

**SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)**

**The role of heme oxygenase-1 enzyme system on ischaemic myocardium**

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## Introduction

Despite a slight improvement on statistical data, cardiovascular diseases are still among the highest risk factors of mortality rates all over the world. The number of patients die due to cardiovascular (CV) diseases is doubled than due to cancer. Since the treatment of CV patients an increasing burden on the national economy, it is clear that better (cost-effective) treatment options and new prevention strategies are becoming increasingly important, which are less expensive than treatment of the disease. Nowadays, the secondary prevention strategies, e.g. slowing or stopping the progression of the disease, and identification of the „high risk” populations to reduce their risk factors, are becoming more relevant than the primer prevention strategy itself. Recently, an emerging trend shows that beside the synthetic drugs the use of bioactive compounds as preventing purposes or as pharmaceutical agent become more important. A cheaper production process of plant derivatives or more favorable side effects could be in the background. An increasing number of studies on animal models report that prophylactic administration of certain plant extracts or natural substances significantly reduce ischemia / reperfusion (I / R) injuries. Sour cherry seed is a possibly active natural material and it is available in large quantities as an industrial byproduct in Hungary.

Oxidative stress, which biochemical process could be characterized by a great amount of oxygen and nitrogen species, and it is one of the most cardinal cellular damaging factors. Materials generated by partial reduction of oxygen are extremely instable and reactive agents. At normal conditions in eukaryotes they are generated by different metabolic processes, respiration, and phagocytosis and through the effect of growth factors and cytokines. In order to balance the toxic effects of reactive oxygen species (ROS) cells utilize both enzymatic and non-enzymatic processes. The latter applies e.g. glutation, ascorbic acid,  $\alpha$ -tokoferol, while superoxide dismutase, catalase, glutathion and heme oxygenase are among the applied enzymatic conditions.

Heme oxygenases are evolutionarily conserved enzymes which catalyse the first step of degradation of heme. Certain endproducts of the catabolism play a crucial role of several important physiological processes of the organisms as an antioxidant, antiapoptotic-, anti-inflammatory-, anti proliferatory-, vasoactive, antithrombotic and angiogenesis supporting agent. These processes assist the cell survival after different injuries.

Previously we have investigated the effect of the sour cherry seed extract (SCSE) against injury caused by I/R. Based on our findings, SCSE decreases infarct size and the incidence of ventricular fibrillations (VF) as a dose dependent manner and it improves postischaemic heart functions and decreases caspase-3 expression.

Nevertheless, more exact underlying mechanisms were still unclear. Furthermore, another investigation of our team showed that SCSE was capable to reduce the I/R injury of rat retina at least by part of inducing heme oxygenase-1 (HO-1) expression. Therefore we hypothesized whether SCSE is susceptible to induce HO-1 expression in the heart tissue as well. Additionally, we aimed to research the exact role of HO-1 preventing myocardial injury caused by I/R and to examine the responsibility of the possible HO-1 overexpression to the potent cardioprotective effect of SCSE.

## Materials and methods

### Animals

Male Sprague Dawley rats (220-300 g) and wild-type non-transgenic (NTg), HO-1 Tg and HO-1 knock out (KO<sup>-/-</sup>) mice (25–35 g) (Charles Rivers Laboratories, Sulzfeld, Germany), were used for the studies. All animals were housed and 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). Maintenance and treatment of animals used in this study was additionally approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. Rats and mice were fed with commercial food pellets and water *ad libitum*.

### Transgenic mice

Tg were generated as described by Araujo et al.. In brief, CFY mouse eggs were injected with 52 kb rat HO-1 construct containing 27.7 kb of the 5' upstream region, 8.3 kb of HO-1 gene and 16 kb of the 3' downstream region. Cloning was carried out by PCR of rat genomic P1 library with two sets of PCR primers corresponding to the first exon (5'-GCT-TCG-GGGT-TAT-CTG-CCG-TTA-T-3' and 5'-CAG-TCT-TAC-AGG-CGG-GGA-ATGTGA-G-3'), and the fifth exon of rat HO-1 gene (5'-GAG-ACG-CCC-CGAGGA-AAA-TCC-CAG-AT-3' and 5'-CCC-AAG-AAA-AGA-GAG-CCA-GGCAAG-AT-3'). Two clones were identified as positives, and restriction mapping showed that both of them included the same insert. The 52 kb band was equivalent to the HO-1 gene and flanking regions. This 52 kb fragment, including the native HO-1 gene and its promoter, was excised and digested with  $\beta$ -agarase, and then microinjected. Mice were genotyped by PCR using the set of primers specific for rat HO-1 exon 5.

HO-1 knock out (KO) mice were also used during these experiments. This model were created by targeted mutation of HMOX1 gene. Homozygotes (-/-) for this mutation were generated by breeding heterozygotes. These mice were also genotyped by PCR.

