, IVSd), left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs), and aortic root diameter (Ao). End-systolic volume (ESV), end-diastolic volume (EDV), stroke volume (SV) and mass of left ventricle (LV mass) were calculated. Fractional shortening was computed by using the equation (LVIDd − LVIDs)/LVIDd × 100%, and the ejection fraction (EF) was calculated by computer using the Teiholz formula. Mitral annular peak systolic excursion (MAPSE) and tricuspidal annular peak systolic excursion (TAPSE) were assessed with M-mode, measuring the distance of mitral or tricuspid annular movement between end-diastole to end-

systole. From the mitral inflow velocity image (Doppler), the following measurements were obtained: left ventricular peak E and peak A waves (mitral early and late filling velocities) and the E to A ratio (E/A). Left ventricle outflow tract (LVOT) parameters were also determined: maximal- and mean pressure gradients (LVOT maxPG, LVOT meanPG), as well as maximal- and mean outflow velocities (LVOT Vmax, LVOT Vmean, see Figure 5.). Tissue velocities at the lateral annulus of mitral valve were estimated using spectral tissue Doppler by determining systolic (S') waves. Visualization of the right ventricle and estimation of systolic function by using TAPSE was accessible in many rats, however, measuring right ventricle diastolic function was unattainable in most cases.

2.4. Measurement of Serum Parameters

Blood samples were collected with EDTA-K2 evacuated tubes (BD Vacutainer, USA) from the marginal ear vein of the animals, at the endpoint of the treatment. The samples were collected and processed aseptically to minimize hemolytic activity. Serum level of total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and value of Apolipoprotein A-I (ApoA), Apolipoprotein B (ApoB), C reactive protein (CRP), Aspartate transaminase (also called Glutamic oxaloacetic transaminase, GOT), Lactate dehydrogenase (LDH), and Creatine kinase (CK) were detected by automated analyzers in the Department of Laboratory Medicine at the University of Debrecen.

2.5. Isolated Heart Parameters

Each of the animals were anaesthetized with a mixture of ketamine/xylazine (50/5 mg/kg, intramuscularly). A bolus of heparin was administered (1,000 U/kg of body weight, intravenously) 20 minutes before thoracotomy, to avoid thrombosis. Next, the chest cavity was opened and the pericardium incised. The heart was excised and immediately transferred to ice-cold modified Krebs-

Henseleit (mKH) buffer (pH 7.4 on 37°C, gassed with 95% O_2 and 5% CO_2 mixture). The aorta was then cannulated and the heart perfused for 10 minutes, retrogradely in Langendorff mode with mKH buffer to clear residual blood from each harvested organ. The perfusate had the following composition: NaCl, 118 mmol/l; $NaHCO_3$, 25 mmol/l; KCl, 4.8 mmol/l; $CaCl_2$, 1.8 mmol/l; Mg_2SO_4, 1.2 mmol/l; KH_2PO_4, 1.2 mmol/l; and 10 mM glucose. A dual-headed peristaltic pump controlled the rate of perfusion of mKH buffer. The left atrial appendage was incised, and the pulmonary veins were ligated. A small incision was made at the bifurcation of pulmonary arteries; thus all coronary effluent was collected by the pulmonary artery. Next, the circulation was switched to anterograde perfusion, in order to set the baseline parameters in working heart mode for 20 minutes.

The following parameters were recorded and resulting data analyzed using a pressure transducer attached to the aortic outflow line: aortic pressure (AoP), heart rate (beats/min), left ventricular developed pressure (LVDP). Aortic flow (AF, ml/min) and coronary flow (CF, ml/min) were measured by using flowmeter. In *HC study*, hearts were then subjected to 30 minutes of global ischemia, then perfused for 15 minutes in Langendorff mode and converted to working heart mode for 105 minutes. The above-listed outcomes were measured and recorded during the reperfusion at the 30-, 60-, 90-, and 120-minute timepoints. Immediately following 120 minutes of reperfusion, small myocardial biopsies from LV heart tissue were removed and frozen for subsequent molecular biological analysis.

2.6. Histological Analysis

Lipid staining was carried out with Oil red O (Sigma Diagnostics, St. Louis, MO, USA) by use of the following protocol: aortic tissues were frozen in OCT medium (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cryostate tissue sections were cut to a thickness of 6.0 μm and applied to Superfrost

Plus slides (Daiggers, Vernon Hills, IL, USA). Atherosclerotic lesions in the aortic root were examined at 3 locations and each separated by 120μm. 4 to 5 serial sections were prepared from each location, starting beyond the aortic arch. The sections were stained, as described previously, with Oil red O, followed by analysis of the lipid composition of the lesions, by calculating the percentage of Oil red O positive area, relative to the total cross-sectional vessel wall area. Nuclei were counterstained with hematoxylin (Sigma Diagnostics), using routine methods. All images were captured with a binocular light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a video camera and analyzed using Scion Image software (Scion Corp., Torrance, CA, USA).

