

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**The possible beneficial effects of different plant derived
biologically active compounds against ischemia/reperfusion-
induced injury**

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Introduction

Myocardial ischemia/reperfusion-induced damage is one of the leading cause of morbidity and mortality in the Industrialized Society. In Hungary, some kinds of cardiovascular diseases are responsible for more than half of the deaths. In the last few decades painstaking work have been done to understand the underlying mechanisms and to indentify the risk factors. Despite of the recognitions of many different risk factors such as smoking, obesity, diabetes, high bood pressure, lack of exercise, and the intake of excess amount of alcohol and food rich in fat and cholesterol, the incidence of the cardiovascular diseases is not decreasing and what is more it is increasing, and younger generations are affected. For these facts, the topic of the cardiovascular diseases needs further investigation to understand the cause and effect relationship, and to develop more alternative treatment for the patients, and the peoples with high risk factors.

Most of the time, the root cause of the ischemia is some kind of occlusion of blood vessels or vasospasm, which decrease or stop the blood perfusion of tissues proximal to the occlusion, leading to the disturbance of the oxygen and nutrients supply of the area. The impaired oxygen and nutrients supply induce the piling up of different harmful byproducts, which ultimately lead to apoptosis or necrosis. One of the underlying mechanisms is the stop of the aerobe glycolysis due to the lack of the oxygen. During ischemia the aerobe glycolysis is switched to the anaerobe glycolysis, which can not supply the required

amount of ATP for cells. The other important result of the anaerobe glycolysis is the elevated level of the lactic acid and the decreased pH in the cells. The reduced pH diminishes the Ca^{++} sensitivity of the miofilaments. Furthermore, the falling pH accelerates the operation of the $\text{Na}^+/\text{H}^{++}$ antiporter, which increases intracellular Na^+ levels. The enhanced level of the intracellular Na^+ reduces the activity of the $\text{Na}^+/\text{Ca}^{++}$ channels leading to the intracellular accumulation of Ca^{++} ion.

For the survival of the ischemic myocardium the reperfusion is essential, when the resorted blood flow resupplies the oxygen and nutrient to the ischemic tissue. However, the reperfusion is essential it is also harmful. At the beginning of the reperfusion when the reduced mitochondrial respiratory chain meets with resupplied oxygen, excess amount of ROS is generating, which can not be neutralized by the weakened antioxidant systems in cells. The excess amount of ROS can oxidized lipids damaging different membrane functions. Damages of the different proteins impaired their function. Furthermore, not only the lipids or proteins, but the DNA also can be damaged by ROS, leading to mutation or DNA breakage. The above mention different damages can induce cell death in the ischemic and reperfused myocardium.

During reperfusion pH and ATP levels are rapidly normalized, but the intracellular calcium is remained high, and it is further increased via the $\text{Na}^+/\text{Ca}^{++}$ antiporter, which at the beginning of the

reperfusion works in “reverse mode” due to the high intracellular Na^+ level. During processes when cells are rearranging their metabolisms, calcium overload is switched to the oscillation of the calcium. The calcium overload results in a hypercontractive state, moreover, enhances the incidence of ventricular tachycardia, and activates some calcium dependent proteolytic enzymes.

In the last decade, it is becoming clear that mitochondrial dysfunction also plays a major role in the ischemia/reperfusion-induced damage. The opening of the mitochondrial permeability transition pore (MPTP) induces the depolarization and the swelling of mitochondria, which leads to the breakage of the outer mitochondrial membrane and the induction of apoptosis or necrosis. During ischemia the MPTP is closed due to the low pH, later during reperfusion the circumstances such as calcium overload, normal pH and the oxidative stress, are ideal for opening of MPTP.

The oxidative stress changes the metabolisms of the cardiomyocytes. Furthermore, it affects the expression of different genes and pathways. This changed redox environment plays a major role in the I/R-induced injury. It is known for some time that the antioxidants can protect the heart against I/R-induced damage. The cells are equipped with intracellular antioxidant systems, which are capable to neutralize the generating ROS under normal condition. Beside the intracellular antioxidant system, many plant extract were shown to possess antioxidant properties and cardioprotective effects.

Withania somnifera could protect the myocardium against I/R-induced injury and isoprenaline-induced damage. Similar to the *Withania somnifera*, *Aegle marmelos* was found to be protective against isoprenaline-induced injury. Beside these two examples many other different plant extracts were found to possess cardioprotective effects. The protective effects of plants are related to their flavonoid, anthocyanin, polyphenol, unsaturated free fatty acid and the high fiber contents.

In Hungary, sour cherry seed is mainly considered as a byproduct of the cherry utilization. However, during the analysis of the sour cherry seed extract many different biologically active components were identified such as cyanides, polyphenols, flavonoids, vegetable acids, pro- and anthocyanidines, trans-resveratrol, stilbenes and catechins. Szabo and her colleagues have shown the ability of the sour cherry seed extract to protect the retina against I/R induced damage. Sour cherry seed extract induces hemoxygenase 1, on the other hand it prevents the intracellular K^+ loss and Na^+ , Ca^{++} gains after I/R. The cardioprotective effect of hemoxygenase system is well known; furthermore, to keep the intracellular ion balance in a proper stage is also essential for the heart. For these reasons, we have decided to check whether the sour cherry seed extract has the same effect on the heart as it has on the eye.

The investigations of different plant extracts were accelerated when the phenomenon of “French Paradox” was first recognized.

According to the existing reports the “French kitchen” also uses a lot of fat and unhealthy ingredients, but the French peoples live 4 years longer than the American peoples. Moreover, the prevalence and the incidence of coronary heart diseases and myocardial infarction are also 40 % lower than in other peoples live in the western countries. At least by part the red wine consumption is the responsible for this phenomenon. A mild-to-moderate wine consumption habit attenuates cardiovascular, cerebrovascular, and peripheral vascular risk due to reduced platelet and monocyte adhesion, attenuates the risk of prostate as well as a variety of cancers including pancreatic, gastric, and thyroid cancer, and slows down some neurodegenerative diseases like Alzheimer disease. Among approximately 500 different antioxidants, recent studies revealed that resveratrol and proanthocyanidins are the two most important polyphenolic antioxidants present in wine that attenuate various health problems. In fact, most believe that resveratrol is the secret compound present in red wine that is responsible for French Paradox.