## **Treatment of rats with sour cherry seed extract**

Animals were segregated into 2 test groups as described: Group 1—Animals were treated orally with 30 mg/kg SCSE (suspended in 2% hydroxyethylcellulose solution) daily for a time period of 8 weeks; Group 2—Animals in this group received vehicle solution (2% hydroxyethylcellulose solution) for the same time period.

## **Treatment of mice**

Transgenic mice were segregated into 2 groups. A group received 50  $\mu\text{mol/kg}$  tin-protoporphyrin-IX (SnPPIX) intraperitoneally 24 hours before performing isolated working heart experiments. The SnPPIX was used as a specific inhibitor of HO-1.

## **Isolated working heart experiments**

Rats were anesthetized by ketamine–xylazine (50/10 mg/kg, intraperitoneally), while mice received sodium pentobarbital (60 mg/kg, intraperitoneally) and all animals were heparinized (1000 IU/kg). Thoracotomy was subsequently performed under terminal anesthesia, followed by excision of hearts and placement of the organs in ice-cold modified Krebs–Henseleit buffer containing 118 mM NaCl, 5.8 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 0.36 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$  and 5 mM glucose. After the thoracotomy, the hearts were cannulated through the aorta and perfused in a Langendorff apparatus in “nonworking” mode (100 cm  $\text{H}_2\text{O}$ ) for 5 minutes to cleanly flush blood from vessels of the organ. During the Langendorff perfusion, the pulmonary vein was cannulated, and the preparation was switched to working mode by closing aortic inflow then opening the pulmonary flow into the heart with a standard pressure (17 cm  $\text{H}_2\text{O}$ ). The perfusion buffer was previously saturated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , pH 7.4 at 37 °C.

## **Induction of ischaemia**

After aerobic perfusion in experiments with rat hearts global ischemia was induced for 30 min. while the experiments with mice hearts ischaemia induced for 20 min. followed by 2 hrs of reperfusion. To prevent the myocardium from drying out, the heart chamber, in which hearts were suspended, was covered and the humidity was kept at a constant level (90–95%). The first 10 minutes of reperfusion was conducted in Langendorff mode to avoid the fatal

ventricular arrhythmias. If VF developed and the sinus rhythm did not spontaneously return within the first 2 min. of reperfusion, hearts were electrically defibrillated by a defibrillator using two silver electrodes and 15 V square-wave pulse of 1 msec. duration and reperfused.

### **Cardiac function assessment on rat hearts**

To examine the recovery of the left ventricle, cardiac function was assessed after 30-, 60-, and 120-minutes reperfusion. During the entire experimental procedure, aortic pressure (AOP) was measured by computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia). Heart rate (HR) and the first derivative of the aortic pressure (AOP/dt) were calculated from the continuously registered AOP. Coronary flow (CF) was measured by the timed collection of the effluent dripping from the heart. Aortic flow (AF) was measured using a calibrated flow meter. Cardiac output (CO) was generated as a sum of AF and CF. Stroke volume (SV) was calculated as the quotient of CO/ HR. Decrement of SV was calculated as a ratio of SV at reperfusion divided by baseline SV and multiplied by 100.

### **Registration of VF and measurement of cardiac function on mouse hearts**

Epicardial electrocardiograms (ECGs) were recorded throughout the experimental period by two silver electrodes attached directly to the heart and connected to a data acquisition system (ADInstruments, Powerlab, Castle Hill, Australia). ECGs were analysed to determine the presence or absence of VF. Hearts were considered to be in VF if an irregular undulating baseline was apparent on ECGs. If the duration of VF was longer than 2 min. the VF was defined as sustained VF, otherwise, the VF was non-sustained. If VF developed and the sinus rhythm did not spontaneously return within the first 2 min. of reperfusion, hearts were electrically defibrillated by a defibrillator using two silver electrodes and 15 V square-wave pulse of 1 msec. duration and reperfused. AF and CF rates were measured by a timed collection of the aortic and coronary effluents that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), CF and AF were registered. AOP and AOPdp/dt were measured by a computer acquisition system (ADInstruments).

### **Protein isolation and Western blot analysis**

Approximately 300 mg of the previously freezed and kept at -70 °C heart tissue were homogenized using a polytron homogenizer in 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 $\times$  protease inhibitor cocktail) and centrifuged at 2000 rpm at 4 °C for 10 minutes. The supernatants were transferred to a new tube and centrifuged at 10,000 rpm at 4 °C for 20 minutes, after which the resulting supernatant was used as cytosolic fraction. The protein concentration was measured by a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). A total of 50–100 mg of protein in each sample were loaded and resolved using SDS-PAGE electrophoresis and then transferred to a nitrocellulose membrane. After blocking the membranes with 5% of nonfat dry milk in TBST for 1.5 hours, membranes were incubated overnight with primary antibody solution at 4 °C (Bcl-2 1/1000, Akt 1/1000, p-Akt 1/1000, HO-1 1/1000, and GAPDH 1/20,000; all antibodies were obtained from Cell Signaling Technology, Boston, MA). Subsequently, the membranes were washed in TBST 3 times and incubated with the horseradish peroxidase-conjugated secondary (Cell Signaling Technology) antibody solution containing 1% of nonfat dry milk in TBST for an hour at room temperature. After washing, the membranes were treated with Western blot Enhanced Chemiluminescent HRP substrate (Millipore, Billerica, MA) to visualize the bands. After the Enhanced Chemiluminescent treatment, the membranes were exposed on x-ray films (Agfa-Gevaert N. V., Belgium). The films were then digitalized and analyzed using ImageJ program.