Following isolated working heart procedure, hearts from the rats were dissected transversely at mid-LV level and samples were fixed for 24 h in 4% neutral buffered formalin (pH = 7.4) and further prepared for paraffin sectioning. From the formalin-fixed, paraffin-embedded (FFPE) blocks, 7 μm thick sections were created and stained with hematoxylin-eosin (HE) to visualize tissue architecture. RV hypertrophy (RHV) was assesed on a whole transverse section of the heart and was described as the ratio RV/(LV + interventricular septum (IVS)).

The formalin-fixed, paraffin-embedded left lung sections (7 μm) were stained with HE, and Elastica van Gieson (EVG) for morphometric analysis of vascular dimensions. The external diameters of small pulmonary arteries were measured along the shortest curvature. The relative medial thickness of muscular arteries and lumen-carrying capacity (ranging in size from <50 μm in external diameter) was calculated with the formula ((external diameter − internal diameter)/external diameter × 100) (% MWT = (M1 + M2)/ED × 100) in EVG-stained slides. Fifteen arteriole images per lung section were examined by light microscopy using a Leica DM2500 microscope with DFC 420 camera and Leica Application Suite V3 software (Leica Camera AG,

Solms, Germany). The microscope slides were analyzed in a blinded fashion and both the size of the lumen and vessel wall thicknesses were calculated using Scion for Windows Densitometry Image program Version Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

2.7. Western blot

Deep-frozen rat LV and lung tissue samples were homogenized and then boiled in sample buffer for 10 min. The adjusted final protein concentration was 25 μg mL−1. BCA reagent and BSA standard were used for protein quantification assay. The separating gel consisted of 30% glycerol, 6% acrylamide-bis, 0.2 mM Tris, 4 mM Ethylenediaminetetraacetic acid (EDTA) and 0.4% Sodium dodecyl sulfate (SDS) and the polymerization of the gels was induced by ammonium persulfate (10 m/m%) and tetramethylethylenediamine. The upper running buffer was composed of 0.1 mM Tris, 150 mM glycine and 0.1% SDS, and gel was composed of 50 mM Tris, 75 mM glycine and 0.05% SDS. Constant voltage (70 V) was used for separation. A prestained molecular weight standard (ProSieve QuadColor, Lonza; Rockland, MA, USA) was used as loading marker. Separated proteins in the gels were electrophoretically transferred onto nitrocellulose membrane at 380 mA for 45 min. The blotted membrane was blocked with 5% low-fat milk in Tris-buffered saline (TBS) containing 1% Tween 20 (TBS-T buffer). After washing the membrane with TBS-T, primary PDE5A, heme-oxygenase 1 (HO-1), superoxide-dismutase 1 (SOD1), vascular endothelial growth factor A (VEGF), cytochrome c oxidase III (COXIII), cytochrome c oxidase IV (COXIV) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Sigma-Aldrich Co., St. Louis, MO, USA) diluted in TBS-T with 1% low-fat milk were added and incubated overnight at 4 °C. All antibodies were indicated to Western blotting, and were used in dilutions suggested by the manufacturers. The bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit Ig secondary

antibody followed by ECL detection (Western Lightning Plus ECL, PerkinElmer; Waltham, MA, USA) and the signals were recorded by an imaging system (MF-Chemibis 3.2, Central European Biosystems; Budapest, Hungary). Quantitative analysis of scanned blots was carried out using the Scion for Windows Densitometry Image program Version Alpha 4.0.3.2 (Scion Corporation). Signal intensity for bands corresponding to each protein of interest was estimated and reported in arbitrary units ± SEM.

2.8. Statistical Analysis

All data are presented as the average magnitudes of each outcome in a group ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni post-testing (when normality test was passed) or by Kruskal–Wallis test with Dunn's post-testing (if the normality test was not passed). Probability values (p) less than 0.05 were considered statistically significant.

3. Results

3.1. Bioanalytics

Alliin (S-Allyl-L-cysteine sulfoxide) is a nonprotein amino acid abundant in most of the Allium species. It is the natural substrate of alliinase. Therefore its content in the pure form is commonly analyzed by HPLC. The percentage of total alliin was analyzed by HPLC. Analysis of a representative lyophilized sample revealed the leaf to contain 0.261% alliin by weight (RSD% = 0.450%, data not shown). Comparison of peaks obtained by analysis of representative samples of known alliin content with material used in the present study allowed estimation of this compound as described above.