Resveratrol (3,5,4'-trihydroxystilbene), a member of a family of polyphenols called viniferins possesses diverse health beneficial effects. First, it was isolated in 1940 from *Veratrum grandiflorum*. Many investigators studied the effects of resveratrol on different diseased models. Resveratrol was found to bind to oestrogen receptors and through that it can modify DNA synthesis and different cell cycle related proteins such as p53, Rb/E2F, cyclins and cyclin dependent

kinases. Moreover, resveratrol influences some transcription factor related to stress response and proliferation for instance NF- κ B, AP1 and Egr1. These effects of resveratrol are being mediated, at least by part, via MAK kinases and tyrosine kinases, leading to the modification of the survival and apoptotic pathways. Furthermore, resveratrol modifies the expression and activity of some enzymes such as cyclooxygenase, nitric oxid synthase or hemeoxygenase; it also changes the expression of transcriptional cofactors like p300 and sirtuin-1.

From the existing literature it appears that many different mechanisms contribute to the cardioprotective effects of resveratrol. Resveratrol was shown to enhance the expression of the i-NOS, e-NOS, n-NOS and relax the aortic smooth muscle cells and reduce myocardial damages. It was found that resveratrol possess antioxidant and anti-inflammatory effects; after I/R resveratrol reduced the level of pro-adhesion molecules. Moreover, resveratrol can precondition the heart via the A₁ and A₃ receptors. A₁ receptor related effect is the activation of the PI3K-Akt-Bcl-2 survival pathway, while the CREB-dependent activation of Bcl-2 is connected to the A₃ receptor. Enhanced levels of HO-1, Trx-1 (thioredoxin-1) and VEGF (vascular endothelial growth factor) were detected after resveratrol treatment in the ischemic myocardium indicating that resveratrol helps the neovascularisation of the ischemic area.

Diabetes is one of the major risk factor for the ischemic heart disease, which can worsen the outcome after an ischemic heart attack. For the detrimental effect of diabetes many factors are responsible; here I would like to highlight the role of the enhanced oxidative stress, increased platelet aggregation and the endothelial dysfunction. Under experimental circumstances carbohydrate rich diet induces insulin resistance and endothelial dysfunction in normal and “Zucker Obese” animals. As resveratrol can prevent endothelial dysfunction and protect the heart against I/R-induced damage in intact animals, we attempted to see whether it has the same effect in the diseased “Zucker Obese” rats. To study the effect of the resveratrol in a diseased model, hearts obtained from resveratrol treated “Zucker Obese” animals were underwent to 30 min ischemia followed by 120 min reperfusion in the presence or absence of an extra glucose load; body weight, serum insulin and glucose levels, postischemic cardiac functions were monitored.

As previously it has been mentioned, due to the functional and energetic disturbances caused by I/R, cells can die either via apoptosis or necrosis. Beside these, there is another type of programmed cell death called autophagy, among the three types of death the role of autophagy is the less clear. Autophagy is an evolutionary conserved catabolic process in which the cell digest it’s own constituents via lysosomal pathway. This process can be observed in normal cells, when the cell digests and reuses some long-lived proteins,

macromolecules or even whole organelles such as mitochondria, ribosomes and peroxisomes.

There are three types of autophagy such as macroautophagy, microautophagy and chaperon-mediated autophagy. Macroautophagy (hereafter referred as autophagy) is the most active form of autophagy. At the beginning, an isolation membrane is formed, which is elongating to form the typical double layer membrane structure called autophagosome. The autophagosome can enclose cytoplasmic particles, damaged proteins or different organelles of the cells, after this the autophagosome fuses with a lysosome to form autophagolysosome. Under normal circumstances autophagy plays an important role in the homeostasis, and serves as a catabolic energy source during fasting. Autophagy was first described in the myocardium in 1976. In that early study, after nutrients and oxygen deprivation and their resupply an increased autophagy was observed. Later, Decker and Wildenthal found correlation between enhanced autophagy and postischemic recovery of ischemic/reperfused rabbit myocardium. Yan and colleagues observed increased number of autophagosomes in the chronically ischemic swine myocardium. The autophagy was elevated in the survival area and reduced in the death portion; furthermore parallel to the increased autophagy reduced apoptosis were seen. Oipari and co-workers demonstrated that resveratrol is capable of inducing autophagy in different cancer cell lines. Based on this, we have decided to examine whether autophagy

contribute to the cardioprotection induced by the resveratrol; furthermore, we investigate the effects of another plant derived natural compound called γ -tocotrienol. Recently, we have found that γ -tocotrienol possesses cardioprotective effects. In the present investigation we aimed to examine the possible synergetic effect between resveratrol and γ -tocotrienol.

Aims

During the work we attempted to examine the effects of different plant derived biologically active compounds on the I/R-induced ventricular fibrillation and postischemic injury.

I, The goal of the first study was to investigate the effect of sour cherry seed kernel extract on postischemic recovery of left ventricle, and on the I/R-induced ventricular fibrillation. Furthermore, we examined the infarct size, myocardial apoptosis and caspase-3 expression.

II, The aim of the investigation was to determinate whether resveratrol is capable for improving the postischemic cardiac function, reducing the infarct size in the genetically modified “Zucker Obese” rat; and to clarify the underlying mechanisms.

III, In the final study, we compared the effects of resveratrol and γ -tocotrienol separately and in combination on the postischemic

recovery; we studied the role of autophagy and the survival pathways in the cardioprotection induced by the resveratrol and γ -tocotrienol.

Materials and methods

All animals received humane care in compliance with 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). The use of animals in our experiments was approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary.

1. Experimental protocols

A. Preparation of the sour cherry seed extract and the feeding protocol:

Sour cherry seeds were dried, and the wall was removed. The kernel was then ground and extracted with n-hexane by Soxhlet extractor. The solvent was evaporated under vacuum, resulting in the oil fraction (fraction 1) of the kernel (32–36%). The remaining (64–68%) solid fraction (fraction 2) was dried, and the oil-free kernel extract was used for the analyses of its cardiovascular effects. UV,

infrared, and gas chromatography/mass spectrometry analyses in composition with HPLC were carried out to identify the components. Before the isolation of hearts, rats were treated orally with 1, 5, 10, and 30 mg/kg/day of sour cherry seed extract for 14 days, respectively. Age-matched control rats received a daily dose of saline solution (0.9% of NaCl) for 14 days.