Mouse myocardial samples from LV, RV and S were homogenized in Tris-HCl (13.2 mM/l), glycerol (5.5%), SDS (0.44%) and  $\beta$ -mercaptoethanol. The same amount of soluble protein (50  $\mu$ g) was fractionated by Tris-glycine- SDS-PAGE (12%) electrophoresis, and Western blot was carried out as described by Pellacani et al. by using an antibody for recombinant rat HO-1 protein.

### **Infarct size assessment**

The estimations of infarct size were carried out using the triphenyl tetrazolium chloride (TTC) method. Briefly, after 30 minutes of ischemia and 120 minutes of reperfusion, rat hearts were perfused with 40 ml 1% (wt/vol) solution of TTC in phosphate buffer, and the samples were stored at -70 °C for subsequent analysis. The frozen samples were sectioned, weighted, and blotted dry. The dried sections were scanned on an Epson J232D flat-bed scanner. The infarcted area (identifiable by white coloration) and the risk area (entire scanned section) were measured using planimetry software (ImageJ). Estimates of infarcted zone magnitude were subsequently obtained by multiplying infarcted areas by weight of each

slice. The resulting numbers represent 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). We carried out the same protocol at mice hearts, but 10 ml of TTC solution was enough to stain the hearts.

## **Immunohistochemistry**

After reperfusion, the hearts were fixed in 4% of buffered paraformaldehyde solution (pH 7.4), embedded in paraffin, and sectioned into 5  $\mu$ m slices. After deparaffinising the sections in xylene, a graded series of alcohol rinses were used to rehydrate the samples. Antigen retrieval was accomplished by boiling the slides in 10 mM sodium citrate (pH 6.0) containing 0.05% of Tween 20 for 25 minutes in a pressure cooker. H<sub>2</sub>O<sub>2</sub> containing methanol was used for 30 minutes at room temperature to block endogen peroxidases. Slides were then blocked with 5 % FBS in TBST for 1 hour at room temperature. After blocking, the slides were washed with TBST and incubated overnight with a primary antibody (Covalab, France) diluted in PBST (1/100) at 4 °C. After washing, the slides were incubated with HRP-conjugated secondary antibody for 1 hour at room temperature (1/300 dilutions). To visualize, DAB solution (Novolink Polymer Detection System; Leica Biosystems, Newcastle, United Kingdom) was used. The slides were then washed and covered with mounting medium. Light microscopic images were obtained by a Zeiss Axioscope microscope.

## **RT-PCR**

RNA was prepared from the left ventricles (LVs), right ventricles (RVs), and septum (S; about 50 mg) by guanidine isothiocyanate acid/phenol method, and 5  $\mu$ g total RNA was used to synthesize first strand cDNA by the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA, USA). The product of cDNA was amplified by PCR with specific primers for rat HO-1 exon 5 (CCC-TTC-CTG-TGT-CTT-CCT-TTG and ACAGCC-GCC-TCT-ACC-GAC-CAC-A). The product of RT-PCR was separated using 1.5% agarose gel, and visualized by ethidium bromide.

## **Measurement of HO activity**

Fifty milligrams of tissue were homogenized in 10 ml of 200 mM phosphate buffer, and then centrifuged at 19000 g 4 °C for 10 min. The supernatant was removed and recentrifuged at 100000 g 4 °C for 60 min., and precipitated fractions were suspended in 2 ml of 100 mM K-phosphate buffer. Biliverdin reductase was crudely purified and HO activity was then assayed. Reaction mixtures consisted of (final volume 2 ml) 100 μM K-phosphate (pH 7.4), 15 nM hemin, 300 μM bovine serum albumin, 1 mg biliverdin reductase and 1 mg microsomal fraction of cardiac tissue. The reaction was allowed to proceed for 1 hr at 37 °C in dark in a shaking water bath and was stopped by placing the test on ice. Incubation mixtures were then scanned using a scanning spectrophotometer, and the amount of bilirubin was calculated as the difference between absorbance at 464 and 530 nm. Proteins were determined by the method of Lowry et al., in microsomal fractions.