The quasi molecular ions cationized by potassium and sodium were observed. It can be assumed that, based on the the mass of [M + K]+ or [M + Na]+ peaks, the following compounds may be present in the plant: kaempferol-3-O-rutinoside (at m/z 617.4 [M + Na]+ and m/z 633.3 [M + K]+); quercitrin (at

m/z 471.2 [M + Na]+ and m/z 487.2 [M + K]+); juglanin at m/z 441.4 [M + Na]+ and m/z 457.3 [M + K]+; dracorubin (at m/z 511.2 [M + Na]+ and m/z 527.2 [M + K]+); and blumeatin (at m/z 325.2 [M + Na]+, m/z 341.2 [M + K]+ and m/z 303.1 [M + H]+).

Calculations were made on the basis of molar mass of the K and Na adducts. The exact molar mass of each compound was calculated as follows: measured m/z minus the molar mass of the adducted ion equals the molar mass of the compound.

3.2. Transthoracic Echocardiography (TTE)

All echocardiographic examinations were completed within a 20-minute time interval.

HC modell

Left ventricular end-systolic diameter (LVESD) of the left ventricle measured in M-mode, exhibited significant increases in HC animals. Nevertheless, no change in this outcome was observed in HCT animals in comparison with this parameter in the Control group.

Fractional shortening (FS) and ejection fraction (EF) data correlated strongly with measurements of both the parasternal long and short axis views. FS and EF of HC animals were significantly decreased in comparison with this outcome evaluated in animals in the control group. Additionally, significant increases in fractional shortening were observed in in the WGLL-treated (HCT) group in comparison with the HC group. Diastolic function of the left ventricle was evaluated by E/A ratios measured in Doppler (PW) mode. E/A ratios were significantly lower in the HC group in comparison to the Control animals. These results notwithstanding, no significant changes were observed in the E/A ratios of treated animals in comparison to Controls. Deceleration time of the E wave (DecT) exhibited significant lengthening in the HC animals. However, DecT values of WGLL-treated animals were significantly lower compared to the HC rabbits. Tissue velocity imaging (TDI) revealed a

non-significant trend toward decreased lateral E'/A' ratios in WGLL-treated animals. Surprisingly, right ventricle function characterized by measuring peak systolic velocity (S') waves and tricuspidal annular plane systolic excursion (TAPSE) exhibited significant improvement in WGLL-treated animals. Amplitudes of S' waves were significantly increased in WGLL-treated animals, compared to the HC group, and TAPSE values were also significantly elevated in the WGLL-treated HCT animals compared to the HC rabbits. Additionally, right ventricle E'/A' ratios of WGLL-treated animals were slightly decreased.

PAH modell

Fractional Shortening (FS) and Ejection Fraction (EF) values did not show any changes among groups. Systolic function estimated by measuring mitral annular plane systolic excursions (MAPSE) showed no changes among treatment groups, a result also observed for Heart rate (HR). Estimation of right ventricle systolic function was attainable by measuring TAPSE (tricuspid annular plane systolic excursion) values. TAPSE parameters of Control animals remained at normal range, but significant decreases were found in values of PAH group animals compared to the Controls. Values of WGLL-treated and sildenafil-treated animals were elevated in comparison to PAH group.

Diastolic function of the left ventricle was estimated using Doppler (PW) techniques, by determining E/A ratios at the mitral valve. E/A ratios were unaffected either by monocrotaline-injection, or by sildenafil- or WGLL-treatment after 8 weeks.

3.3. Isolated working heart model

HC modell

Cardiac functions evaluated included: aortic flow (AF), coronary flow (CF), aortic pressure (Aop), heart rate (HR), cardiac output (CO) and stroke volume (SV). Measurement of these functions in animals in the HC and HCT groups

revealed decreases in AF, HR, and CO for basal functions of the perfused hearts, compared to Controls ($P<0.05$). There were significant increase under preischemia AoP, both in hypercholesterolemic and WGLL treated hypercholesterolemic groups, compared to the Control group ($P<0.05$). After 60 minutes of reperfusion, animals in all groups showed decreases in AF, CF, HR, and CO compared with preischemic values. Significant increases in recovery of AF and HR were observed in the WGLL-treated group, compared with the other groups ($P<0.05$). Subsequent correlation with results of echocardiographic measurements further supported the cardioprotective capacity of WGLL.