B. Protocol for resveratrol treatment:

Male 23- to 24-wk-old Zucker obese rats fed commercial food pellets were used for all studies. In the first series of the study, Zucker obese rats were treated orally with 5 mg/kg/day of resveratrol [obese + resveratrol (O+R)] for 2 wk. The results obtained in the O+R group were compared with the resveratrol-free obese control (OC) group. The second group of rats received tap water containing 10% of glucose ad libitum for 3 wk, and, during the 2nd- and 3rd-wk period, rats were orally treated with 5 mg/kg/day of resveratrol [obese + glucose + resveratrol (O+G+R group)]. The results obtained in the O+G+R group were compared with the obese + glucose resveratrol-free group (O+G). Resveratrol was dissolved in ethanol and diluted with water (1:10), and 10 ml/kg of final volume were used for oral treatment of rats as a gavage each day.

C. protocol for resveratrol and γ -tocotrienol treatment:

Male Sprague-Dawley rats were randomly assigned to one of the following groups: control I/R, resveratrol treated-I/R, tocotrienol treated-I/R, resveratrol and tocotrienol treated-I/R. 2.5 mg/kg/day

resveratrol was gavaged to the animals for 15 days, γ -tocotrienol was gavaged for 30 days at a dose of 0.3 mg/kg/day. In the dual treated group, during the first 15 days γ -tocotrienol was given while for the second 15 days both resveratrol and γ -tocotrienol were administered.

2. Isolated working heart preparation:

After completing the feeding protocol, the animals were anesthetized with sodium pentobarbital (60-80 mg/kg, i.p.), and intraperitoneally heparin sodium (500 IU/kg., i.v.) was used as an anticoagulant. After the deep anesthesia was conformed, hearts were excised, the aorta was cannulated, and the hearts were perfused through the aorta in Langendorff mode at a constant (100 cm of water) perfusion pressure at 37 °C with the KHB for a 5 min washout period as described previously. The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer (millimolar concentration: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium dihydrogenphosphate 0.36, magnesium sulfate 1.2 and glucose 10), and after its oxygenization pH was 7.4 at 37 °C. During the washout period left atria was cannulated, and the Langendorff preparation was switched to the working mode for 10 min with a left atrial filling pressure of 17 cm H₂O, aortic afterload pressure was set to 100 cm of water.

3. Induction of ischemia and reperfusion:

After aerobic perfusion of the heart, both the aortic outflow and pulmonary inflow lines were clamped at a point close to the origin of

the aortic and pulmonary cannulas, thus the global ischemia could then be maintained for any desired period by clamping the inflow line. Reperfusion could be initiated by unclamping and removing the occluders.

4. Left ventricular function measurement:

An epicardial ECG was recorded by a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia) throughout the experimental period with the use of two silver electrodes attached directly to the heart. ECGs were also recorded for the incidence of ventricular fibrillation (VF) and ventricular tachycardia (VT) during the first 2 min of “nonworking” Langendorff reperfusion. The heart was considered to be in VF if an irregular undulating baseline was present on the ECG. VT was defined as five or more consecutive premature ventricular complexes, and this classification included repetitive monomorphic VT, which is difficult to dissociate from rapid VT. The heart was considered to be in sinus rhythm if normal sinus complexes in a regular rhythm were present on the ECG. The first 10 min of 2-h reperfusion period was initiated by “nonworking” Langendorff mode. If VT and VF developed and the sinus rhythm did not spontaneously return within the first 2 min of “nonworking” Langendorff reperfusion, hearts were electrically defibrillated by a defibrillator using two silver electrodes and 15-V square-wave pulse of 1-ms duration and reperfused. Then, after the first 10 min of Langendorff reperfusion, hearts were further reperfused by switching

to “working” mode for an additional 110 min. After 10 min of working mode perfusion baseline parameters were recorded. To monitor the recovery of the heart, the left ventricular cardiac function was recorded after 30, 60 and 120 min of reperfusion. A calibrated flow-meter was used to measure the aortic flow (AF). Coronary flow (CF) was measured by timed collection of the coronary effluent dripping from the heart. During the entire experiment aortic pressure was monitored using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH, USA) connected to a side arm of the aortic cannula, the signal was amplified using a Gould 6600 series signal conditioner.

5 a. Measurement of infarct size (during experiment with the sour cherry seed extract and resveratrol):

The myocardium for infarct size determination was perfused, at the end of each experiment, with 25 ml of 1% triphenyl tetrazolium solution in phosphate buffer (Na_2HPO_4 88 mM, NaH_2PO_4 1.8 mM) via the side arm of the aortic cannula, and stored at -70°C for later analysis. Frozen myocardium was sliced transversely (38) in a plane perpendicular to the apical-basal axis into 2- to 3-mm-thick sections, weighted, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). Using the National Institutes of Health Image 1.16 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction,

brightness, and contrast enhancement for improved clarity. The infarct zone in each slice was traced, and the respective area was calculated in terms of pixels. The areas were measured by computerized planimetry software, and these areas were multiplied by the weight of each slice, then the results were summed up to obtain the weight of the risk zone (total weight of the left ventricle; mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

5 b. Infarct size estimation (during the experiment with resveratrol and γ -tocotrienol):

Infarct size was measured according to the TTC method. After the 2 h of reperfusion, 40 ml of 1% (w/v) solution of triphenyl tetrazolium chloride (TTC) in phosphate buffer was infused into aortic cannula, and the heart samples were stored at -70°C for subsequent analysis. Sections (0.8 mm) of frozen heart were fixed in 2% paraformaldehyde, placed between two cover slips and digitally imaged using a Microtek ScanMaker 600z. To quantitate the areas of infarct in pixels, standard NIH image program was used. The infarct size was quantified and expressed in pixels.

6 a. Determination of cardiomyocyte apoptosis (during experiment with the sour cherry seed extract and resveratrol):

The formaldehydefixed left ventricle was embedded in paraffin, cut into transverse sections (4 μm thick), and deparaffinized with a graded series of xylene and ethanol solutions.

Immunohistochemical detection of apoptotic cells was carried out using terminal deoxynucleotidyl transferase- mediated dUTP nick-end labeling (TUNEL) in which residues of digoxigenin-labeled dUTP are catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotide was incubated with a sheep polyclonal anti-digoxigenin antibody, followed by a FITC-conjugated rabbit anti-sheep IgG as a secondary antibody, as described by the manufacturer. The sections (n = 6) were washed in PBS three times, blocked with normal rabbit serum, and incubated with mouse monoclonal antibody recognizing-sarcomeric actin, followed by staining with tetra-rhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (1:200 dilution). For detection of apoptosis in endothelial cells, the sections were first stained with TUNEL (FITC staining). The sections were then incubated with rabbit polyclonal anti-von Willebrand factor as a primary antibody, followed by incubation with tetra-rhodamine isothiocyanate-conjugated goat anti-rabbit IgG as a secondary antibody. The fluorescence staining was viewed with laser confocal microscopy. For quantitative purposes, the number of TUNEL-positive cardiomyocytes and endothelial cells was counted on x 100 highpower fields (HPF, magnification x 600) from the endocardium through the epicardium of the midportion of the left ventricular free wall in five sections from each heart.

