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

Conventional drug therapies from new perspective in the cardiomyocyte

by Kitti Szőke

Supervisor: Istvan Lekli PharmD, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

DEBRECEN, 2022

Conventional drug therapies from new perspective in the cardiomyocyte

By Kitti Szőke, PharmD

Supervisor: Istvan Lekli PharmD, PhD

Doctoral School of Pharmaceutical Sciences, Pharmacology Program, University of Debrecen

Head of the **Defense Committee**:	Lajos Gergely, PhD, DSc
Reviewers:	Anikó Pósa, PhD
	Attila Kertész, PhD
Members of the Defense Committee:	Zoltán Csiki, PhD
	Tamás Bálint Csont, PhD

The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostic Building, University of Debrecen; 1 p. m. June 22, 2022

Table of Contents

1. Introduction and aims

Mortality rates from cardiovascular diseases (CVDs) show remarkable improvement due to modern successful drug therapy and interventional therapies, although nowadays CVDs are still the leading cause for death. According to the World Health Organization (WHO) in 2019 an estimated 17.9 million people died from CVDs, which is 32 % of all global death. Out of these deaths, 85% were due to stroke and heart attack. According to Hungarian Central Statistical Office hypertension is the leading health problem among adults aged 18 and over. The prevalence of hypertension increased with increasing age. Ischemic heart disease is one of the most common diseases that affect the circulatory system include ischemic heart disease.

During ischemic heart disease an imbalance occur in the supply and demand of myocardial oxygen, which leads to contraction and relaxation abnormalities, increase preload and altered diastolic volume, and diastolic blood pressure. During ischemia dramatic changes occur also in cardiac energy metabolism. The only way to rescue ischemic myocardium from myocardial infarction is the quick reperfusion in time. However, reperfusion not only salvages ischemic myocardium from infarction but also induces a specific additional component of reversible and irreversible injuries.

Reactive oxygen species (ROS) is a phrase to describe atoms or molecules which has unpaired electrons. Under physiological conditions, ROS play an important role in the normal redox signaling. However, under pathophysiological conditions, increased levels of ROS lead to dysfunctions and remodeling, through oxidative damage. Antioxidant defense system try to eliminate the increased level of ROS. The endogenous defensive mechanism consists of different enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). During reperfusion the ischemic myocardial goes through several changes, such as intracellular Ca^{2+} overload, restoration of physiologic pH, inflammation and increased production of ROS.

Heme oxygenases (HO) are evolutionary conservated enzymes, which catalyze the rate-limiting step in heme degradation converting heme to biliverdin. The heme catabolism's end products are biliverdin, carbon monoxide (CO) and ferrous iron. Then biliverdin is reduced to bilirubin by the biliverdin reductase enzyme. Besides endogenous heme, HO-1 expression can be induced by several stimuli such as ischemia, glutathione depletion, hypoxia, endotoxins, heavy metals, ultraviolet radiation, hyperthermia, sulfhydryl agents, and nitrogen monoxide.

Thereby HO-1 and their products play a beneficial role in the protection against oxidative injury and have important antioxidant, anti-inflammatory, antiapoptotic, antiproliferative, and immunomodulatory effects. HO-1 induction plays a significant role in cardioprotection evidenced by several studies.

Allium sativum, commonly known raw garlic, has a long history as a spice and medical plant. It is mentioned in ancient literature from different parts of the Word including Egypt, India, and Greece. Health benefits of garlic has known for decades, which is confirmed by scientific literature. Recent evidence suggests that a processed form of garlic, called aged black garlic, also possesses beneficial to health properties including antidiabetic and antiatherogenic effects. Black garlic is obtained from fresh garlic that has been fermented. During the aging process the offensive and harsh odor fresh garlic receives new properties, such as the taste change to very mild with sweet and sour tones, the texture turns to jelly-like and the color of garlic cloves change from white to black due to the Maillard reaction. The Maillard reaction is a non-enzymatic process that occurs between reducing sugars and the amino group found in amino acids, peptides, and proteins. Black garlic has enhanced biological activities, especially antioxidant activity. The heating process denaturates alliicinase enzyme, which is responsible for the harsh odor of fresh garlic. The major active components of garlic are near 33 organosulfur compounds, such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), E/Z-ajoene, S-allyl-cysteine (SAC), and S-allyl-cysteine sulfoxide (alliin). Furthermore, it contains enzymes (like allinase, peroxidase, myrosinase). Allicin is not found in fresh garlic, but it is formed when raw garlic is crushed or chopped by alliinase enzyme

Garlic and aged black garlic have been documented to have several biological impacts on health promotion. Garlic has been used for centuries to combat infectious disease due to its antibacterial activity. It also exhibits antioxidant and anti-inflammatory effect, by scavenging ROS, enhancing the cellular antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase. Furthermore, garlic has been widely recognized as agents for prevention and treatment of cardiovascular diseases as it can reduce blood pressure and total cholesterol. The mechanism of the antihypertensive effect of garlic is inhibiting the angiotensin converting enzyme and increasing the production of NO and hydrogen sulfide (H_2S). Garlic can stimulate the production of NO and arginine from garlic also crucial in the NOS-mediated NO production. Large number of pharmacological studies found that garlic can lower blood lipids and inhibit platelet aggregation.