## **Measurement of CO**

Tissue CO content was detected using gas chromatography. Hearts were homogenized in 4 volumes of 0.1 M phosphate-buffer (pH 7.4) using an 520 homogenizer (Ingenieurburo CAT, M. Zipperer GmbH, Staufen, Germany). The homogenates were centrifuged at 4 °C for 15 min. at 12,800 g and the supernatant fractions were used for the determination of tissue CO content. The reaction mixtures contain: 150 μl of supernatant, 60 μl of NADPH (4.5 mM) and 50 μl of 3.5/0.35 mM methemalbumin, and for blank samples 60 μl phosphate buffer was used instead of NADPH. Samples were pre-incubated at 37 °C for 5 min., then the headspace was purged and the incubation was continued for 1 hr in dark at 37 °C. The reaction stopped by placing the samples on ice and the headspace gas was analysed. One thousand microlitres of the headspace gas from each vial was injected into the gas chromatograph using a gastight syringe (Hamilton Co., Reno, NV, USA) in hydrogen gas flow with a speed of 30 ml/min.. Analysis took place during the next 150 sec. on a 200 cm stainless-steel column with a 0.3 cm inner diameter. The detector was a thermal conductivity detector with an AC current of 80 mA. The individual value was expressed in millivolts. The column was packed with Molselect 5 Å and maintained at 30 °C. The temperature of the injector and detector was controlled and kept at 50 °C.

## Measurement of intracellular Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup>

Cellular cations were measured as previously described. In brief, hearts were rapidly cooled to 0–5 °C by submersion in, and then perfused for 5 min. with an ice-cold ion-free buffer solution containing 100 mmol/l of trishydroxy-methyl-amino-methane and 220 mmol/l of sucrose to wash out ions from the extracellular space and to stop enzyme activities responsible for membrane ion transport processes. Five minutes of cold washing of the heart washed out > 90% of the ions from the extracellular space. Following the wash out period, left ventricular tissues were dried for 48 hrs at 100 °C, and made ash at 550 °C for 20 hrs. The ash was dissolved in 5 ml of 3 M nitric acid and diluted 10-fold with ion-free deionized water. Cellular Na<sup>+</sup> was measured at a wavelength of 330.3 nm, K<sup>+</sup> was measured at 404.4 nm, and Ca<sup>2+</sup> at 422.7 nm in air-acetylene flame using an atomic absorption spectrophotometer (Perkin-Elmer 1100-B, Perkin-Elmer, Waltham, MA, USA). This method for the measurement of cellular ion contents has been previously described in the myocardium and central nervous system.

## Statistical analyses

Infarct size, HR, CF, AF, AOP, AOPdp/dt, COUT, SV, cellular Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and infarct size were expressed as mean ± standard error of mean (S. E. M.). The student's t-test was performed, and a level of p <0.05 was considered to be statistically significant. For Western blot analysis, repeated measures of ANOVA followed by Tukey's post-hoc test were accomplished. If differences were established the values of NTg group were compared to those of HO-1 Tg, and HO-1 KO<sup>-/-</sup> groups by multiple t-test followed by Bonferroni test. Because of the non-parametric distribution of the incidence of VF (sustained and non-sustained), the  $\chi^2$  test was used to compare individual groups. A change of P <0.05 was considered to be statistically significant. For statistical analysis, we have used the Graphpad Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

## **Results**

### **Augmentation of postischemic left ventricular function by SCSE treatment**

The cardioprotective effect of SCSE treatment was assessed in hearts from rats pretreated with the extract for a period of 8 weeks. Each heart was subjected to 30 min of global ischemia and 120 min of reperfusion as described above. Enhanced postischemic ventricular function was observed in the SCSE-treated group. For example, after 30 minutes of ischemia and 120 minutes of reperfusion, CO<sub>2</sub>T was significantly greater in SCSE-treated hearts with a value of  $59.9 \pm 3.4$  mL/min than in hearts from vehicle-treated control animals, which exhibited CO<sub>2</sub>T values of  $44.2 \pm 6.0$  mL/min. Similar improvements were noted in measurements of AF, AOP, and AODP/dt and decrement in SV in hearts from SCSE-treated animals. Moreover, SV showed a trend, albeit nonsignificant, towards enhancement in the SCSE-treated hearts. However, no significant difference was observed in HR and CF between groups.

### **SCSE-mediated effects on infarct size**

To further confirm the cardioprotective effect of SCSE, infarct size was measured using the TTC method. The extent of infarction zones in hearts from SCSE-treated animals were  $11.8\% \pm 3.6\%$ , which was significantly lower in comparison with the vehicle-treated control value of  $27.7\% \pm 4.1\%$ , further supporting the cardioprotective properties of SCSE.

### **SCSE-mediated effects on survival signaling**

Assessment of cell survival signaling in this study was made through measurements of the level of antiapoptotic protein Bcl-2 and the ratio of phosphorylated to nonphosphorylated Akt (p-Akt/Akt) in cardiac tissue. These experiments were conducted based on outcomes of a previous study suggesting that SCSE might modulate these and related homeostatic signaling processes. This investigation shows that SCSE treatment upregulates Bcl-2 expression in the heart, thereby suppressing apoptosis and inducing the survival of the cardiac tissue after I/R challenge. Moreover, a nonsignificant trend toward enhancement of p-(473)Akt/Akt ratio in SCSE-treated hearts was noted, but it did not reach the significant level. Cell death as a result

of I/R injury may occur as a result of both necrotic and apoptotic processes. Previous studies demonstrate the ability of plant polyphenols to suppress apoptosis and infarct size. Results of this study further validate these outcomes and lend additional support for the possibility for use of SCSE in cardioprotection.