6 b. Assessment of apoptotic cell death (during the experiment with resveratrol and γ -tocotrienol)

Immunohistochemical detection of apoptotic cells was carried out using the TUNEL method. Briefly, after the isolated heart experiments the heart tissues were immediately put in 10% formalin and fixed in an automatic tissue fixing machine. The tissues were carefully embedded in the molten paraffin in metallic blocks. Prior to analysis of tissues for apoptosis, the samples were sectioned and placed on glass slide. The tissue sections were deparaffinized with xylene, washed and rehydrated by sequential washing with different concentrations of ethanol. Then the TUNEL staining was performed according to the manufacturer's instructions. The fluorescence staining was viewed with a fluorescence microscope at 520 ± 20 nm for green fluorescence of fluorescein and at 620 nm for red fluorescence of propidium iodide. The number of apoptotic cells was counted and expressed as a percent of total myocyte population.

7. Measurement of caspase-3 expression by immunohistochemistry:

The free-floating sections of the heart were first incubated with biotinylated goat anti-caspase-3 antibody (diluted 1:1,000) for 2 days at 4°C. The immunological and immunocytochemical characteristics of antibody have been published earlier. Sections were then transferred into a solution of biotinylated rabbit antibody (diluted 1:200) for 50 min at room temperature, then into avidin-biotinylated-peroxidase

complex (diluted 1:100) for 4 h at room temperature, and were completed with a diaminobenzidine chromogen reaction. Before, sections were kept in 10% normal goat serum for 50 min. All incubations were performed under continuous gentle agitation, and all of antibodies were diluted in 10 mM PBS (pH 7.4) to which 0.1% Triton X-100 and 1% normal rabbit serum (Vector) were added. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium.

8. Western-blot analysis:

Left ventricles from the hearts were homogenized in 1 ml of buffer and proteins were isolated. A total of 50 µg protein were separated in SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and probed with primary antibody 1:1000 dilution overnight. The protein bands were detected using horseradish peroxidase conjugated secondary antibody and Western blot luminol reagent. The bands were digitized, subjected to densitometric scanning and normalized against the loading control.

9. Measurements of serum glucose and insulin levels:

Blood samples were obtained from rats before the excision and isolation of hearts, and serum glucose levels were measured by a spectrophotometer at a wavelength of 340 nm using standard assay kits. Insulin levels were also determined from the same samples using radioimmunoassay kits in all untreated, glucose-treated, and resveratrol-treated groups.

10. Determination of ET in the coronary effluent:

Coronary effluents (100 ml) were collected in polystyrene containers spiked with EDTA and Triton X-100 to give a final concentration of 5 mM and 0.5% (vol/vol), respectively, in each sample, and then samples were frozen at -70°C until their use. Perfusates were loaded with 3 ml of methanol and 5 ml of water conditioned by Sep-Pak C18 cartridges, and ET was eluted with 2 ml of 60% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid, yielding a mean final concentration of 60%. Eluates were freeze-dried and kept at -20°C until further radioimmunoassay determination. Residues were re-dissolved in 0.5 ml of assay buffer and concentrated 200 times. ET immunoreactivity was then determined by radioimmunoassay using commercial ET radioimmunoassay kits according to the manufacturer instruction.

11. Immunofluorescence techniques and image analysis:

Heart tissue samples collected at the end of experiments were fixed in 4% buffered paraformaldehyde (pH 7.4), embedded in paraffin and sectioned. After deparaffinizing the sections the antigen retrieval treatment was performed using 10 mM sodium citrate containing 0.05% Tween 20 at 90–95 °C for 30 min. After washing with PBS, the slides were blocked with Powerblock for 10 min. Slides were washed with PBS and incubated with primary antibodies (rabbit LC3II; (1:50 dilution) in PBS containing 1% BSA for 2 h. After washing, the slides were incubated with fluorescein-conjugated

secondary antibodies (1:1000 dilutions) in the dark for 45 min. For nuclear staining To- Pro 3 iodide (1:1000 dilution) was used for 45 min in the dark. The slides were washed and covered with mounting medium. Confocal microscopic images were obtained using a Zeiss LSM 510 (Thornwood, NY) confocal laser scanning microscope with 40 × 1.3 oil immersion objective.

12. Transmission electron microscopy:

Small sample of myocardium were fixed in 4% glutaraldehyde. Membranes contrast was enhanced using an osmium-ferrocyanide mixture in 0.1M cacodylate buffer in the postfixation step. Subsequently, the samples were dehydrated, infiltrated and embedded in Epon 812 at 60 °C for 48 hours. Light microscopy was performed on 1 µm semithin section stained with 1% toluidine blue and digital images were recorded using a CCD AxioCam HRc Zeiss camera with AxioVision software on Nikon Eclipse E600 microscope (Nikon Instruments, Inc.). Routine 60 nm ultrathin sections were cut with a diamond knife, mounted on formwar-coated grids and stained with 1% uranyl acetate and Reynolds's lead citrate. Ultrathin sections were examined using a Morgagni 286 TEM (FEI Company, Eindhoven, Nederland) at 60 kV. Digital electron micrographs were recorded with a MegaView III CCD using iTEM-SIS software (Olympus, Soft Imaging System GmbH, Germany).

13. Statistic:

HR, CF, AF, LVDP, infarct size, and apoptotic cells were expressed as means \pm SEM. A two-way analysis of variance was first carried out to test for any differences in mean values between groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free group by Dunnett's or modified t-test. A different procedure, because of the nonparametric distribution, was used for the distribution of discrete variables, such as the incidence of VF and VT. An overall khi -square test was used to compare individual groups.

Results

I, The effect of sour cherry seed extract treatment on the ischemia/reperfusion-induced injury.

I a. The effect of sour cherry seed extract on the incidence of left ventricle arrhythmias.