Basic therapy of ischemic heart disease are the antiplatelet and antithrombotic treatment. Acetylsalicylic acid (ASA), also known as aspirin - the oldest nonsteroidal anti-inflammatory drug (NSAID) - is extensively used for the treatment of pain and inflammation and because of its antithrombotic properties, it is also commonly used for the prophylaxis against myocardial infarction and stroke. A multicenter, randomized placebo-controlled trial, ISIS-II, verified that low dose aspirin started immediately increase survival after suspected acute myocardial infarction. According to PCI-CURE, low dose aspirin (≤100 mg) appeared to be as effective to prevent major cardiovascular events as the higher doses in preventing ischemic event. The anti-inflammatory action of ASA is based on the inhibition of cyclooxygenase (COX1 and COX2) enzymes involved in prostaglandin (PG) biosynthesis. However, ASA is a much more potent inhibitor of COX1 isoenzyme than that of COX2. Moreover, ASA irreversibly inhibits COX1 in platelets, consequently resulting in the inhibition of thromboxane A2 biosynthesis. Since thromboxane A2 is a potent platelet aggregator and causes vasoconstriction, this inhibitory process affects the antiplatelet-aggregation property of ASA. Low-dose, long-term prophylactic use of ASA is limited by its strong local irritant effects and gastro toxicity and ulcerative ability. There is an increasing number of experimental data supporting basic physiological and protective roles of nitrogen monoxide, also called nitric oxide (NO), and nitrogen monoxide-releasing molecules (NMRMs) in injured tissues. The main source of endogenous NO is nitrogen monoxide synthase (NOS). NOS/NO system was proved to play an important role in signaling mechanisms and several physiological processes, including the maintenance of neuronal, immune, and cardiovascular functions. Moreover, NO acts as a crucial signaling molecule and an effector mediator to regulate the coronary artery function in the myocardium. However, overexpression of inducible NOS and its consequence, an extensive increase in endogenous NO production may not be beneficial for the myocardium. On the other hand, molecules releasing NO including molsidomine (MOL) are used as antihypertensive and antianginal drugs. The strategy for avoiding the systemic gastrointestinal damage, ASA prodrugs bearing nitrogen monoxide (NO)–donating moiety covalently attached to the carboxylic function of ASA were designed since locally released NO is able to trigger anti-inflammatory effects. Nitrate ester, furoxan, or diazenium-diolate derivatives have been attached covalently to ASA to form ester-type prodrugs. The biological activity of these hybrid aspirins has been evaluated extensively. Thus, nitrate ester derivative NCX4016 prevented thromboembolism and restenosis and protected the heart from ischemia/reperfusion injury in animal models displaying no gastro toxicity in the stomach. Further beneficial effects include the inhibition of platelet COX1 activation and favorable influence on platelet-activation

function in healthy volunteers. Additional advantageous properties of ASA and NMRMs include anti-inflammatory and gastro sparing activities. Furthermore, various NO donors have been developed as pharmacological tools to induce the protective effect of the ischemic myocardium. The NMRMs release NO into biological systems for therapeutic purposes in a controlled and safe manner. The cardiovascular effects of NMRMs are currently under intensive investigation and various classes of compounds are being developed with the goal of exploiting therapeutic potentials in the treatment of inflammatory and cardiovascular diseases. Thus, it is quite rational to hypothesize that an NMRM bearing ASA and molsidomine may have vasoactive, and COX inhibitor activities. Moreover, molsidomine excels from NO donor compounds since during long-term treatment with it, tolerance development is not a clinically relevant problem. The colleagues of our University, Department of Pharmaceutical Chemistry, synthetized a new nitric oxide (NO) donating acetylsalicylic acid (ASA) derivative, ERJ-500. The ERj-500, ASA ester covalently attached to the NO donor linsidomine, an active metabolite of molsidomine, by a tetraethyleneglycol chain to improve the water solubility of the product and a carbamate group.

We aimed to study from a new aspect the conventional plant, garlic and aspirin. The major objective of the present study was to compare the effect of aged black garlic and raw garlic on the pre- and postischemic cardiac function, and investigate the underlying molecular mechanisms. Next, our goal was to study ERJ-500 toxic and vasodilator effects, in the highlight of coronary artery dilation in the myocardium.

2. Materials and methods

2.1. Methods I. Aged garlic and Raw Garlic against Ischemia/Reperfusion-Induced Cardiac Complications

2.1.1. Animals

Male Sprague Dawley (SD) rats with an average weight of 575 ± 45 g (n = 9, in each group) were used. Animals were nutrified with standard rodent chow pellets (R/M-Z+H, ssniff Spezialdiäten GmbH, Soest, Germany) ad libitum with free access to water and kept at an ambient temperature of 25 ± 2 °C, with a relative humidity of 55 ± 5%, and a 12-h light-dark cycle. All animals were treated according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication number 86–23, revised in 1996). Breeding and handling of the animals was approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary (May 2012; 12 March 2012).

2.1.2. Treatment protocol

Rats were randomly devided into three treatment groups: GROUP I: control rats, gavage-treated with the mucin-water vehicle (2% hydroxyethylcellulose solution). GROUP II: raw garlic-treated rats, gavaged with mucin-water, supplemented with 300 mg/kg/day dose of raw garlic. GROUP III: aged black garlic-treated rats, gavaged with mucin-water, supplemented with 300 mg/kg/day dose of aged black garlic. The dose of 300 mg/kg is approximately equal with 15–20 cloves of garlic. All animals were treated every day for a period of 4 weeks. Body mass was measured at the beginning and end of treatments.

2.1.3. Preparation of Aged Garlic

Separated and peeled raw garlic cloves were vacuum sealed in heat-resistant plastic bags. After 3 weeks incubation at 75 °C, conversion was completed and the aged black garlic cloves were used for the treatment and GC-MS analyses.