PAH modell

Monocrotaline treatment produced reduction in aortic flow (AF) compared to the Control group, and aortic flow of animals after WGLL treatment reached the Control values. Coronary flow (CF) and Aortic pressure (AoP) were observed to be unaffected by the treatments ($p < 0.05$). Heart rate of isolated hearts increased in WGLL-treated animals in comparison to Control group.

3.4. Western blot

HC modell

The outcomes of treatments administered to rabbits in these experiments revealed that expression of HO-1 protein was significantly greater in tissue harvested from HCT animals, compared to the levels observed in the HC group ($P<0.05$). Tissue expression of SOD-1 in the HC group was observed to be significantly higher compared to Control and HCT animals ($P<0.05$). COXIII and VEGF proteins were expressed at lower levels both in HC and HCT groups versus quantities of these proteins found in hearts harvested from the Control animals ($P<0.05$).

PAH modell

When measured in cardiac tissues, expression of PDE5A in PAH and WGLL

groups was elevated compared to levels detected in Sildenafil and Control groups. Additionally, sildenafil treatment showed marked inhibition of PDE5A expression in RV tissue. There were no changes observed in values of WGLL and PAH groups from right vetricle homogenates. In contrast, PDE5 expression levels in MCT-treated lung tissues showed no differences when compared to Control group, however, a significant increase was observed in WGLL in comparison to PAH group.

3.5. Histology

HC modell

No atherosclerotic lesions were observed in sections of these blood vessels harvested from the Control animals fed normal rabbit chow. At the end of the 8 week-treatment period, up to 35% of the total aortic area harvested from HC animals was Oil red O positive. The extent of atherosclerotic lesions observed in animals within the HC group was significantly increased in comparison to lesional coverage in aortic sections taken from animals in the Control group ($P<0.05$). Discrete lesion formation was visualised by oil red O stain and consecutive quantitative analysis in aortas form HCT group rabbits. Aortas harvested from WGLL-treated animals in HCT group, exhibited significantly reduced atherosclerotic lesional coverage in comparison with the HC group.

PAH modell

Right and left ventricle (RV + LV) sections stained with HE showed clear signs of tissue damage in all MCT-treated groups (PAH, WGLL, Sildenafil groups), whereas the Control group is morphologically normal. Tissue damage in PAH group samples is characterized by contractures of cardiomyocites, hyperbasophilia, hypereosinophilia, wavy arrangement of myofibers, cell edema, lesion and apoptosis of cardiomyocytes. Samples of PAH group show the most dramatic RV hypertrophy in myocardial structure, meanwhile only moderate macroscopic hypertrophy is visible in WGLL-treated RV tissues compared to PAH group. Right vetricular hypertrophy (RVH) was presented

as RV/(LV + S) ratio. RV/(LV + S) ratio was increased in PAH group compared to Control and WGLL groups. Medial wall thickness percentage (MWT%) was determined in pulmonary arteries/arterioles (<50 μm diameter) of each group by using the equation: MWT% = (M1 + M2)/ED × 100. Medial wall thickness increased in PAH animals when compared to all other groups. We observed no differences in values of WGLL- and Sildenafil-treated animals compared to the Control group.

3.6. Serological Correlates of Experimental Treatments

Fasting plasma TC and LDL cholesterol were two orders of magnitude higher, and HDL cholesterol concentration was 8-fold higher, in HC and 6-fold higher in HCT groups, compared to levels of these analytes in the serum of the Control group animals ($P<0.05$). However, significantly lower plasma TC and LDL cholesterol levels were observed in HCT, versus the HC groups ($P<0.05$), showing a possible protective effect of the WGLL. Moreover, ApoA levels detected in blood of HC animals were significantly lower, versus those of the Control rabbits fed diets with normal cholesterol content and WGLL-treated rabbits in the HCT group ($P<0.05$). No significant differences were noted between serum ApoA content of serum from animals in the Control group, versus HCT groups ($P<0.05$). Serum levels of ApoB in rabbits from both HCT and HC groups were significantly higher as compared to the Control group ($P<0.05$). Moreover, ApoB levels detected in the serum of rabbits in the HCT group were significantly lower compared with the content of this analyte in the HC group. Additionally, no significant differences were observed between the serum TG content of these three groups. It was further noted that the serum content of the pro-inflammatory biomarker c-reactive protein (CRP), was increased significantly in blood from HC animals, compared to the Control group. GOT liver enzyme levels were elevated in blood of HC group animals, as compared to the Control and HCT groups. LDH and CK serum levels were significantly decreased in the HCT group

compared to the HC group ($P < 0.05$).