We have determinate the incidence of reperfusion-induced VF in isolated hearts obtained from rats treated with 0, 1,5, 10, and 30 mg/kg of sour cherry seed extract for 14 days, respectively, and subjected to 30 min of ischemia followed by 120 min reperfusion. Thus, with the doses of 1, 5, 10, and 30 mg/kg of sour cherry seed kernel extract, a significant dose dependent reduction in the incidence of reperfusion-induced VF was detected from its control value of 92% to 92%, 75%, 50%, and 17%, respectively. In the reduction of the

incidence of reperfusion induced VT the same protection was observed.

I b. The effect of sour cherry seed extract on the postischemic recovery:

The protection against the development of reperfusion-induced VF and VT in rats treated with various doses of sour cherry seed kernel extract reflected in the improvement in the recovery of postischemic cardiac function. Thus, in hearts obtained from rats treated with 10 and 30 mg/kg of sour cherry seed kernel extract, a significant recovery in CF, AF, and LVDP was observed compared to the drug-free control values. For instance, after 30 min of ischemia followed by 60 min of reperfusion, AF was significantly increased from its drug-free ischemic-reperfused control value of 9.5 ± 0.7 to 22.0 ± 1.5 and 27.2 ± 2.1 ml/min with the concentrations of 10 and 30 mg/kg of extract, respectively. The same extent of postischemic recovery was observed in CF and LVDP. Interestingly, HR was not substantially and significantly changed using various doses of the extract. The lower doses of sour cherry seed kernel extract (1 and 5 mg/kg) failed to significantly improve the postischemic function.

I c. The effect of sour cherry seed extract on the infarct size:

The infarct size was significantly reduced from its drug-free control value of 38.3 ± 1.3 to $26.5 \pm 2\%$ and $21.8 \pm 1.8\%$, in hearts obtained from rats treated with the concentrations of 10 and 30 mg/kg

of sour cherry seed kernel extract for 14 days and subjected to 30 min of ischemia followed by 120 min of reperfusion. Lower concentrations of the extract (1 and 5 mg/kg) failed to significantly reduce infarct size.

I d. The effect of sour cherry seed extract on the caspase 3 expression and apoptosis:

It is clear from our results that numbers of apoptotic cells were significantly reduced in hearts treated with the kernel extract of sour cherry seed compared with the extract free ischemic-reperfused control group. In the drug free ischemic we have detected 21 ± 4 % apoptotic cells, whereas only 15 ± 3 and 12 ± 3 % apoptotic cells were detected with the concentrations of 10 and 30 mg/kg of sour cherry seed kernel extract for 14 days, respectively.

Caspase-3 is an aspartate-specific cysteine protease, which can be found in a zimogen form in cells. At the beginning of apoptosis procaspase-3 is cleaved to form the active caspase-3. Based on our results we can conclude that 10 and 30 mg/kg sour cherry seed extract treatment reduce the expression of the caspase-3.

II. The effect of resveratrol on the ischemia/reperfused “Zucker Obese” myocardium.

II a. The effect of resveratrol on the bodyweight serum insulin and glucose level:

The results show body weight, serum glucose, and insulin levels in Zucker obese age-matched rats treated with glucose and resveratrol. Thus, in obese rats treated with 5 mg/kg of resveratrol for 2 wk, a significant reduction was observed in body weight and serum glucose from their control values of 414 ± 10 g and 7.08 ± 0.41 mmol/l to 378 ± 12 g and 6.11 ± 0.44 mmol/l. However, insulin levels were not changed in the resveratrol treated group. In Zucker obese rats that obtained 10% of glucose ad libitum for 3 wk, a significant increase in body weight and serum glucose levels was detected compared with the glucose-free Zucker obese group. In another group, Zucker obese rats obtained 10% of glucose for 3 wk, and, during the 2nd and 3rd wk, rats also received orally 5 mg/kg/day of resveratrol. Thus a significant reduction was observed in the body weight and serum glucose level from 504 ± 16 g and 9.02 ± 1.20 mmol/l (in the glucose-treated group) to 428 ± 11 g and 7.21 ± 0.51 mmol/l in the resveratrol-treated group. However, a change in serum insulin levels was not detected in any of resveratrol-treated groups.

II b. The effect of resveratrol on the postischemic recovery:

The results clearly show that postischemic recovery of CF, AF, and LVDP was significantly improved in the resveratrol-treated groups in the presence or absence of glucose intake compared with the resveratrol-free group. Thus, for instance, after 60 and 120 min of reperfusion, AF was significantly increased from their obese control values of 5.2 ± 0.4 and 5.1 ± 0.6 ml/min to 7.9 ± 0.6 and 7.8 ± 1.0

ml/min in the obese rats treated with 5 mg/kg of resveratrol, respectively. The same improvement in CF and LVDP was obtained in the obese rats treated with 5 mg/kg of resveratrol in the presence of 10% of glucose intake. However, HR was not significantly changed in hearts subjected to ischemia and reperfusion, in either the presence or absence of glucose intake in the resveratrol-treated groups.

II c. The effect of resveratrol on the incidence of ventricular tachycardia:

The reduction in the incidence of reperfusion-induced VF in the resveratrol-treated groups is probably related to the recovery of postischemic cardiac function and the beneficial effects of resveratrol on the ischemic myocardium. Thus the incidence of reperfusion-induced VF was significantly reduced in rats treated with 5 mg/kg of resveratrol from its obese drug-free control value of 100% to 17% and 33% in the presence or absence of glucose intake, respectively.

II d. The effect of resveratrol on the infarct size:

Our results show that the resveratrol treatment is capable to decrease the infarct size in ischemic-reperfused myocardium. Thus resveratrol significantly reduced the infarct size from $41 \pm 6\%$ (in the OC group) and $42 \pm 7\%$ (in the O+G group) to $21 \pm 5\%$ in the O+R group and $26 \pm 6\%$ in the O+G+R group, respectively.

II e. The effect of resveratrol on the endothelin release:

The ET concentration was significantly increased from 0.39 \pm 0.13 fmol/min/g (lean control group) to 1.49 ± 0.25 fmol/min/g (OC

group) and 1.79 ± 0.35 fmol/min/g (O+G group). ET release was significantly attenuated in all groups treated with 5 mg/kg of resveratrol. Our results clearly show that attenuation in ET release in resveratrol-treated groups can lead to an elevated cardiac perfusion, which is indicated by an increase in postischemic CF and AF, leading to a better recovery in LVDP (contractility) during reperfusion.