2.1.4. GC-MS Analyses

Ground raw garlic and aged black garlic cloves were placed into head space vials and were thermostated at 50 °C for 1 h. Solid phase micro-extraction was carried out by using a Supelco fiber assembly with 85 µm polyacrylate-fused silica fiber.

Chromatograms of RG and ABG were taken by a Hewlett-Packard 5890 Series II gas chromatograph-5971A mass spectrometer. Samples were injected into HP-5 stationary phase containing capillary column (25 m × 0.25 mm × 0.25 μm) where the eluent gas was 40 °C helium with 1 mL/min constant flow. Temperatures during analyses were the following: 55 °C for 2 min followed by the scan period to 200 °C with 20 °C/min heating. The overall time of one analysis was 27 min. The temperature of the injector was 200 °C and contained an unpacked liner. Transfer line temperature was 280 °C. Ionization was reached at 70 eV and 10–500 AMU weighed particles were analyzed. Operation of the GC-MS setup, data collecting, and evaluation process was carried out with Hewlett-Packard GC-MS Chemstation rev.3 software (Hewlett-Packard Company, Wilmington, DE, USA. Regarding the mass spectra, components were identified by databases of Nist98 and Wiley.

2.1.5. Isolated Working Heart Preparation and Cardiac Function Assessments

Following a 4-week treatment period, 24 h after the last treatment, rats were anesthetized with an intraperitoneal pentobarbital sodium injection (60 mg/kg), with heparin as an anticoagulant (1000 U/kg). Following the induction of deep anesthesia, chest cavities were opened, hearts were excised and placed in ice-cold modified Krebs-Henseleit bicarbonate (KHB) buffer (containing 118 mM NaCl, 5.8 mM KCl, 1.8 mM CaCl2, 25 mM NaHCO3, 0.36 mM KH2PO4, 1.2 mM MgSO4, and 5.0 mM Glucose) to prevent damage of cardiac tissue. Subsequent to excision, aortas were cannulated, and each heart was perfused with modified KHB buffer at a filling pressure of 100 cm of water, using the non-working (Langendorff) mode of the isolated working heart apparatus for 5 min to flush blood out from the hearts. During the washout period, pulmonary veins were cannulated and heart functions were assessed in working mode at a filling pressure of 17 cm of water with KHB buffer. A total of 10 min of working mode activity was sustained to stabilize the cardiac activity. Upon conclusion of 10 min of working mode perfusion, baseline cardiac parameters were registered, including heart rate (HR), aortic flow (AF), and coronary flow (CF); cardiac output (CO) and stroke volume (SV) were calculated. Next, 30 min of ischemia was induced by closing off atrial inflow and aortic outflow. Upon completion of the ischemic period, reperfusion was initiated by opening the aortic cannula. The first 10 min of reperfusions were conducted in the non-working mode to prevent the development of fatal ventricular arrhythmias. The heart was defibrillated with a square wave impulse if ventricular fibrillation was observed at the onset of the reperfusion. Following the first 10 min of Langendorff reperfusion, hearts were switched to working mode for an additional 110 min. Cardiac parameters were recorded at 30, 60 and 120 min of the

reperfusion period to monitor the postischemic recovery of the myocardium. A continuous pressure signal was recorded during the whole experiment with the help of a pressure transducer (ADInstruments, PowerLab, Castle Hill, Australia). HR and AOdP/dt were calculated from the continuously recorded pressure signal. AF was measured by a calibrated flow meter, while CF was assessed by time-collecting the coronary effluent. Cardiac output (CO) was calculated as the sum of AF and CF, while stroke volume (SV) was the ratio of CO and HR. Stroke volume alteration was calculated as a ratio of SV and the baseline of SV.

2.1.6. Infarct Size Measurements

To monitor the degree of infarction, triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich, Inc., St. Louis, MO, USA) staining was carried out. Hearts were perfused with 35 mL of 1% TTC solution via the aortic cannula at the end of the reperfusion. Following 10 min, hearts were stored at −20 °C for 24 h, to allow each heart to solidify. A total of 2–3 mm thick sections were made from the stained frozen hearts. Sections were subsequently scanned on an Epson J232D flat-bed scanner, blotted dry and weighed. The infarcted area (unstained tissue remained white) and the risk area (entire scanned section) were measured using planimetry software (Image J, National Institutes of Health, Bethesda, MD, USA). Estimates of infarcted zone magnitude were subsequently obtained by multiplying infarcted areas by the weight of each section. The resulting numbers represented the weight of the risk zone and the infarcted zone. Infarct size was expressed as a ratio of the weight of infarcted tissue and the weight of risk zone (whole heart). The entire area of each section was considered to be an infarcted risk zone, while the numerical extent of each infarcted area was planimetrically calculated and multiplied by the weight of the section. Outcomes were expressed as the ratio of the total infarcted tissue volume to volume of at-risk tissue.

2.1.7. Blood Enzymes

Following 4 weeks of treatment with different garlic preparations or mucin-water vehicle prior to sacrifice, peripheral blood was collected from a left external jugular vein of each animal. Analyses of selected serum analytes was conducted using the Cobas 8000 modular analyzer series (Roche Diagnostics GmbH, Mannheim, Germany). The samples were assayed for content of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (ASTL) total cholesterol (CHO), low-density lipoprotein cholesterol (LDL), triglycerides (TRIG), high-density lipoprotein cholesterol (HDL), lactate dehydrogenase (LDH), C-reactive protein (CRP), and creatine-kinase-myoglobin (CK-MB). Testing was conducted in the Department of Laboratory Medicine, University of Debrecen, Hungary.