### **SCSE-mediated effects on HO-1 expression**

Our team previously demonstrated that SCSE-mediated induction of HO-1 in the retina strongly protects against ischemia/reperfusion injury. This study measured SCSE dosage effect on HO-1 expression in I/R-injured rat myocardium. The results of Western blot analyses reveal elevated HO-1 protein expression in the myocardium of SCSE-treated rats before and after I/R injury in comparison with the vehicle-treated control hearts. Consistent with these outcomes, immunohistochemical analysis of heart tissue also revealed significantly higher levels of HO-1 in the SCSE-treated group versus that of the control group.

### **Confirmation of the expression of rat HO-1 in transgenic mice**

For the confirmation of rat HO-1 expression at mRNA level, RT-PCR was used and total RNA was isolated from the non-ischemic LVs and RVs, and S of Tg and NTg littermates. The PCR product was rat HO-1 specific and was detected selectively in Tg mouse samples by RT-PCR and Western blot. The specific band was amplified in tissues obtained from LV, RV and S, respectively by PCR from RNA samples of Tg mouse from different lines, but was absent in RNA samples of NTg mice. Tg1 and Tg2 proteins were also detected by Western blots in the LV, RV and S of mouse hearts, but the expression of rat HO-1 proteins were absent in the NTg mouse myocardium. HO enzyme activities were also measured in the LV, RV and S obtained from NTg and Tg mice. The results show that HO activities were significantly increased in samples obtained from the LV, RV and S of Tg mouse hearts in comparison with NTg values indicating the function of Tg1 and Tg2 genes in the mouse myocardium. It is important to note that HO-1 mRNA and protein were not detected in NTg mouse myocardium either by PCR or Western blot, but HO enzyme activities were present in the samples of NTg mouse myocardium including LV, RV and S, respectively, indicating that measured HO enzyme activity involves all isoforms such as HO-1, HO-2 and HO-3. Thus, the differences in HO enzyme activities between NTg and Tg in the LV, RV and S were clearly related to the rat HO-1 transgene in the mouse myocardium.

## **The effect of HO-1 expression on the functions of left ventricles**

Before the induction of ischemia significant changes were not detected between NTg and HO-1 Tg groups in HR, CF, AF, AOP and AOPdp/dt. Upon reperfusion, the post-ischemic recovery of CF, AF, AOP and AOPdp/dt were observed in the HO-1 Tg group in comparison with the NTg values without any significant differences in HR. Thus, for instance, after 30 and 120 min. of reperfusion, AF was significantly increased from its NTg control values of  $0.9 \pm 0.1$  ml/min. and  $0.8 \pm 0.1$  ml/min. to  $2.3 \pm 0.2$  ml/min. ( $p < 0.05$ ) and  $2.2 \pm 0.2$  ml/min. ( $p < 0.05$ ) in the Tg group, respectively. The same pattern was observed in the post-ischemic recovery in CF, AOP and AOPdp/dt. The results also show that 50  $\mu$ mol/kg of SnPPIX, an HO enzyme inhibitor, abolished the post-ischemic recovery of cardiac function in comparison with the observed protection in the HO-1 Tg group. Thus, the values obtained in the HO-1 Tg + SnPPIX group were not statistically significant in comparison with the NTg values.

## **Evaluation of changes in tissue carbon monoxide level**

It is shown that in HO-1 Tg hearts subjected to 20 min. of ischemia followed by 120 min. of reperfusion, a substantial increase in CO production was observed in comparison with the NTg myocardium. However, the endogenous production of CO in the HO-1 Tg myocardium treated with SnPPIX was detected at relatively low level.

## **Evaluation of changes in the intracellular ion contents affected by different HO-1 expression levels**

Left ventricular tissue  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  contents were not significantly varied between NTg, HO-1 Tg and HO-1 Tg + SnPPIX groups before the induction of ischemia. However, the results also depict that left ventricular tissue  $\text{Na}^+$  and  $\text{Ca}^{2+}$  contents were significantly reduced after 20 min. of ischemia followed by 120 min. of reperfusion in the HO-1 Tg group in comparison with the NTg and HO-1 Tg + SnPPIX values. In addition, the left ventricular tissue  $\text{K}^+$  content was significantly elevated ( $261 \pm 8$   $\mu$ mol/g dry weight,  $p < 0.05$ ) in the HO-1 Tg group compared to the  $\text{K}^+$  loss measured in the NTg group ( $229 \pm 5$   $\mu$ mol/g dry weight). In hearts treated with SnPPIX, the ischemia/reperfusion resulted in the same maldistribution in cellular  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  contents to those of the NTg group. In other words, SnPPIX

completely abolished the cardiac protection detected concerning the cellular Na<sup>+</sup> and Ca<sup>2+</sup> gains, and K<sup>+</sup> loss in the HO-1 Tg group in the ischemic/reperfused myocardium. The values measured in the SnPPIX group were the same to the NTg mouse hearts after 20 min. of ischemia followed by 120 min. of reperfusion.