II f. The effect of resveratrol on the expression of ET-1 and Glut-4:

ET-1 expression was significantly increased in the OC and O+G group compared with the lean control, and resveratrol substantially reduced ET-1 expression compared with the resveratrol-free OC and O+G groups. In contrast to ET-1, the repression of GLUT-4 was detected in isolated hearts obtained from Zucker obese rats in the absence (OC) or presence (O+G) of glucose. When 5 mg/kg of resveratrol were administered, the expression of GLUT-4 was significantly increased in the ischemic-reperfused myocardium. These changes induced by resveratrol in ET-1 and GLUT-4 signaling could be responsible for the recovery of postischemic cardiac function.

II g, The effect of resveratrol on apoptosis:

Similar to myocardial infarct size, the numbers of apoptotic cardiomyocytes were significantly reduced when rats were pretreated with resveratrol and hearts were isolated and subjected to ischemia and reperfusion. Total numbers of cardiomyocytes at $\square 100$ HPF, which covers almost all of the midportion of the left ventricular free wall,

were examined for detecting apoptotic cells. The data are expressed in counts/100 HPF and not in percentage of apoptotic cells. For subjects treated with 5 mg/kg of resveratrol, the numbers of cardiomyocyte apoptotic cells were significantly reduced after ischemia-reperfusion in the presence or absence of glucose intake.

III. The effect of resveratrol and/or γ -tocotrienol on the I/R-induced injury:

III a. The effect of resveratrol and/or γ -tocotrienol on the postischemic cardiac function:

As expected, both resveratrol and γ -tocotrienol treatment protected the hearts against ischemia/reperfusion injury as evidenced by improved postischemic AF, LVDP, and LVdp/dt in comparison with the vehicle treated control group. However, there were no significant differences between the resveratrol and γ -tocotrienol groups. The hearts obtained from the resveratrol + γ -tocotrienol treated group exhibited enhanced cardiac function after 2 hrs of reperfusion compared to either resveratrol or γ -tocotrienol alone, suggesting an additive effect between resveratrol and γ -tocotrienol on improving cardiac function. Thus, for instance, after 120 min of reperfusion aortic flow was $7,8 \pm 1,7$ ml in control group, it was $22,1 \pm 1$ ml in resveratrol treated, $21,2 \pm 0,9$ ml in γ -tocotrienol treated group and $27,1 \pm 0,7$ ml in the dual treated group. In dual treated group, besides

the other functional parameters, coronary flow was also significantly higher compared to the control group. Wortmannin treatment abolished the protective effect of both the mono therapies and the dual treatment. Similar to Wortmannin, 3-MA also suppressed the postischemic functional parameters; however it should be noted that the effect of 3-MA on the suppression of cardiac function was to a lesser extent than that of Wortmannin in our experimental circumstances.

III b. The effect of resveratrol and/or γ -tocotrienol on the infarct size and apoptosis:

Both resveratrol and γ -tocotrienol treatments significantly reduced the I/R-induced infarct size compared to the drug free control ($22,2 \pm 1,9$ %, $23,7 \pm 0,8$ vs. $38,2 \pm 1,5$), and further reduction in infarct size was observed in the hearts with dual therapy ($17,7 \pm 1,4$ %), although it was not statistically significant compared to the resveratrol or γ -tocotrienol treated group alone. As the infarct size is contributed from both necrosis and apoptosis, we estimated the apoptosis employing TUNEL assay. In consonance with the infarct size both of the mono therapies significantly decreased the percentage of apoptotic cells, and a more pronounced decline in case of dual treatment was observed, although it was not statistically significant compared to mono therapies. Similar to infarct size, Wortmannin abolished the protective effect of resveratrol or tocotrienol on apoptosis induced by ischemia/reperfusion. Consistent with our

TUNEL assay results, we have detected higher level of procaspase 3 in the mono treated groups as well as in the dual treated group, which were decreased after Wortmannin treatment, indicating the cleavage and activation of caspase 3.

III c. The effect of resveratrol and/or γ -tocotrienol on the induction of survival signals in the ischemic reperfused heart:

In order to examine the effect of the mono and dual therapies on survival signal, we examined the level of Bcl-2 and the activation of Akt, which was studied by measuring the ratio of phosphorylation of Akt at 473 to total Akt by Western blot. Both resveratrol and γ -tocotrienol induced extensive activation of Akt. However, dual treated hearts showed more pronounced activation of Akt and dramatically increased Bcl-2 level compared to the monotherapies, further suggesting a synergic effect on the induction of survival by these two compounds. It appears from our results that resveratrol has more prominent effect on activation of the Akt where as γ -tocotrienol enhances the level of Bcl-2. Furthermore, perfusing the hearts with the PI3 kinase inhibitor Wortmannin diminished the activation of Akt as well as decreased the level of Bcl-2 induced by mono and dual treatments.

III d. The effect of resveratrol and/or γ -tocotrienol on the induction of autophagy:

To determine whether autophagy contributes to the cardioprotective effect of resveratrol or γ -tocotrienol, we determined the level of Beclin-1 and the ratio of LC3II/LC3I. As expected, ischemia/reperfusion slightly enhanced the ratio of LC3II/LC3I and the level of Beclin-1. Significant increment in the ratio of LC3II/LC3I as well as in the level of Beclin-1 was found in the hearts treated with resveratrol or γ -tocotrienol alone. Similar to survival signaling molecules, Wortmannin treatment abolished the effect of resveratrol or γ -tocotrienol on the level of Beclin-1 and ratio of LC3II/LC3I. Significant and more extensive increase in the level of Beclin-1 and LC3II/LC3I ratio were found in the dual treated hearts, which further supports the existence of the synergic effects between resveratrol and γ -tocotrienol. Consistent with our Western blot data, our fluorescent microscopy revealed that normal myocardium contained a few LC3 stained cells, which were enhanced after ischemia/reperfusion. The hearts obtained either from resveratrol or γ -tocotrienol treated animals exhibited more LC3 positive cells, and further increment was detected in the dual treated animals' hearts after ischemia and reperfusion. Wortmannin treatment reduced the number of LC3 stained positive cells. The light microscopy on semithin section of samples treated with resveratrol, γ -tocotrienol and resveratrol + γ -tocotrienol showed almost normal morphology with very few degenerative changes on isolated cardiomyocytes. However, the myocardium in hearts treated with 3-methyladenine clearly showed oncotic changes with

myofibrillar contraction bands and vacuolar degeneration. To visualize the autophagosomes, transmission electron microscopy was employed. Autophagosomes have been identified as intracellular structures with double limiting membrane (smooth ribosome free double membrane) that contain morphologically intact cytoplasmic material. Our results revealed that control myocardium showed a normal morphology without ultrastructural changes. Ischemia/reperfusion induced oncotic changes in the myocardium with myofibrils disorganization, mitochondrial swelling and cellular lyses. Few small autophagosomes were seen in both cases. Electron microscopy examination of resveratrol, γ -tocotrienol and resveratrol + γ -tocotrienol treated samples showed an almost normal ultrastructure and the presence of numerous autophagosomes in different stages of maturation. Early autophagic vacuoles contain still identifiable organelles. The autophagosomes enclose single or grouped mitochondria, lamellar structures and cytoplasmic content. Few small autophagosomes have been seen in samples with 3-methyl adenine but ultrastructural ischemic changes (mitochondrial swelling, dilated cisternae of endoplasmic reticulum, myofibrils contraction or disruption) were more prominent.