2.1.8. Protein Isolation

Approximately 300 mg of heart tissue was lysed in 1 mL isolating buffer (25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail) using a polytron homogenizer. Homogenates were centrifuged at 2000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube and further centrifuged at 10,000 rpm at 4 °C for 20 min; the resultant supernatant was used as a cytosolic extract. The protein concentration was determined by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard. Samples were mixed with Laemmli buffer and boiled for 10 min.

2.1.9. Western Blot Analyses

A total of 100 µg of protein in each sample was loaded and separated on 12% SDS–PAGE gels (Sigma Aldrich, Schnelldorf, Germany) and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Following blocking the membranes with 5% of nonfat dry milk powder dissolved in tris-buffered saline buffer with 0.1% Tween 20 (TBST) for 1 h, membranes were incubated with primary antibody solution at 4 °C overnight (HO–1 1/500, Abcam, Cambridge, UK; iNOS, Cell Signaling Technology, Boston, MA, USA). The membranes were washed with TBST 3 times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody solution (1/2000, Cell Signaling Technology, Boston, MA, USA) for 1 h at room temperature. Subsequent to washing, the membranes were developed using Luminate Forte Western HRP substrate (Millipore, Billerica, MA, USA). Chemiluminescence was detected as well as band intensities were measured by ChemiDocTM Touch Imaging System and Image Lab software (Bio-Rad Inc., Hercules, CA, USA) [8] and normalized against total protein.

2.1.10. Statistical Analyses

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), followed by Kruskal–Wallis or Dunnett's multiple comparison tests with GraphPad Prism software for Windows (GraphPad Software Inc., La Jolla, CA, USA). Probability values (p) less than 0.05 were considered statistically significant.

2.2. Methods II. Evaluation of cardiovascular effects of ERJ-500

2.2.1. In vitro experiments

2.2.1.1. Oxidative stability:

Chemical Fenton system

The chemical Fenton system was carried out based on a method reported by Jurva et al. The reaction mixture contained the following components: 400 µL 2.5 mM ERJ-500 in acetonitrile, 50 µL 20 mM FeCl3 and 50 µL 20 mM EDTA-Na2 in acetonitrile/water (50:50), 500 µL 10 mM ascorbic acid in acetonitrile/water (50:50) and 1 µL 30% hydrogen peroxide. The blank sample was prepared without ERJ-500, meanwhile the control sample was prepared in the absence of hydrogen peroxide. The mixtures were stirred at room temperature at 600 rpm. Samples were drawn at 1 h in the Fenton reactions prior to injecting them instantly to the HPLC and further investigation. Then samples were analysed immediately by an API 2000 Triple Quadrupole mass spectrometer (Applied Biosystems, Waltham, MA, USA) equipped with a syringe pump. Flow rate was set to 100 µL/min, curtain gas 10 PSI, declustering potential 20 V, ion spray voltage 4000 V, focusing potential 400 V, ion source temperature 200 ◦C. ESI mass spectra were recorded in the range of m/z 100–500 in positive-ion mode with analyst 1.5.1. Software (AB SCIEX, Concord, ON, Canada).

Synthetic porphyrin system

The porphyrin system was composed based on a method reported by Johansson et al. The reaction mixture contained the followings: 50 µL 10 mM ERJ-500 in acetonitrile, 35 µL acetonitrile, 315 µL 100 mM formic acid, 50 µL 10 mM Fe(III) meso-tetra(4-sulfonatophenyl)porphine chloride and 50 µL 30% H2O2. Reaction mixtures for blank contained acetonitrile only without ERJ-500. The control mixtures contained no peroxide.
The mixtures were shaken at 37 ◦C for 30 min at 700 rpm in a shaking water bath. After that one hundred microlitres of the reaction mixture were diluted with 2 mL with acetonitrile/water (50:50). After HPLC separation (column: Kinetex XB-C18 2.6 µm, eluent: 0.1% formic acid in water and acetonitrile with 0.1% formic acid, gradient elution), the samples were introduced into an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: sheath gas flow rate 35.00 a.u., spray voltage 5000 V, capillary temperature 300 ◦C, capillary voltage 31 V, tube lens voltage 150 V, skimmer voltage 34 V.

2.2.1.2. Determination of cytotoxicity by MTT assay

Assessment of the cytotoxicity of the ERJ-500, ASA, and MOL on cellular survival was accomplished using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. H9c2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin at 37 ◦C in a 5% CO2 and 95% air humidified incubator. Cells were cultured for one day, next day the cells were treated. H9c2 cells were treated with 100 nM, 1 μM, 10 μM, 100 μM of ERJ-500, 100 μM of MOL, 100 μM of ASA and 1% H_2O_2 (positive control) containing medium for 24 h on 96 well plates. Then, MTT solution was added to the medium and incubated for 3.5 h at 37 °C. After eliminating the solution from the cells, isopropanol was added and incubated for 0.5 h at 37 °C to dissolve formazan product to dissolve the formazan aggregates. Absorbance was measured at 570 nm and 690 nm.

2.2.1.3. Determination of hemolytic activity

Hemolysis tests were performed as described by Roka et al. (Roka et al., 2015) with some minor modifications. Rat blood samples were collected to K3EDTA containing vacuum tubes (BD, Plymouth, UK) and were treated with 100 nM, 1 μM, 10 μM, 100 μM ERJ-500, 100 μM of MOL and the same concentration of ASA in phosphate buffered saline (PBS). The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of the different chemical solutions related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water.

2.2.2. Ex vivo experiments:

2.2.2.1. Animals

Female Sprague Dawley (SD) rats with an average weight of 248 ± 6 g were used in the present study. Animals were nutrified with standard rodent chow pellets (R/M-Z + H, ssniff Spezialdiäten GmbH, Soest, Germany) ad libitum with free access to water and kept at an ambient temperature of 25 ± 2 °C, with a relative humidity of 55 ± 5%, and a 12-h light-dark cycle. All animals were treated according to the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication no. 86–23, revised in 1996). Breeding and handling of animals were approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary.