### **Differences in infarct size and incidence of ventricular fibrillation depending on HO-1 expression**

The infarct size was markedly reduced from its NTg control value of  $37 \pm 4\%$  to  $20 \pm 6\%$  (\*p <0.05) in the HO-1 Tg group. However, in the HO-1 KO hearts, the infarct size was significantly increased to  $47 \pm 5\%$  (\*p <0.05) in comparison with NTg group. We had measured the incidence (%) of reperfusion-induced VF as total (sustained and non-sustained) and sustained VF in NTg, Tg and KO mouse groups. The incidence of reperfusion-induced total and sustained VF was significantly reduced from their NTg control values of 92% and 83% to 25% (\*p <0.05) and 8% (\*p <0.05), respectively, in the HO-1 Tg group. In the KO group, the incidence of reperfusion induced total and sustained VF were 100% and 100%, respectively, showing the important role of HO-1 in arrhythmogenesis.



## Discussion

Cardiovascular disorders affect millions of people all over the world. Despite the improvement of our knowledge of clinical sciences especially on the pathogenesis of different diseases and possible risk factors, mortality statistical data show only a very slight improvement in the past decades. Recently, an unspoken but considerable call has appeared for treating diseases not only with synthetic, but natural compounds and also to prevent disorders by functional foods and dietary supplements, especially in cardiovascular cases. Previous results of investigation of our research team show that SCSE protects the retina against I/R at least by part via the induction of hemeoxygenase-1 (HO-1). This protein, which is also known as heat shock protein-32 plays a crucial role in stress response of the cells and helps to the cell survival mechanisms upon oxidative stress acting against reactive oxidative species (ROS). This oxidative biochemical process result in an imbalance between oxidative species and antioxidants. It is connected to almost uncountable pathophysiological processes, mainly cardiovascular diseases. ROS plays role in signaling under physiological conditions, but if the generation of these species is greater than the antioxidant capacity, then cellular injury or even cellular death may happen. Beside endogen antioxidants, applying outsource antioxidants under such conditions could be critical in order to protect the tissue. These substances are able to reduce I/R caused cardiovascular injury through several biochemical pathways.

During the analysis of the sour cherry seed extract (SCSE) many different biologically active components were identified such as cyanides, polyphenols, flavonoids, vegetable acids, pro- and anthocyanidines, trans-resveratrol, stilbenes and catechins. Many of these are powerful natural antioxidants. In our present investigations we have tested rat hearts after 8 weeks of SCSE treatment. This study demonstrates that administration of SCSE to animals at a dose shown in previous work to mediate cardioprotection, for a time period double that previously used, resulted in superior postischemic cardiac function versus drug-free control animals. The significant improvement in multiple cardiac functions, may have particularly strong relevance to the development of post-I/R clinical interventions to reduce severity of cardiovascular endothelial function. In these experiments, no sign of any adverse effect of SCSE treatment was observed. It is acknowledged that animals in this study were not specifically monitored for toxic effects. However, none was expected based on outcomes of toxic studies demonstrating negligible liver and kidney toxicity of the material in animals, even at dosages in excess of therapeutic value. Reduction in postischemic infarct size was also observed in

SCSE-treated animals. Moreover, consistent with our previous work, Western blot data show the enhanced level of Bcl-2, supporting the antiapoptotic and cytoprotective effect of SCSE. Adaptive/protective responses mediated by SCSE have previously been demonstrated to correlate with HO-1 induction as described in studies by the authors showing the capacity of SCSE to protect against ischemic damage to both retinal and cardiac tissue. The protective effects of the extract on tissue correlated significantly with elevated HO-1 protein detected by Western blotting and immunohistochemistry. Experiments to determine the effect of SCSE treatment on antiapoptotic signaling were conducted with the objective of providing insight into how the product might be used to modulate rates of cell death in cardiac tissue so as to optimize stable tissue homeostasis. Of particular importance to the accomplishment of these research goals is identification of how redox balance and metabolic energy utilization by cardiomyocytes is altered by I/R injury in ways that promote inflammatory tissue damage and remodeling of heart tissue. Examples of such strategies are provided by demonstrations that pharmacological interventions that negatively regulate apoptosis, particularly through effect on mitochondrial function are cardioprotective after I/R injury. An antiapoptotic property of SCSE was suggested by an earlier study by the authors. This study reveals increased Bcl-2 levels as a result of SCSE treatment, which support the author's previous findings. Some particularly interesting results have been obtained by examination of the effect of resveratrol, a cytoprotective polyphenol on cardiac tissue. This compound was shown to inhibit cardiomyocyte apoptosis induced by doxorubicin treatment in lymphoma nude mice. The aforementioned study revealed enhanced Bcl-2 and reduced Bax expression levels in hearts of mice treated with resveratrol before and during doxorubicin treatment in comparison with animals treated with doxorubicin alone. The protective effect and the modified ratio of Bcl-2/Bax of resveratrol were reversed by zinc protoporphyrin IX (HO-1 inhibitor) indicated a correlation of HO-1 and Bcl-2. Our study also indicates a HO-1/Bcl-2 axis in the protective effect of SCSE. Additional demonstration of the protective effect of HO-1 increased by plant extract induction was observed by the anti-inflammatory action of genipin, an aglicon of geniposide. In this (aforementioned) study, the authors demonstrated a PI3-kinase-JNK1/2-Nrf2 cascade as a possible underlying mechanism in genipin-induced HO-1 expression. The role of PI3K was also suggested in the oleanolic acid-induced HO-1 expression, which contributes to the possible protective effect of oleanolic acid in vascular smooth muscle cells against oxidative stress induced cellular damage. The study revealed the contribution of the activation of Akt and Erk to the Nrf2 nuclear localization and the subsequent HO-1 induction. The role of Nrf2 in the HO-1 induction by Ginkgo biloba whole-leaf extract was also reported