III e. Differential effects of resveratrol and γ -tocotrienol on activation of mTor.

To examine the mechanisms of initiation of the autophagy, we studied the level and the phosphorylation status of mTOR. We found extensive phosphorylation of mTOR at baseline condition, which was slightly lowered after ischemia/reperfusion. In case of resveratrol treatment, the phosphorylation of mTOR was almost identical to the vehicle treated I/R control, and enhanced after Wortmannin treatment. In contrast, γ -tocotrienol treated hearts displayed lower level of p-mTOR as well as mTOR. Similar to the γ -tocotrienol treated hearts, the dual treated hearts also showed lower level of p-mTOR.

Discussion

Sour cherry seed is a byproduct of the industry in Hungary. It has been suggested that sour cherry seed extract possesses retinoprotective effect, and it maybe can prevent I/R induced injury in the heart. During the first period of my work we have studied the effect of the sour cherry seed extract in ischemic/reperfused myocardium. We have determined the incidence of ventricular fibrillation and the pre- and postischemic heart function. Furthermore, at the end of experiments, infarct size, apoptosis, and caspase-3 expression were measured. In the light of our results, we concluded that resveratrol is capable of reducing ischemia/reperfusion induced ventricular fibrillation in a dose dependent manner. Moreover, after the sour cherry seed extract pretreatment, hearts are resistant to I/R induced damage as evidenced by the improved postischemic cardiac

function, reduced infarct size and apoptosis. Our investigations revealed that sour cherry seed extract reduces the expression of caspase-3.

Caspases are the members of a conserved cysteine protease enzyme family (caspase = cystein-dependent aspartate-specific protease), which play a key role in the evolutionary conserved process of programmed cell death. They are produced as precursors and activated via two different well regulated pathways. One of the two pathways is the so called death receptor, and the other is the mitochondrial pathway, which is activated by cellular stress and the release of cytochrom c from the mitochondria. The caspase-3 belongs to effector caspases, which are responsible for the destruction of cells. According to early studies, hypoxia or ischemia was thought to be enough to induce apoptosis, but latter this concept has been changed. It has been shown that not the ischemia or hypoxia, but the acidification, the reoxygenisation and reperfusion are accountable for the apoptosis in the myocardium. Studies with different specific and nonspecific capsase inhibitors have proved that inhibition of caspases lead to reduced apoptosis and cardiac damage induced by I /R. Based on our results we can conclude that sour cherry seed extract is capable of reducing apoptosis via inhibition of caspase-3, but we cannot rule out that inhibition of other caspases also play a role in the cardioprotective effect of sour cherry seed extract. The chances are that, beside caspase inhibition, other mechanisms also contribute to the beneficial effect of

the sour cherry seed extract. Many components of the sour cherry seed possess antioxidant effects. Furthermore, it has been shown that sour cherry seed extract protects the retina against ischemia/reperfusion-induced injury, via the induction of hemeoxygenase-1 expression, which ultimately leads to enhanced endogenous CO levels. It should be noted that the examination of the HO-1 system was not the aim of the present study, but we can suppose that the HO-1 system also one of the contributing factor.

In the present study, the kernel extract of sour cherry seed was applied before the induction of ischemia, thereby slowing the rate of development of ischemic injury so that, at the time of reperfusion, the myocardium in the treated group is less severely injured. Because the extent of damages, including postischemic cardiac function, is proportional to the severity of the antecedent ischemic period, it is impossible to ascertain whether the observed protection by sour cherry seed extract is a direct consequence of a reduction of reperfusion-induced damage or is secondary to some of its antiischemic effect.

In the second study we have investigated the effect of resveratrol, one of the possible bioactive components of sour cherry seed. During the experiments, we were seeking for the answer whether resveratrol also can protect the “diseased” myocardium against I/R-induced damage. In the light of our results it appears that resveratrol is capable of protectinf the diseased myocardium against I/R-induced

injury evidenced by the improved postischemic cardiac function, reduced infarct size and apoptosis.

It is well known that diabetes and metabolic syndrome X are important risk factors in different cardiovascular diseases. Moreover, among the diabetic patients the incidence of the ischemic heart diseases and its severity is more pronounced. The mortality after myocardial infarction is 2-3 times higher compared to the non-diabetics. Furthermore, the incidence of heart failure after myocardial infarction is also doubled. One of the most important differences between the diabetic and non-diabetic myocardium is the altered metabolism especially the decreased glucose and enhanced fatty acid utilization. The switch from glucose to fatty acid increases the severity of the ischemia, and reduces the postischemic recovery. It has been shown that the restoration of insulin sensitivity by rosiglitason improve the postischemic recovery in “Zucker Obese” rat. The animals, used in their experiments, had insulin resistance along with a reduced Glut-4 expression, which were restored after rosiglitason treatment. Another related study, using a Glut-4 knock out mouse model, has found reduced glucose uptake in the hearts of Glut-4 KO mouse, which was vulnerable to the I/R damage. These results highlight the importance of Glut-4 transporter in diabetic as well as in non-diabetic patients.

In our experiments we examined whether Glut-4 plays any role in the cardioprotection induced by resveratrol. Our results confirm that

the resveratrol is capable for restoring the Glut-4 expression, and at least by part this effect is responsible for the observed protection. One of the experimental groups received an extra sugar load to further aggravate the metabolic changes induced by diabetes. We found that the resveratrol is able to induce the level of Glut-4 in these circumstances also. Furthermore, our results revealed that resveratrol treatment reduced bodyweight and blood sugar level without any effect on serum insulin levels.