2.2.2.2. Langendorff heart preparation and assessment of heart rate and coronary flow

Rats were anesthetized with an intraperitoneal pentobarbital sodium injection (60 mg/kg), with heparin as an anticoagulant (1000 U/ kg). Following the induction of deep anesthesia, chest cavities were opened, hearts were excised and placed in ice-cold modified KrebsHenseleit bicarbonate (KHB) buffer (containing 118 mM NaCl, 5.8 mM KCl, 1.8 mM CaCl2, 25 mM NaHCO3, 0.36 mM KH2PO4, 1.2 mM MgSO4, and 5.0 mM glucose). After excision, aortas were cannulated and each heart was perfused with modified KHB buffer at a filling pressure of 100 cm of water, using the "non-working" Langendorff mode for 5 min in order to flush blood out from the myocardium. The setup was assembled with two buffer-chambers at the same constant pressure. The one contained the KHB buffer only, the other contained ERJ-500 dissolved into the KHB buffer at different concentrations (1 μM, 10 μM, 30 μM, 100 μM). At the end of the washout period, baseline cardiac parameters were registered, including coronary flow (CF) and heart rate (HR), and the inflow was switched to serve the hearts from the chamber containing ERJ-500 for 10 min. Next, 10 min of washout period, followed by 10 min of adding once more the ERJ-500 containing buffer. A continuous pressure signal was recorded during the whole experiment with the help of a pressure transducer (ADInstruments, PowerLab, Castle Hill, Australia), which was calibrated before each experiment. HR was calculated from the continuously recorded pressure signal. CF was assessed by the time-collecting of the coronary effluent.

2.2.2.3. Isolated working heart preparation to assess cardiac parameters

Sprague Dawley female rats divided into two groups n = 11 in the control group, n = 6 in the treated group. After completing the isolated working heart preparation procedure followed by 10 min washout period, we registered the baseline working heart parameters such as aorta flow (Catella-Lawson et al., 2001a), coronary flow (CF), aortic pressure (AOP), heart rate (HR) and derivated aortic pressure (AOdP/dT). Cardiac output (CO) was calculated by the sum of AF and CF and we got stroke volume (SV) by dividing the CO with HR. In the treated group, ERJ-500 was added to the KHB buffer by a dilution of a previously prepared stock solution, creating a 100 μM concentration of ERJ-500 in the heart inflow. The molecule-containing KHB buffer was presented after the washout and baseline registration period for 5 mins, followed by a 30 min ischemia followed by 90 min reperfusion

2.2.2.4. TTC staining

To determine the degree of the infarcted area in the myocardium, triphenyl tetrazolium chloride (TTC) staining was performed. Following ischemia and reperfusion, 50 ml of 1% TTC solution was perfused through the myocardium. Then, hearts were frozen, sectioned, digitalized and all heart sections were blotted dry and weighed. Risked and infarcted areas were quantified by an open-source planimetry software Fiji (Schindelin et al., 2012). Percentage of the infarcted area compared to the whole risked area of the myocardium is represented on a bar chart.

2.2.2.5. Statistical analyses

All data are presented as the average magnitudes of each outcome in a group ± standard error of the mean. Statistical analysis was performed using t-test or one- or two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test with GraphPad Prism software for Windows (GraphPad Software Inc., La Jolla, CA, USA). Probability values (p) < 0.05 were considered statistically significant.

3. Results

3.1. Aged garlic and Raw Garlic against Ischemia/Reperfusion-Induced Cardiac Complications

3.1.1.Analysis of the Composition of Raw and Aged Black Garlic

The amounts and numbers of different sulfur-containing compounds are higher in ABG. The study failed to detect any allicin in ABG. During the aging process, the Maillard reaction also occurred, which was evidenced by the presence of the 2-acetyl-1-pyrroline in ABG and its absence in raw garlic.

3.1.2. Effect of Raw and Aged Garlic on Body Weight

Initial body weight were similar in all groups. After 4 weeks of raw garlic or aged black garlic treatment there were no significant differences in body weight compared to the vehicle-treated group.

3.1.3. Effect of Raw and Aged Garlic on Blood Enzymes

Most of the levels of the investigated blood markers showed no alterations. The level of LDL resulted in a slight increase. howver remained in the normal range in all groups.

3.1.4. Raw and Aged Garlic Treatment effects on cardiac function

To compare the beneficial to cardiovascular effects of raw and aged black garlic, isolated hearts originating from treated animals were taken to 30 min of global ischemia and 120 min of reperfusion. No significant alterations were seen in preischemic values of the studied cardiac functions including CF, AF, AOP, AOdP/dt, CO, and SV alteration. Significantly increased postischemic cardiac function in the presence of raw and aged garlic, respectively, was detected. Thus, after 30 min of ischemia and 120 min of reperfusion, aortic flow was significantly enhanced in the treated groups compared to their control value of 4.3 ± 2.0 mL to 24.1 ± 4.6 mL for raw garlic, and 22.7 ± 3.8 mL for aged garlic. Interestingly, in comparison with the control values of 13.6 ± 1.2 mL superior coronary flow values in the treated groups was observed, in the raw garlic group a value of 19.3 ± 2.8 mL and for aged garlic a value of 19.9 ± 1.4 mL.