in vascular smooth muscle cells, and this pathway is suggested to play an important role of the antiatherogenic effect of this plant material. Based on our results, we cannot rule out the additional role of Akt to the protective effect of SCSE, but this study did not confirm an existence of a potential HO-1/Akt axis. There were no significant increases in the p-Akt/Akt ratio in hearts from SCSE-treated animals. It is nevertheless acknowledged that this study would have benefitted by a more comprehensive evaluation of signaling mechanisms by which SCSE-mediated increases in HO-1 expression occurred.

Furthermore, transgenic mice expressing high levels of HO-1 exhibit reduced susceptibility to I/R-induced damage. In recent years, the concept of the modification in various gene expressions or repressions has emerged as new mechanisms and therapeutic tools for the induction of protection in ischemic/reperfused tissues. It is now also relatively well accepted that many human vascular diseases such as hypertension, heart failure, organ transplantation and arrhythmias can be treated with various interventions at a level of underlying genetic mechanisms. Certain studies show a substantial reduction in the expression of HO-1 mRNA and its protein with a causative reduction in the HO-1 enzyme activity in fibrillated ischemic/reperfused rat myocardium. Furthermore, HO-1 KO mouse hearts displayed a reduced left ventricular function after ischemia/reperfusion. If HO-1 system has a crucial role in the protection against ischemic/reperfusion-induced injury in the myocardium, with the enhanced expression of the HO-1, more ischemic tissue can be salvaged. Therefore, in the present study, because the HO-1 system can play a crucial role in the pathology of the ischemic/reperfused myocardium, we decided to approach the question from a different angle using HO-1 Tg mouse hearts. We generated HO-1 overexpressing Tg mice with an elevation in baseline HO enzyme activity by about 50% in the LV, RV and S, respectively. Thus, the application of a genome-based transgene resulted in expression levels in relation with increased HO enzyme activities in comparison with those of HO activities levels detected in the LV, RV and S obtained from NTg mice. Although, in our studies, the very similar homology between the mouse and rat HO-1 made it impossible to differentiate the HO-1 and Tg-induced enzyme activities, but we were able to distinguish them at mRNA level. In addition, our results obtained in NTg, HO-1 Tg and Tg-SnPPIX treated mouse hearts, we favour the idea that increased endogenous CO production levels in the myocardium represents the major signal transduction responsible for HO-1 related protection reflected in the improvement of the recovery in post-ischemic cardiac function, reduction of the incidence of reperfusion induced VF and infarct size connected to the recovery of cellular ion contents. Our results further supported by the complete loss of cardiac protection in the Tg

ischemic/reperfused mice treated with SnPPIX, the inhibitor of HO enzyme. The exact mechanism by which HO-1 induces cardiac protection remains to be elucidated; however, one of the most significant protective mechanisms of the HO system can be explained by the generation of endogenous CO and its signalling mechanism. CO could suppress cell apoptosis suggesting that the anti-apoptotic effect of HO-1 is mediated via the generation of CO. Another possible cellular signalling mechanism of CO is associated with the guanylyl cyclase activation and tissue levels of guanosine monophosphate (cGMP) and adenosine monophosphate (cAMP) in isolated ischemic/reperfused myocardium. Very low concentrations of CO, in the perfusion buffer, afford a significant protection against the ischemia/reperfusion-induced damage in isolated buffer perfused hearts. The isolated buffer perfused heart could be an ideal model to study the direct effect of exogenous CO on cardiac function and molecular signalling because the blood and its elements are excluded from the model, thus the oxygen transport to cells and tissues is not directly damaged via the CO/blood/haemoglobin system. Previous results have shown that low concentrations of exogenous CO protect the isolated ischemic/reperfused myocardium and act by increasing tissue cAMP and cGMP levels. Indeed, a moderate increase in cAMP content could lead to arrhythmogenesis and development of various arrhythmias by elevating cytosolic calcium levels in the ischemic and post-ischemic myocardium. It is of interest to note that multiple increases in cGMP levels could mask and interfere with the arrhythmogenic effects of cAMP leading to the suppression of reperfusion-induced VF in the myocardium. The significant increase in cGMP levels can be related to guanylate cyclase activities in CO-treated myocardium, and suggests that induction of guanylate cyclase-cGMP system via CO signalling is essential for cardioprotection in ischemic/reperfused hearts. However, not only the absolute levels of cGMP and cAMP but also the ratio of these two nucleotides are critical factors that determine the recovery of post-ischemic cardiac function after ischemia and reperfusion. Thus, the lower the cAMP/cGMP ratio is during ischemia, the better is the post-ischemic cardiac recovery achieved in isolated ischemic/reperfused hearts. Although not specifically studied in the present investigation at molecular level, it is of interest to note the findings of Piantadosi et al. with myocardial cells. In their studies, Piantadosi et al. implicated the role of HO-1/CO pathway in mitochondrial biogenesis through Akt1 activation involving nuclear factor erythroid 2-related factor (Nrf-2) expression, downstream GSK-3 $\beta$  blockade and Nrf2 nuclear translocation leading to Nrf2-dependent activation of nuclear respiratory factor (NRF)-1 transcription. Their finding suggest HO-1/CO, by sequentially activating the

aforementioned two transcription factors, as a remarkable component of a prosurvival program of mitochondrial biogenesis connected to cellular antioxidant defence mechanisms. Today, when so many advances and efforts are being made in molecular biology and genetics, we tend to lose sight of the importance of basic ions such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  in both clinical and experimental studies. As a gap, in changes between various gene expression and/or repression and the function of myocardial tissue, could closely be connected with changes in myocardial tissue ion contents. An important finding such a final endpoint of our present study was the myocardial  $\text{K}^+$  – conserving effect related to the prevention of cellular  $\text{Na}^+$  and  $\text{Ca}^{2+}$  gains in Tg myocardium, and this cardiac protection can be reversed by SnPPiX, an HO-1 inhibitor. HO-1–bilirubin system and one of its by-products, CO, are essential for regulation of cardiac function and ion transport across cell membranes via the stabilization of various Na, K and Ca ion channels possibly by an effect exerted on Na-K-ATPase, and these transient outward and inward currents are thought to underline the delayed after depolarization that can be observed after the action potential in Ca-loaded cardiac tissue propagating rhythm disturbances. There are significant differences in the cellular signalling mechanisms induced by CO when they are compared with the actions of other interventions that elevate cyclic nucleotides in tissues. In our study we have shown that CO level was significantly increased in the HO-1 Tg mouse myocardium. However, we did not measure CO levels during reperfusion; therefore, we believe that the elevated CO production can be responsible for the protection during reperfusion in Tg myocardium. Besides this, we cannot rule out the contribution of other HO-1 related molecules such as biliverdin and bilirubin in the observed cardiac protection.

In conclusion, using HO-1 Tg, we have demonstrated the direct causative role of HO-1 in protection against ischemia/reperfusion induced injury. Specifically, our results show a significant recovery of post-ischemic cardiac function, prevention of the development of reperfusion-induced VF and reduction in infarct size. In addition, we have provided new insights into the ionic mechanisms by which HO-1 may contribute to the attenuation of reperfusion-induced arrhythmias in HO-1 Tg. We believe that these studies would help in developing novel therapies based on targeting HO-1 for the treatment of ischemia/reperfusion injury in patients with coronary artery disease.

## Summary

Despite a slight improvement on statistical data, cardiovascular diseases are still among the highest risk factors of mortality rates all over the world. Hungarian mortality indexes are far above than in the EU, therefore our resources should be focused on the prevention and diagnosis at the exact time.

HO-1, a cytoprotective, antioxidant heat shock protein plays a crucial role in the pathogenesis of several human disorders, especially cardiovascular diseases. Based on our experiments carried out on HO-1 transgenic mice model HO-1 overexpression leads the restoration of functioning state of left ventricles after I/R injury. An unspoken but considerable call has appeared recently for treating diseases not only with synthetic, but natural compounds and to prevent disorders by functional foods and dietary supplements, especially in cardiovascular cases. Sour cherry seed extract experimented by our team induced the intracellular level of HO-1, therefore it can mediate cardioprotection.



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Neptun ID: ZJFTHO  
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### List of publications related to the dissertation

1. **Czompa, A.**, Gyöngyösi, A., Czeplédi, A., Csépanyi, E., Bak, I., Haines, D.D., Tósaki, Á., Lekli, I.:  
Cardioprotection afforded by sour cherry seed kernel: The role of heme oxygenase-1.  
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### List of other publications

3. , Csépanyi, E., **Czompa, A.**, Haines, D., Lekli, I., Bakondi, E., Balla, G., Tósaki, Á., Bak, I.:  
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