The endothelial dysfunction has been connected to various cardiovascular diseases such as arteriosclerosis and other ischemic cardiac diseases. It is well known that endothelial cells play an important role in the regulation of the cardiovascular system via the production of many different vasoactive compounds. An elevated endothelin level was found in the coronary effluent after bypass surgery in diabetic patients. Moreover, micro vessels originated from diabetic patients gave a more pronounced contractive response after exposing them to ET-1 than the vessels obtained from non-diabetic patients.

In the current study, we also investigated endothelin levels. An increased endothelin level was found in the coronary effluent of the obese animals after I/R, which was further enhanced in the sugar loaded group. After treating the animals with 5 mg/ttkg resveratrol, the level of endothelin was found to be decreased. Similar results were obtained from the Western-blot analysis of ET-1. In these endothelin

signal suppressing effect of resveratrol presumably both direct and indirect mechanisms play a role. It has been demonstrated that resveratrol is able to reduce the strain-induced ET-1 release and ET-1 mRNA level in Human Umbilical Vein Endothelial Cells. The authors concluded that the responsible factors are the reduced ROS level and the reduced ERK1/2 signaling. At least by part the above mechanism explains the results of the present investigation. Beside this, another indirect mechanism also could contribute to the endothelin lowering effect of resveratrol. Namely, the elevated glucose level also induces the secretion and the expression of the ET-1. Moreover, a related study showed that the high glucose levels enhance ET-1 levels, and it can be reversed by the reduction of the glucose concentration. Based on these results and our observations, that resveratrol reduced the blood sugar in the treated groups, it is likely that this indirect effect also contribute to the decreased endothelin signaling. Thus, we can conclude that resveratrol can protect the heart of the obese and diabetic animals against I/R-induced injury evidenced by the improved postischemic cardiac function, reduced infarct size and apoptosis. This effect of resveratrol can be explained by the improved glucose utilization, reduced endothelin signaling.

In our third set of experiments we were seeking for the answer whether resveratrol has any synergetic effect with γ -tocotrienol. We can conclude that there is a synergetic effect between resveratrol and γ -tocotrienol. As it was expected, based on our previous experiments,

both resveratrol and γ -tocotrienol possess cardioprotective effects evidenced by the improved postischemic cardiac function. In the dual treated group further increment was observed in the postischemic cardiac function, which was significant in comparison with the mono therapeutic groups. Similar results were seen in the case of infarct size and apoptosis. We found reduced infarct size and apoptosis in the treated group among which in the dual treated group was the smallest, but statistically not significant compared to the mono treated groups. Similar results were observed in the procaspase-3 expression.

We also studied the expression of the antiapoptotic Bcl-2 protein. Bcl-2 was shown to protect the myocardium via different mechanisms. For instance Bcl-2 can inactivate the Bax/Bak proteins and with this can block the permeabilization of the mitochondrial outer membrane. Moreover, decreased numbers of apoptotic cells were indentify in the hearts of Bcl-2 Tg mice along with an improved postischemic cardiac function and reduced infarct size after ischemia/reperfusion. Our Western-blott results revealed that all treatments can enhance the Bcl-2 expression; among them the strongest induction was observed in the dual treated group. Similar results were observed when we studied the survival protein Akt. Based on this, it appears that the Akt-Bcl-2 survival pathway play an important role in the cardioprotective effect of resveratrol and γ -tocotrienol, moreover, this survival pathway is responsible, at least by part, for the synergistic effect.

Another aim was to study the role of the autophagy in the cardioprotective effects of resveratrol and γ -tocotrienol. Initially, autophagy was considered as the non-apoptotic form of programmed cell death. Nowadays, this concept is changing and increasing number of evidence support that beside the death under some circumstances autophagy also can induce cell survival. Enhance autophagy was described in the chronically ischemic swine myocardium; the autophagy was pronounced in the living area and almost absent in the death myocardium. In our previous study we found enhanced autophagy after ischemic precondition (IPC), which was mediated via the induction of Bag-1. Silencing the Bag-1 suppressed the autophagy and the protection induced by IPC. In the current study, we evaluated the level of Beclin-1 and the ratio of LC3II/LC3I with Western-blot. The LC3II is one of the best markers for the identification of the autophagosomic membrane. Beside the LC3 we have used the Beclin-1 as a marker. According to our results, all of the treatments enhanced the level of Beclin-1 and the ratio of LC3II/LC3I, but the most extensive changes were observed in the dual treated group. In line with the literature, our immunohistochemistry staining revealed slight LC3II staining in the I/R group. Further increment was detected in the treated groups, which were reversed by Wortmannin.

The Wortmannin treatment reduced the number of LC3II staining, the ratio of LC3II/LC3I and the level of Beclin-1. Moreover, the Wortmannin treatment abolished the beneficial effect of both of the

compounds, evidenced by the poor postischemic cardiac function, the enhanced infarct size and apoptosis. In line with the poor cardiac recovery, low level of Bcl-2 and reduced activation of Akt pathway were detected in the Wortmannin treated groups, suggesting that autophagy plays a role in the cardioprotection induced by these two natural products.

Consistent with these results, light microscopic data showed the presence of oncotic myocytes characterized by contraction band necrosis and vacuolar degeneration in tissues obtained from ischemia/reperfusion and those treated with 3-methyladenine indicating that resveratrol and γ -tocotrienol mediated cardioprotection was inhibited by 3-MA. Transmission electron microscopy echoed these results, which clearly demonstrate that the normal structure was disrupted after ischemia/reperfusion resulting in disorganization of myofibrils and mitochondria with swelling and dense structures. Both resveratrol and tocotrienol resulted in autophagy as indicated by the presence of autophagosomes containing mitochondria enclosed in a double membrane.

To check how the autophagy induction is being mediated, we have studied the phosphorylation status of mTOR. It seems from our results that ischemia/reperfusion itself decreases the p-mTOR/mTOR ratio indicating slight activation of autophagy. Resveratrol further decreased the ratio of mTOR, and the treatment with Wortmannin brings back the ratio closer to the normal value. We found lower ratio

of p-mTOR/mTOR in case of tocotrienol treated hearts suggesting that autophagy induced by tocotrienol occurs through mTOR pathway. Further increment was noticed in dual treated hearts which was lowered by Wortmannin. It appears from our results that γ -tocotrienol-induced autophagy at least in part is being mediated through mTOR pathway, and resveratrol-mediated autophagy is less dependent on mTOR.

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