3.1.5. Raw and Aged Black Garlic Treatment effects on Infarct Size

To confirm the cardioprotective effect of the raw and aged black garlic at the end of I/R TTC, staining was carried out to measure the size of infarcted tissue. At the control group 27.5 ± 8.4% infarcted volume was, and both raw and aged garlic were able to significantly decrease the infarct volume to 5.9 ± 2.0% and 6.2 ± 1.2%. There were no statistically significant differences between the two garlic treated groups.

3.1.6. Raw and Aged Black Garlic Treatment effects on expression level of HO-1 and iNOS

To explore the molecular mechanisms by which raw and aged black garlic protected the heart against I/R injury, the levels of HO-1 and iNOS were analyzed by Western blot. Before I/R enhanced level of HO-1 was found in both garlic treated groups However, after I/R, HO-1 showed a significantly increased expression level in both garlic treated groups compered to control.

After evaluation of iNOS no significant differences were seen before I/R between the groups. However, after I/R a significantly reduced protein level of iNOS was seen in the control and raw garlic treated hearts. At the same time aged black garlic was able to prevent the reduction of iNOS after I/R.

3.2. Evaluation of cardiovascular effects of ERJ-500

In vitro experiments

3.2.1. Oxidative stability assays

Classical Fenton reaction:

The peaks on the chromatogram were not changed notably after 1 h of oxidation compared to the control chromatogram.

Synthetic porphyrin system:

In this experiment a synthetic porphyrin, Fe(III) meso-tetra(4-sulfonatophenyl) porphine chloride was used. The total ion chromatograms of the control and ERJ-500 after oxidation by synthetic porphyrin were almost the same.

3.2.2. Safety evaluation of ERJ-500

MTT assays

To assess the direct cytotoxic effects of ERJ-500, we carried out MTT assays at different concentrations of the studied molecule, and its two constituents, ASA and MOL in H9c2 cells. A slight decrement can be seen in all treated groups compared to the control, but all treated groups resulted in a significantly higher cell viability compared to the positive control group, which was treated with 1% H2O2. No significant differences can be observed between the groups treated by ERJ-500 or other molecules studied.

Hemolytic activity studies

To confirm our previously demonstrated cytotoxicity results, we performed hemolytic activity studies in blood cells isolated from Sprague Dawley rats. The hemolytic activity in rat erythrocytes at different concentrations of ERJ-500, ASA and MOL were significantly lower compared to the positive control group. Samples of the latter group received sterile water, which induced 100% hemolysis. No significant differences can be observed among the groups treated by ERJ-500, ASA, and MOL.

3.2.3. Vasoactive effects of ERJ-500

To study the vasoactive effects of the ERJ-500 in the myocardium, the drug was dissolved in the perfusion buffer at a concentration rate of 1 μM to 100 μM, and isolated hearts were perfused. During Langendorff perfusion, the ERJ-500 did not produce any incidence of ventricular tachycardia or ventricular fibrillation. In addition, heart rate was not significantly changed in comparison with the drug-free control group. Coronary flow was significantly increased by about 50% in the group treated with 100 μM ERJ-500.

To further confirm vasoactive effects of ERJ-500 and to study any possible beneficial effects of the compound on the mechanical activity of the hearts, we tested the molecule on the isolated working heart perfusion system as well, at a concentration, which seemed the most advantageous previously. In the working heart perfusion, when other mechanisms also involved to compensate measurable vasoactive effects, coronary flow was still significantly elevated in treated hearts with 100 μM ERJ-500 (Fig. 2.D.). Stroke volume was also significantly increased, thus, ERJ-500 can be an additive effect to improve myocardial contraction force. As previously measured in Langendorff heart preparation, heart rate did not change notably in working heart preparation also.

3.2.4.Anti-ischemic effect of ERJ-500

Triphenyl-tetrazolium-chloride staining method (TTC) was used to further analyze the effect of ERJ-500 on the rat myocardium. After 30 min of ischemia and 90 min reperfusion, infarct zones of TTC-stained hearts were expressed in a percentage of the whole myocardium. Hearts perfused with ERJ-500 containing buffer resulted in a significantly decreased infarct size. This result indicates that ERJ-500 has a cardioprotective effect, which could be a consequence of the vasorelaxant property, however, other mechanisms may also contribute to this effect.

4. Discussion

Cardiovascular diseases, especially ischemic heart disease, nowadays still a leading cause of death, despite of greater focus on primary prevention and improved diagnosis and treatment. Our aim was to study from a new aspect the conventional plant and drug, garlic and aspirin. The major objective of the present study was to compare the effect of aged black garlic and raw garlic on the pre- and postischemic cardiac function and investigate the underlying molecular mechanisms. Next, our goal was to study ERJ-500 toxic and vasodilator effects, in the highlight of coronary artery dilation in the myocardium.

In the first part of our study the major aim was to compare the cardiovascular effect of raw and aged black garlic in an experimental model. Consistent with earlier studies, these results clearly demonstrate that both raw garlic and aged black garlic have similar very significant cardioprotective effects in I/R-ed myocardium, as evidenced by superior postischemic cardiac functions and smaller infarct size. The study failed to find any significant differences between the two treated groups, indicating that the aging process of garlic does not alter the cardioprotective ability of the preparation. Indeed, different studies have compared the antioxidant and anti-inflammatory effects of raw garlic and aged black garlic under certain conditions. Lee and colleagues have shown superior antioxidant properties for aged black garlic over raw garlic in diabetic animals. Earlier, a long term garlic administration was shown to enhance the level of endogen antioxidants such as catalase, SOD in the myocardium in a dose dependent manner. The authors have suggested the contribution of the enhanced antioxidant defense mechanism to the cardioprotective ability of garlic. Consistently, enhanced antioxidant activity was found in aged black garlic by Jeong and co-workers, however, the antioxidant capability was not directly proportional to the anti-inflammatory property of the different garlic preparations in an LPS-stimulated inflammatory model. The authors found that pyruvate and other polyphenols, flavonoids and organosulfur compounds, enriched during the aging process, act synergistically as antioxidants in aged black garlic. Furthermore, the anti-inflammatory effects of pyruvate found in aged black garlic might be perturbed by the sugar component in aged black garlic. The study did not find any analyzed biomarkers out of the physiological range; since all remained under the physiological level any effect on metabolism cannot be concluded. However, it must be noted that the possibility is quite reasonable since rats were also symptom-free during the treatment period. The same reasoning might explain the unaltered body weight during the experiments. Thus, the metabolic effect of the garlic preparations should be studied in a diabetic or atherosclerotic animal model.