4. Discussion

One accomplishment of this present study is characterizing the flavonoid content of Allium ursinum leaves, liophylised by the method used for our standard investigations, and comparing it to data available in literature, where chemical constituents were determined from fresh leaves. Our results are well-correlated with previous findings, since the dry liophilysate contains several phenolic compounds, i.e., kaempherol derivatives, juglanin, dracorubin, blumetain and quercitrin; thus we successfully demonstrated that Allium ursinum is exceptionally rich in flavonoid compounds. These outcomes have high value, because if a natural product is intended to be used for therapeutic purposes according to evidence-based medicine, investigators need to be aware of its main components and their chemical structure. Furthermore, many pharmacological studies have confirmed the fact that these polyphenols have high potential in prevention and treatment of several disorders.

As shows the bioanalytical analysis of a representative WGLL sample revealed the leaf to contain 0.261% alliin by weight, a property of this material that contributes to its ability to mitigate expression of other biomolecules described here, which are involved in the atherosclerotic pathophysiologic processes. This natural component of fresh garlic is a sulfoxide derived and forms from the amino acid cysteine, and is a major contributor to the capacity of garlic extracts to scavenge hydroxyl radicals, along with a wide variety of other antioxidant properties that counteract oxidative tissue damage. Alliin has also been demonstrated to potently stabilize and strengthen immunoregulation, contributing to well-known curative properties of garlic.

The outcomes of echocardiographic analyses conducted on live animals reveal the effect of elevated dietary cholesterol and WGLL treatment. The physiologic significance of these results may be stated according to 4 major

interpretations of the data shown. These may be summarized as follows:

(1) The observed stability throughout the evaluation period of heart rates, respiratory frequencies, M-mode and Doppler measurements demonstrate that basal cardiopulmonary activity was not substantially disrupted by hypercholesteremia, an outcome for which preliminary evidence was provided by an earlier study conducted in the laboratory of the authors.

(2) Moreover, in comparison to untreated control rabbits fed a normal diet, left ventricular end-systolic diameter (LVESD) measured in HC animals was significantly increased, with or without WGLL treatment, along with decreased fractional shortening and ejection fraction in HC (ii) animals – and a strong correlation was found between fractional shortening (FS) and ejection fraction (EF) data measured on both the parasternal long and short axis views. Also, the effects of WGLL treatment included observations that, relative to HC animals not receiving the lyophilisate, HCT rabbits showed significant increases in fractional shortening. Pathologically increased ESD is associated with greater risk of mortality in heart disease, and decreased FS and ES have recently been implicated as contributors a to fibrotic disease.

(3) Diastolic function datas, generated in Doppler (PW) mode also reveals that elevated dietary cholesterol resulted in significantly lower left ventricular E/A ratios relative to those observed in control animals. WGLL-treated animals showed values close to Controls. Moreover, significant lengthening was observed in deceleration time of the E wave (DecT) in HC animals, showing an abnormal diastolic filling pattern of the ventricle, which was counteracted by WGLL-supplemented diet, indicates slightly improved diastolic function.

(4) Finally, surprisingly significant increases were shown in right ventricular function mediated by WGLL treatment of animals fed elevated cholesterol diets, which were obtained through evaluation of peak systolic velocity (S') waves and tricuspidal annular plane systolic excursion (TAPSE).

Reduction of peak systolic velocity identifies the presence of RV dysfunction with high sensitivity. This reduction was significant in HC animals but was counteracted fully by WGLL treatment, showing that the aforementioned beneficial effects of WGLL supplementation can be seen on right ventricle function as well. In heart failure patients, the reduction of tricuspidal annular systolic velocity is associated with the severity of RV dysfunction.

Echocardiographic data revealed that after 8 weeks of experiment, deterioration was not observed in left ventricular diastolic or systolic functions of pulmonary hypertensive or either WGLL- or sildenafil-treated animals. Tricuspid annular plane systolic excursion (TAPSE) correlates well with right ventricular dysfunction in heart failure patients, and can be easily determined by using M-mode echocardiography. TAPSE parameters were reduced in pulmonary hypertensive rats, but improved after 8 weeks of WGLL treatment. Our findings support the results of other experiments; for instance, Rajkumar et al. showed very similar TAPSE values of MCT-induced PAH rats (around 1.6 mm), as well as healthy animals (above 2 mm). According to our former and recent findings, Allium ursinum liophylisate treatment improves right ventricular function in hypercholesterolemic rabbits, as well as in a rat model of MCT-induced pulmonary hypertension. A limitation of this current study is that we failed to consequently visualize pulmonary artery and measure parameters such as pulmonary arterial pressure, acceleration time and pulmonary ejection time by echocardiography, as hallmarks of PAH, formerly described by Thibault et al.

Evaluation of cardiac functions in Langendorff-mounted isolated working hearts revealed significantly increased preischemic AoP values in both the hypercholesterolemic and WGLL-treated hypercholesterolemic groups, relative to untreated Control rabbits. Moreover, ischemic-reperfusion injury-associated decreases in AF, CF, HR, and CO, versus preischemic values, along with significantly increased recovery of AF and HR in animals fed the

lyophilisate, were observed. These effects are consistent with previous studies by the authors, in which interventions that decrease oxidative stress on cardiac tissue, dramatically improved recovery from ischemic events. An interpretation of the significance of these outcomes to the cardioprotective ability of WGLL should be considered in the context of the fact that myocardial ischemic events typically reduce cardiac aortic blood pressure (AoP), along with reduction in myocardial metabolic requirements, coronary blood flow, and left ventricular tension. For these reasons, influences that decrease AoP may be either detrimental or beneficial. Thus, whereas increased preischemic AoP in HC animals indicates that such an increase is pathological for animals maintained on a high cholesterol diet, the failure of WGLL treatment to lower AoP suggests that the extract has negligible effect on this aspect of cardiovascular function.

Isolated heart outcomes of the present study revealed that monocrotaline treatment decreases AF, which characterizes the pump function of the left ventricle. This finding further supports several former outcomes of other authors, where monocrotaline induced PAH, and the consequential right-heart failure eventually leads to left ventricle failure as well. According to our results, sildenafil increased CO when compared to PAH group. Consistent with this, many articles in the scientific literature confirm this finding and verify that sildenafil exerts significant protection in monocrotaline-induced PAH. Comparably, treatment with WGLL also exerted protection against monocrotaline-induced changes: Allium ursinum improved AF and CO compared to the PAH group. Nonetheless, an increase in CO was observed in the WGLL group compared to PAH group; neither HR and non SV parameters changed significantly, due to the power of detection.

Western blot analysis of myocardial tissue, reveals significantly elevated content of HO-1 protein in tissue harvested from HCT animals, versus that taken from rabbits in the HC group. The heat shock protein HO-1 (hsp-32) is a

major antioxidant defense enzyme, which is increased in response to trauma intrinsic to a wide range of diseases, including (and especially) atherosclerotic syndromes. Often, the effects of a disease process overwhelm the protective capacity afforded by endogenous HO-1 expression. However, its cardioprotective effects may be greatly amplified by administration of pharmacological inducers, as demonstrated by the authors of the present report. A major effect of increased levels of HO-1 is attributable, in large part, to the degradation of heme from denatured heme protein to bilirubin/biliverdin, iron, and carbon monoxide. HO-1-derived bilirubin has also been shown to display cytoprotective properties in the cardiovascular system.

SOD1 levels measured by Western blot analysis in myocardial tissue of HC animals after ischaemia/reperfusion injury were significantly elevated compared to the Controls, while SOD1 expression in WGLL treated animals was maintained at the normal (Control) levels. The SOD1 enzyme is an important constituent in apoptotic signaling and oxidative stress, most notably, SOD1 is pivotal in reactive oxygen species (ROS) release during oxidative stress by ischemia-reperfusion injury, specifically in the myocardium as part of a heart attack (also known as ischemic heart disease). During ischemia reperfusion, ROS release substantially contributes to the cell damage and death via a direct effect on the cell, as well as via apoptotic signals. SOD1 is known to have a capacity to limit the detrimental effects of ROS by eliminate O2- to produce H2O2 which is eliminated by Glutathione peroxidase or by Catalase to harmless H2O and O2, but on the other hand, with free iron(II) H2O2 also can form free hydroxyl radicals by Fenton’s reaction. High SOD levels along with considerable amounts of Fe2+ are associated with increased production of the highly toxic hydroxyl radical, and may even enhance the extent of reperfusion injury. An unbalance between the production of prooxidant H2O2 (SOD1) and antioxidants, such as Glutathion peroxidase and

Catalase in the cell might lead to a strengthened production of free radicals which could lead to serious cellular damage. This is supported by the fact that SOD had previously been observed to increase in MI patients in comparison with healthy controls. The high level of SOD enzyme associated with poor prognosis is probably a defensive reaction associated with an extensive myocardial damage and a high level of oxidative stress.

Western blot analyses conducted in this investigation, revealed that COXIII and VEGF, which are both implicated in the pathophysiology of cardiovascular syndromes were expressed at lower levels both in HC and HCT groups, versus quantities of these proteins found in hearts harvested from the Control animals. In COXIII and VEGF protein levels, there were no significant changes between WGLL-treated and hypercholesterolemic groups. Our results suggest that wild garlic may develop its cardioprotective activity via heme/HO system, and has no effect on COXIII and VEGF proteins.

Pulmonary vascular remodeling is the first and critical component of PAH pathogenesis, with significant involvement of NO/cGMP system. Isoforms of phosphodiesterase enzyme are responsible for hydrolysis of cGMP in pulmonary vasculature. It was previously demonstrated that sildenafil promotes accumulation of cGMP, thus prolonging effects of NO via inhibition of PDE5 enzyme. This study confirms the fact that sildenafil inhibits the elevation of PDE5A expression in right ventricle induced by the disease, affirming previous reports. Upregulation of PDE5 has been reported in conditions like congestive heart failure, pulmonary hypertension and even right ventricular hypertrophy, perhaps as a countering mechanism. Our Western blot findings also verify that in monocrotaline-induced PAH model, expression of PDE5A is highly elevated in the right ventricle. WGLL treatment did not affect raised PDE5A levels in the right myocardial tissues, and possibly exerts its protective effects via other signalling pathways. According to our knowledge, this result is unique, since no other research

team has studied the impact of Allium ursinum extracts on the expression of PDE5 enzyme. Furthermore, levels of PDE5 in lung tissues were elevated after WGLL treatment when compared to the PAH group. Although PDE5A protein levels did not differ between PAH and Control group when measured in lung tissues, this finding does not exclude that the activity of the enzyme may be elevated without a simultaneous rise in its expression. This observation is consistent with previous works, demonstrating that only the phosphorylated form of PDE5 indicates an increase in PDE activity. Incidentally, our investigation was limited to the measurement of only the unphosphorylated PDE5 protein levels, therefore, we did not observe any changes in values of Sildenafil-treated group compared to Control group, as reported by others. Although it is well published that elevated levels of PDE5 enzyme are associated with underlying pathological processes of the disease, we observed no significant deterioration in cardiac functions nor histological features of WGLL-treated animals when compared to the PAH group, which can be partly explained by the fact that many other signaling pathways are involved in molecular pathogenesis of pulmonary arterial hypertension.

The extent of atherosclerotic plaque coverage on the intimal surface of hematoxylin-Oil red O-stained rabbit aortas reveals negligible plaque on sections harvested from Control animals maintained on diets with normal cholesterol content, and lesional extent of approximately 35% in sections from HC rabbits. The significantly reduced lesional coverage observed in WGLL-treated rabbits fed high cholesterol chow (HCT) is an effect also observed in previous work by these authors, in which HO-1 expression increased by adding sour cherry seed kernel extract to rabbit chow. This protected against dietary cholesterol-induced arterial plaque formation.

In this work, we demonstrated that MCT-induced pulmonary arterial hypertension caused RV hypertrophy in animal models, a finding published in previous studies as well. Sildenafil has also been reported to attenuate mean

pulmonary artery pressure (PAP), pulmonary vascular resistance (PVR), and enhance RV functions by inducing changes in gene expression in heart and lung tissues. Our results confirm these findings; furthermore, as reported in microscopic morphometrical findings, WGLL supplementation demonstrates comparable effects to Sildenafil in alleviating symptoms of pulmonary arterial hypertension.

The blood of animals utilized in the present study was evaluated for serum analytes expressed at levels which may be used as diagnostic and therapy effect indicators for cardiovascular disease severity along a wide range of other severe inflammatory syndromes. They reveal significantly elevated fasting plasma levels of TC and LDL cholesterol, which were two orders of magnitude higher, and HDL cholesterol concentration, which was 8-fold higher, in HC and 6-fold higher HCT rabbits versus the Controls, effects that are an expected result of high cholesterol diets. However, elevated levels of HDL cholesterol in WGLL-treated rabbits may indicate a cardioprotective property of the lyophilisate in the context of beneficial effects of HDL. Significantly lower TC and LDL cholesterol levels were observed in WGLL-treated (HCT) animals versus groups fed with high cholesterol chow but no WGLL (HC), demonstrating that the lyophilisate is protective with respect to influence of these analytes. ApoA levels in blood of HC animals were significantly lower versus rabbits fed normal chow. Thus, the lack of significant differences in ApoA content of blood from animals fed normal diets versus content of this molecule in WGLL-treated rabbits maintained on high cholesterol (HCT) indicated a normalizing effect of the lyophilisate on this outcome. The significance of this result is that ApoA-I deficiency causes both hypertriglyceridemia and increased atherosclerosis in animal models, which can be counteracted by WGLL-supported diet.

Analysis of ApoB revealed that systemic levels of this analyte in rabbits from both HCT and HC groups were significantly higher versus its levels in blood

of animals fed chow with normal cholesterol content. Moreover, ApoB levels detected in blood taken from rabbits in the HCT group were significantly lower in comparison to content of this analyte in the HC group. This result is well correlated with LDL levels measured in the two groups. This finding was expected since ApoB is the primary apolipoprotein of chylomicrons, VLDL, IDL, and LDL particles.

Elevation in serum TG of the HC group was tendentious but not significant compared to that of the Controls. One interpretation is that triglyceride metabolism is unaffected by either influence within the constraints of the present study. Further analysis of blood from each of the three test groups revealed significant elevation of c-reactive protein (CRP) in HC animals versus the Controls. Since CRP is a reliable systemic indicator of a wide range of inflammatory pathologies, this result implies that levels of dietary cholesterol administered to animals in this study managed to induce onset of inflammatory processes. Analysis for serum liver enzyme activities demonstrated significantly elevated GOT in blood of HC group animals versus the Control and HCT groups, suggesting a hepatotoxic effect of elevated dietary cholesterol intake, an effect noted by previous investigators. Finally, the significantly lower levels of LDH and CK observed in HCT animals, versus group rabbits maintained on high cholesterol (HC), indicated that effects of dietary supplementation with WGLL may have beneficial effects on impaired liver function caused by hypercholesteroleamic state. Alliin (aka S-Allyl-L-Cysteine Sulfoxide) treatment effectively reduced TNFa-induced ICAM-1 mRNA transcription, associated with inhibiting monocyte adhesion to HUVECs and porcine coronary arterial tissues in vitro. Furthermore, Alliin protected cells from TNF-a-induced depolarization of mitochondrial membrane potential and overproduction of superoxide anion, which may be associated with the inhibition of NOX4 mRNA transcription in HUVECs. In addition, Alliin treatment mitigated the TNF-a-induced phosporylation of

JNK, ERK1/2 and IjB, but not p38, in HUVECs. Therefore, these datas may provide new insights into understanding the mechanism(s) underlying the action of Alliin in inhibiting the pathogenic process of atherosclerosis.

Despite the fact that Allium sativum (cultivated garlic) is much more characterized and described in scientific literature, along with previous studies, our recent work suggests that Allium ursinum is also a valuable herbal product. Moreover, there are a substantial number of comparative reports in which Allium ursinum exerts even more beneficial effects on disease models compared to Allium sativum. Last, but not least, Wild garlic is a readily accessible and affordable plant, distributed widely both in Europe and Asia.

5. Summary

Outcomes of the present report demonstrate that wild garlic leaf lyophilisate improves cardiac functions in hypercholesterolaemic rabbit hearts after ischemia/reperfusion injury and in PAH-induced right ventricular hypertrophy in rats alike. Our studies confirm the relevance of both experimental animal models with deteriorated cardiac functions published by former investigators. To our knowledge, this is the first study to evaluate the possible cardioprotective benefits of Allium ursinum in a model of PAH, and directly compare it with sildenafil, a well-known PDE inhibitor. It is worth mentioning that WGLL supplementation exerts protection in right ventricle as it was shown by significant changes in the value of echocardiography-derived TAPSE in both models. Our research revealed that WGLL supplementation elevate the activity of HO-1-mediated cardioprotective pathway in hypercholesterolemic models. Nevertheless, the exact underlying molecular mechanism of how WGLL exerts protection against MCT-induced PAH requires further investigations. Overall, our hypothesis that supplementation with WGLL, a low-cost and reasonable natural product, suppresses the harmful effect of various factors affecting the cardiovascular system has partly

been confirmed experimentally. Undoubtedly, our findings also indicate that it would be impressive to evaluate the synergistic actions of Sildenafil and WGLL supplementation in the treatment of pulmonary arterial hypertension. Moreover, based on the same principles, we would like to examine the combination therapy of conservative anti-hyperlipidemic drugs with WGLL supplementation as well. Finally, it is among our future plans to isolate and separate the chemical components of the wild garlic leaf lyophilisate and also analyze it in in vitro and in vivo circumstances.

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List of publications related to the dissertation

1. Bombicz, M., Priksz, D., Varga, B., Kurucz, A., Kertész, A. B., Takács, Á., Pósa, A., Kiss, R., Szilvássy, Z., Juhász, B.: A Novel Therapeutic Approach in the Treatment of Pulmonary Arterial Hypertension: allium ursinum Liophylisate Alleviates Symptoms Comparably to Sildenafil.
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