To explore the molecular mechanisms by which the different garlic preparations protect the heart, the levels of HO-1 and iNOS were studied. The study found that treatment with raw garlic can enhance the level of HO-1 before ischemia. Following ischemia in both garlic-treated groups, the level of HO-1 is significantly enhanced. Induction of HO-1 leads to the production of Fe^{2+}, endogen CO and biliverdin/bilirubin as a byproduct of heme metabolism. Enhanced activity of the HO-1/CO system by different natural products has been shown to induce cardioprotection. Upregulation of HO-1 might be an adaptive response to different harmful stimuli such as ischemia, or even excess heme levels. Earlier, sour cherry seed extract was found to protect the heart via upregulation of HO-1 protein. Ginseng derived ginsenoside was proven to activate the Nrf2/HO-1 pathway and protect H9c2 cells against hypoxia/reoxygenation in a recent study. Furthermore, Issan and colleagues have demonstrated that pharmacological induction of HO-1 by CoPP protects H9c2 cells against hypoxia and also diabetic hearts from ischemia via the modulation of the AKT/GSK3β pathway. However, it must be noted that from this study's results, the authors cannot conclude which component is the major HO-1-inducer since the composition of the two garlic preparations is not the same. NOSs, possessing three isoforms including eNOS, nNOS, and iNOS, are a group of enzymes producing NO, which a gaseous transmitter is playing a role in different physiological and pathophysiological processes. The level of iNOS was significantly reduced after I/R in the vehicle-treated control and raw garlic-treated groups. A slight decrement was observed in the aged black garlic group, however, it was not at a significant level indicating the ability of aged black garlic to prevent iNOS loss after I/R. Recently, an extract of aged black garlic was shown to possess dose-dependent cardioprotective effects and similarly, an enhanced iNOS expression was found in aged black garlic-treated hearts. Similarly, in a recent study, a reduced level of iNOS mRNA expression was found in hearts obtained from saline-treated animals after infarction. However, fish oil treatment prevented the decreased expression of iNOS after infarction. Furthermore, enhanced expression of iNOS accompanied by smaller infarct size were found in eNOS KO animals in response to I/R-injury in an "ex vivo" model, indicating that NO plays a role in cardioprotection. It must be noted that the authors did not observe any alteration of iNOS expression in WT animals after I/R. Quite the reverse, as "in vivo" studies showed earlier, overexpression of iNOS might contribute to myocardial injury. Thus, cardioprotection afforded by Propofol and Sabiporide (Na^+/H^+ exchanger-1 inhibitor) was found to be mediated via the suppression of iNOS. Furthermore, slightly enhanced expression of iNOS was detected in hearts obtained from overfed animals. Moreover, enhanced iNOS activity was found to play a role in cardiomyocyte dysfunction in a regional ischemia in vivo.

Interestingly, in another study, enhanced expression of iNOS was observed in the ischemic region of the heart after a "sub-lethal ischemic" insult suggesting a regulatory role of iNOS during the late preconditioning. Based on the above-mentioned outcomes, the role of NO and NOSs in ischemic tissue could be both beneficial and harmful, depending on the environment or tissue damage. However, it must be noted that the difference in iNOS expression also might arise from the different experimental models since, in the current study, "ex vivo" 30 min ischemia and 120 min of reperfusion was used to mimic I/R, while in other studies "in vivo" 6–24 h of reperfusion were allowed. Under "in vivo" conditions, the authors cannot rule out the influence of the immune system and platelets on the expression of different proteins.

Int he second part of our study we aimed to evaluate the cardiovascular effects of ERJ-500. The total ion chromatograms of the control and ERJ-500 after oxidation by synthetic porphyrin were almost identical, therefore the ERJ-500 molecule was resistant against simple oxidative conditions, which could possibly change the structure of the molecule in another case. Fenton reaction further confirmed the stability of the new molecule under simple oxidative condition. According to the cell viability MTT assay ERJ-500 is an equally safe compound as the MOL or ASA. By the hemolytic test the safety was further confirmed that our aspirin derivative seems to be a safe compound. During Langendorff perfusion, the ERJ-500 did not produce any incidence of ventricular tachycardia or ventricular fibrillation. In addition, heart rate was not significantly changed in comparison with the drug-free control group. During Langendorff perfusion, the coronary flow is influenced by the heart rate, the perfusion pressure, and the coronary dimension. Since the perfusion pressure used in the present study is constant and the heart rate is not significantly altered, the increased coronary flow could be a result of the coronary relaxation. Although in the present study, the concentration of NO was not directly measured, and it would be the subject of another study, our results support the hypothesis that NO may originate from the aspirin-molsidomine compound (ERJ-500), since salicylic acid shows no vasodilator activity in the myocardium. Cardioprotective effects of aspirin and salicylic acid related derivatives can be attributed to affect the platelet activation related to cyclooxygenase enzyme activities (COX1 and COX2) and heat stress protein expression in the diseased myocardium. To further confirm vasoactive effects of ERJ-500 and to study any possible beneficial effects of the compound on the mechanical activity of the hearts, we tested the molecule on the isolated working heart perfusion system as well, at a concentration, which seemed the most advantageous previously. In the working heart perfusion, when other mechanisms also involved to compensate measurable vasoactive effects, coronary flow was still significantly elevated in treated hearts with 100 μM ERJ-500. Stroke volume was also

significantly increased, thus, ERJ-500 can be an additive effect to improve myocardial contraction force. As previously measured in Langendorff heart preparation, heart rate did not change notably in working heart preparation also. ERJ-500 containing buffer resulted in a significantly decreased infarct size, this result indicates that ERJ-500 has a cardioprotective effect,which could be a consequence of the vasorelaxant property, however, other mechanisms may also contribute to this effect.

5. Summary

In the first part of our experiments, we investigated aged black garlic obtained from raw garlic, which is used for medicinal purposes for thousands of years. Through the aging process under high temperature and humidity the texture, taste and bioactive components are changed. Male Sprague Dawley rats were fed with raw garlic, aged black garlic and with vehicle for a period of 4 weeks. Isolated hearts were undertaken to ischemia/reperfusion. No significant differences in body weight and blood parameters were observed after 4 weeks in neither of the groups. Isolated hearts were undertaken to 30 minutes of global ischemia and 120 minutes of reperfusion. After I/R superior postischemic cardiac function and decreased infarct size were found in both garlic treated groups compared to the drug-free control group. We experienced an alteration in the HO-1 expression levels, which have an important antioxidant property. Moreover, in control and raw garlic samples decreased iNOS levels were observed, meanwhile in samples originated from aged black garlic iNOS levels were not significantly reduced after ischemia/reperfusion. Based on these observations not only raw but also aged black garlic have a cardioprotective effect.

Furthermore, we also investigated the properties of a nitric oxide (NO) donating acetylsalicylic acid (ASA) derivative (ERJ-500), which is a newly synthetized compound at the University of Debrecen. One of the most commonly used drugs for the treatment of ischemic heart disease is the low-dose, prophylactic use of a platelet aggregation inhibitor, acetylsalicylic acid (ASA). However, gastrointestinal toxicity is the major limitation of ASA, which can be avoided by the protective NO. Based on the cell viability assay and hemolysis tests the ERJ-500 not affected negatively the viability of living cells in the concentration ranged between 100nM and 100μM. By the ex vivo Langendorff method on hearts taken from Sprague Dawley rats we experienced that, ERJ-500 displayed a dose-dependent, outwashable vasodilative effect in coronary arteries. Moreover, by focusing on the most potent 100 μM ERJ-500 concentration, we found that vasodilative effect also appears on isolated working heart modell elevated stroke volume. Furthermore, we found that the infarct size significantly decreased in ERJ-500 treated hearts after ischemia/reperfusion. Taken together these results, ERJ-500 may contribute to a new therapeutic tool against ischemic heart conditions and related syndromes.

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/84/2022.PL
Subject: PhD Publication List

Candidate: Kitti Szőke
Doctoral School: Doctoral School of Pharmacy

List of publications related to the dissertation

1. **Szőke, K.**, Czompa, A., Lekli, I., Szabados-Fürjesi, P., Herczeg, M., Csávás, M., Borbás, A., Herczegh, P., Tósaki, Á.: A new, vasoactive hybrid aspirin containing nitrogen monoxide-releasing molsidomine moiety.
Eur. J. Pharm. Sci. 131, 159-166, 2019.
DOI: http://dx.doi.org/10.1016/j.ejps.2019.02.020
IF: 3.616

2. **Szőke, K.**, Czompa, A., Lekli, I., Szabados-Fürjesi, P., Herczeg, M., Csávás, M., Borbás, A., Herczegh, P., Tósaki, Á.: Dataset on structure, stability and myocardial effects of a new hybrid aspirin containing nitrogen monoxide-releasing molsidomine moiety.
Data brief. 25, 1-10, 2019.
DOI: http://dx.doi.org/10.1016/j.dib.2019.104146

3. Czompa, A., **Szőke, K.**, Prokisch, J., Gyöngyösi, A., Bak, I., Balla, G., Tósaki, Á., Lekli, I.: Aged (Black) versus Raw Garlic against Ischemia/Reperfusion-Induced Cardiac Complications.
Int. J. Mol. Sci. 19 (4), 1-13, 2018.
DOI: http://dx.doi.org/10.3390/ijms19041017
IF: 4.183

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

List of other publications

4. Gyöngyösi, A., **Szőke, K.**, Fenyvesi, F., Fejes, Z., Bekéné Debreceni, I., Nagy, B. J., Tósaki, Á., Lekli, I.: Inhibited autophagy may contribute to heme toxicity in cardiomyoblast cells.
Biochem. Biophys. Res. Commun. 511 (4), 732-738, 2019.
DOI: http://dx.doi.org/10.1016/j.bbrc.2019.02.140
IF: 2.985

5. Czompa, A., Gyöngyösi, A., **Szőke, K.**, Bak, I., Csépányi, E., Haines, D. D., Tósaki, Á., Lekli, I.: Effects of Momordica charantia (Bitter Melon) on Ischemic Diabetic Myocardium.
Molecules. 22 (3), 488, 2017.
IF: 3.098

Total IF of journals (all publications): 13,882
Total IF of journals (publications related to the dissertation): 7,799

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

16 February, 2022

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *