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CARDIAC

Cardioprotection afforded by sour cherry seed kernel: the role of heme oxygenase-1

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ABSTRACT

Cardiovascular diseases are primary cause of death worldwide, particularly among populations with sedentary lifestyles and diets rich in animal products and processed foods. Currently, public health countermeasures to these disorders focus on costly and often marginally effective interventions administered only after the development of disease. These countermeasures are mainly palliative and fail to address the underlying causes of cardiac pathologies. Previously, the authors of the present report have demonstrated that sour cherry seed extract (SCSE), a non-toxic low-cost plant material, strongly preserves tissues via induction of heme oxygenase-1 (HO-1), a critical host antioxidant defense enzyme. The present investigation seeks to characterize underlying mechanisms of SCSE-mediated tissue protection. Isolated hearts from Sprague-Dawley rats fed 30 mg/kg/day SCSE for 8 weeks and untreated controls were mounted in a "working heart" apparatus and subjected to ischemia and reperfusion. A panel of cardiac functional evaluations was conducted on each heart. Infarct size assessments were made along with Western blot and immunohistochemical analysis for selected proteins involved in cardiovascular homeostasis. SCSE treatment was observed to improve post-ischemic cardiac functions and suppress infarct size. Analysis of the outcomes produced by the present study is consistent with SCSE cardioprotection that involve interaction of Bcl-2 and HO-1.

Key words: sour cherry seed extract, heme oxygenase-1, ischemia/reperfusion

INTRODUCTION

The development of improved preventive and therapeutic strategies for ischemic heart disease has emerged as a major focus of cardiovascular research during the late 20th and the early 21st centuries. Currently, the incidence of the cardiovascular diseases is increasing to include progressively younger sectors of the human population, particularly in affluent nations (1). Countermeasures to this trend are being actively pursued. During the past several years, there has been steady improvement both short- and long-term management of a wide range of cardiovascular disorders and associated co-morbidities. Examples of these trends are provided in 3 recently published reports, demonstrating impressive gains made in many aspects of this field (2-4), underscoring the fast-paced advance of basic and clinical research in this field. These outcomes notwithstanding, cardiovascular disease, along with obesity/diabetes remain prevalent at epidemic levels in industrialized nations. Thus, programs for their management will benefit from adjuvant therapies which may be administered cost-effectively. For this reason, phytotherapies such as those described here, are particularly attractive, due to their low cost, excellent toxicity profiles and long histories of dietary use (5, 6)

To support development of phytotherapeutic strategies as contributors to sustainable medicine, we have conducted several studies demonstrating significant cytoprotective effects of sour cherry seed kernel extract (SCSE). For example, this material has been shown to reduce ischemia/reperfusion (I/R)-induced injury in the rat retina via SCSE-mediated induction of the endogenous antioxidant enzyme heme oxygenase-1 (HO-1) (7). HO-1 is a ubiquitous enzyme widely distributed among animal species and expressed by most tissues. Increased activity of HO-1 protects the organism against a diverse variety of stressors (8) and pharmacological upregulation of HO-1 is being developed for therapeutic and preventive

medical use (9). Previous work by the authors has demonstrated potent cardioprotective properties of HO-1 (10, 11). These outcomes are consistent with many investigations demonstrating that phytochemical HO-1 induction correlates with improved prognoses of many diseases (12).We have previously shown that SCSE strongly protects the heart against I/R injury, as revealed by enhanced postischemic left ventricle function, reduced infarct size, and decreased apoptosis in rat hearts subjected to I/R-injury (13). Recent animal studies also demonstrated SCSE-mediated enhancement of SCSE-mediated adaptive response in hearts of hypercholesterolemic rabbits (14). In related *in vitro* studies, SCSE-induced upregulation of HO-1 by human leukocytes, suppressed lipopolysaccharide (LPS)-mediated expansion of CD3+TNF-α+ and CD3+IL8+ subpopulations more significantly than in cells from healthy individuals in blood from type 2 diabetics (15). The present study was undertaken with the objective of characterizing elements of the major mechanisms by which SCSE enhances healthy homeostasis and protects cardiovascular tissue. To achieve these goals, isolated working hearts from animals treated with SCSE or placebo were evaluated for major indicators of cardiovascular homeostasis; and physiological signaling known to affect healthy function of these tissues. The major outcome variables assessed following I/R injury as a correlate of SCSE treatment included: (i) cardiac functions; (ii) size of infarcted zones (iii) expression of proteins related to cell death and survival such as Bcl-2, Akt; (iv) and expression of HO-1 in cardiac tissue.

MATERIALS AND METHODS

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 the present study was additionally approved by the Institutional Animal Care and Use Committee of the University of Debrecen, Debrecen, Hungary. All reagents were obtained from Sigma-Aldrich Co. LLC (Schnelldorf, Germany). Sour Cherry Seed Kernel extract was prepared in our laboratory as described previously. Briefly, following removal of external shell components, the dried seed kernels were ground and extracted with n-hexane, followed by vacuum evaporation and defatting to yield a standardized extract suitable for use as a feed supplement (13).

Animals and isolated working heart preparation.

Male 220-280 g Sprague-Dawley rats were fed with commercial food pellets and water *ad libitum*. Animals were segregated into 2 test groups as described: Group 1: Animals were treated orally with 30 mg/kg/day SCSE (suspended in 2 % hydroxyethylcellulose solution) for a time period of eight weeks; Group 2: Animals in this group received vehicle solution (2 % hydroxyethylcellulose solution) for the same time period.

A representative experimental protocol is summarized as follows: Briefly, following 8-week treatment regimens with SCSE or vehicle, the rats were anesthetized (ketamine-xylazine 50/10 mg/kg, ip), and 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, as previously described (13). After the thoracotomy, the hearts were cannulated through the aorta and perfused in a Langendorff apparatus in "non-working" mode (100 cm H_2O) for 5 minutes to cleanly flush blood from vessels of the organ. During the Langendorff perfusion, the pulmonary vein was cannulated and the preparatum was switched to working mode. After 10 minutes working perfusion, 30 min global ischemia followed by 120 min reperfusion was initiated. The first 10 minutes reperfusion was conducted in Langendorff mode to avoid the fatal ventricular arrhythmias.

Cardiac function assessment.

Baseline parameters for each heart were registered following the 10 minutes working perfusion. 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 (AOdP/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 (16). Decrement of SV was calculated as a ratio of SV at reperfusion divided by baseline SV and multiplied by 100.

Infarct size assessment.

The estimations of infarct size were carried out using the triphenyl tetrazolium chloride (TTC) method. Briefly, following 30 minutes of ischemia and 120 minutes of reperfusion, hearts were perfused with 40 ml 1% (w/v) 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 (Image J). 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) (13).

Western blot analysis.

Approximately 300 mg of heart tissues 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 1x 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, USA).

A total of 50-100 μg of protein in each sample were loaded and resolved using SDS-PAGE electrophoresis then transferred to a nitrocellulose membrane. After blocking the membranes with 5 % nonfat dry milk in TBST, membranes were incubated overnight with

primary antibody solution (Bcl-2 1/1000, Akt 1/1000, p-Akt 1/1000, HO-1 1/1000, and GAPDH 1/20000 all antibodies were obtained from Cell Signaling Technology, Boston, MA, USA) at 4 °C. Subsequently, the membranes were washed in TBST three times and incubated with the horseradish peroxidase-conjugated secondary (Cell Signaling Technology, Boston, MA, USA) antibody solution containing 1 % nonfat dry milk in TBST for an hour at room temperature. After washing, the membranes were treated with Western blot Enhanced Chemiluminescent (ECL) HRP substrate (Millipore, Billerica, MA, USA) to visualize the bands. Following the ECL treatment, the membranes were exposed on x-ray films (Agfa, Mortsel, Belgium). The films were then digitalized and analyzed using ImageJ program.

Immunohistochemistry.

Following reperfusion, the hearts were fixed in 4 % buffered paraformaldehyde solution (pH 7.4), embedded in paraffin, and sectioned into 5 µm 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 min in a pressure-cooker. H_2O_2 containing methanol was employed for 30 min 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 visualise DAB solution (Novolink Polymer Detection System, Leica Biosystems, Newcastle, UK) was employed. The slides were then washed and covered with mounting medium. Light microscopic images were obtained by a Zeiss Axioscope microscope.

Statistical analyses

Infarct size, HR, CF, AF, LVDP, CO, and SV were expressed as mean values ± SEM. The student t-test was performed and a level of $p<0.05$ was considered statistically significant. For Western blot analysis repeated measures of ANOVA followed by Tukey post-test was accomplished. For statistical analysis we have used the Graphpad Prism software.

RESULTS

Augmentation of post-ischemic 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 eight weeks. Each heart was subjected to 30 minutes of global ischemia and 120 minutes of reperfusion as described above. Figure 1 shows the pre- and post-ischemic left ventricular functions to include HR, AF, CF, AOP, AOdP/dt, CO, SV and the decrement in SV. Enhanced postischemic ventricular function was observed in the SCSE treated group. For example, as shown in Figure 1, after 30 min of ischemia and 120 min of reperfusion, CO was significantly greater in SCSE-treated hearts with a value of 59.9 ± 3.4 ml/min than in hearts from vehicle-treated control animals, which exhibited CO values of 44.2 ± 6.0 ml/min. Similar improvements were noted in measurements of AF, AOP, AOdP/dt and decrement in SV in hearts from SCSE-treated animals. Moreover, CO showed a trend, albeit non-significant, 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 and survival signal.

To further confirm the cardioprotective effect of SCSE, infarct size was measured using the TTC method. As shown in Figure 2, the extent of infarction zones in hearts from SCSE-treated animals were 11.8 ± 3.6 % which was significantly lower in comparison with the vehicle-treated control value of 27.7 ± 4.1 %, further supporting the cardioprotective properties of SCSE.

Assessment of cell survival signaling in the present study was made via measurements of the level of antiapoptotic protein Bcl-2 and the ratio of phosphorylated to non-phosphorylated 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 (13). The present investigation shows that SCSE treatment upregulates Bcl-2 expression in the heart, thereby suppressing apoptosis and inducing the survival of the cardiac tissue following I/R challenge (Figure 3 A). Moreover, a non-significant trend toward enhancement of p-(473)Akt/Akt ratio in SCSE treated hearts was noted , but it did not reached the significant level (Fig. 4 B).

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 (17). Results of the present study further validate these outcomes and lend additional support for the possibility for use of SCSE in cardioprotection.

SCSE-mediated HO-1 expression.

The authors of the present report have previously demonstrated that SCSE-mediated induction of HO-1 in the retina strongly protects against ischemia/reperfusion injury (7). The present study measured SCSE dosage effect on HO-1 expression in I/R-injured rat myocardium. The results of Western blot analyses shown in Figure 4 reveal elevated HO-1 protein expression in the myocardium of SCSE-treated rats before, as well as 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 (Figure 4).

DISCUSSION

Rapid advances made in understanding the pathogenesis of and risk factors of cardiovascular diseases during past decades have greatly improved strategies for their long-term management, however; mortality and morbidity due to these syndromes still provides incentive for new directions in research. Moreover, despite clear evidence that prevention of cardiovascular disorders is far more cost-effective than therapy after disease develops, high risk lifestyle choices remain prevalent in the human population along with increasing incidence of heart diseases and related disorders (18, 19)

The present study demonstrates that administration of SCSE to animals at a dose shown in previous work to mediate cardioprotection (13), for a time period double that previously used, resulted in superior post-ischemic cardiac function versus drug-free control animals (Fig. 1). The outcomes shown in Figure 1 may which demonstrate significant improvement in multiple cardiac functions, may have particularly strong relevance to 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 the present study were not specifically monitored for toxic effects. However, none was expected based on outcomes of toxicity studies demonstrating negligible liver and kidney toxicity of the material in animals, even at dosages in excess of therapeutic value (20). Reduction in post-ischemic infarct size was also observed in SCSE-treated animals (Fig. 3). Moreover, consistent with our previous work, Western blot data show the enhanced level of Bcl-2, supporting the antiapoptotic and cyoprotective effect of SCSE (Fig. 4 A) (13). 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 (7, 13). The protective effects of the extract on tissue correlated significantly with elevated HO-1 protein detected by Western-blotting and immunohistochemistry (7). Studies by other investigators have shown that the enzyme may also be induced by common dietary phytochemicals (21, 22). Furthermore, transgenic mice expressing high levels of HO-1 exhibit reduced susceptibility to I/R-induced damage (10, 11). Additional evidence for the cytoprotective effect of phytochemical inducers of HO-1 is provided by a demonstration of the ability of chamomile extract-induced HO-1 to protect murine macrophages against oxidative stress (23).

Experiments to determine the effect of SCSE treatment on anti-apoptotic 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 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 (24-26). Examples of such strategies are provided by demonstrations that pharmacological interventions which negatively regulate apoptosis – particularly through effect on mitochondrial function are cardioprotective following I/R injury (27, 28). A very intriguing study of the phenomenon of cardiovascular “necropoptosis” in a guinea pig model demonstrated that simultaneous inhibition of necroptosis and apoptosis exhibited additive – and possibly synergistic resistance to I/R damage (29).

An antiapoptotic property of SCSE was suggested by an earlier study by the authors (13). The present 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 (30). 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 was reversed by zinc protoporphyrin IX (HO-1 inhibitor) indicated a correlation of HO-1 and Bcl-2 (30). 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 (31). 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 (VSMCs) against oxidative stress induced cellular damage (32). The study revealed the contribution of the activation of Akt and Erk to the Nrf2 nuclear localisation 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 VSMCs, and this pathway is suggested to play an important role of the anti-atherogenic effect of this plant material (33). Based on our results we cannot rule out the additional role of Akt to the protective effect of SCSE, but the present 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 the present study would have benefitted by a more comprehensive evaluation of signalling mechanisms by which SCSE-mediated increases in HO-1 expression occurred. Of particular interest for ongoing studies of this material, are the effects of oxidative stressors, including I/R injury on mitogen-activated

protein kinase (MAPK) signaling and activation of the unfolded protein response (UPR). UPR activation is observed to upregulate expression of heme oxygenase-1 **(**HO-1), via the Nrf2 pathway (34). Since modulation of the UPR has recently been shown to have enormous therapeutic potential (35), characterization of how MAPK and critical proteins of the UPR are altered by SCSE will yield insight that may result in identification of novel therapeutic targets and improved clinical application for the material.

Limitations of study.

(**a**) *Applicability of outcomes to human clinical application*: The authors acknowledge that several major differences between rodent and primate physiology limit the use of these animal models in predicting human therapeutic outcomes. Challenges to use of rats in cardiovascular research are underscored by three examples major differences between this model and humans. One major problem is that pathologies induced in rats using surgery or drugs occur rapidly as a consequence of the constraints on timeframe and environmental factors required for performance of experiments, whereas the wide-ranging neurohumoral adaptations that underlie human cardiovascular disease typically occur over years to decades (36). A second confounder to use of the rat model is that whereas heart disease is significantly skewed to elderly human populations, most research using rat models typically use young adult animals (37). A third problem in use of rat study outcomes as predictors for human therapies is that whereas atherosclerosis is a highly significant risk factor for humans and is a major contributor to a wide range of cardiovascular syndromes, it is very rare in rats, even in those with sustained hyperlipidemia (36). Nevertheless the rat model is widely used in cardiovascular research, yielding data that is widely accepted as groundwork for human studies when interpreted conservatively.

(b) *Limitation of the model*: The model (isolated working heart) of ischemia/reperfusion used in the present study possesses limitations: (i) The isolated heart is free from blood and its

constituents; moreover, no circulating hormones and transmitters are presented in the perfusion buffer, which influence the response of cardiac tissue to ischemia and reperfusion; (ii) There is no pre- and afterload during myocardial ischemia.

(**c**) *Relevance of dosage used in study to therapeutic human dosage*: The therapeutic dosage of SCSE for rodents of 30 mg/kg/day of extract scaled up to human equivalent is 2.1 grams. In caplet form, this would not be practical, however, blended into a "functional food" this dosage could be readily delivered in the form of (for instance) a baked good or desert – to name only 2 potential configurations. Preliminary human studies suggest that administration of large amounts of SCSE may not be required to achieve therapeutic endpoints in humans. For example, preliminary human studies with subclinical levels of orally-delivered SCSE (250 mg daily, 14 days), demonstrate that low dosage of the extract did not alter the level of any toxicity-associated enzymes (unpublished data) and additionally suggest beneficial effects of the material on aspects of iron metabolism. These investigations which are ongoing are producing data that in time may be cross-referenced with outcomes of animal studies. Ultimately, these comparisons will allow reasonable human dose ranges as orally-delivered preparations that patients are compliant with, to be selected.

Acknowledgments

The authors confirm that there are no conflicts of interest with respect to any of the topic material presented herein.

Legends for Figures

Figure 1: Effects of sour cherry seed extract treatment on pre- and post-ischemic cardiac functions. Left ventricular function was assessed before ischemia and after 30, 60, and 120 min of reperfusion. Cardiac functions were recorded by a computer acquisition system included: Heart rate (HR); Coronary flow (CF); Aortic flow (AF); Aortic pressure (AOP); First derivative of the aortic pressure (AOdP/dt); Stroke volume (SV); and Stroke volume decrement in SV. Results are expressed as means ± SEM; n=10 in the control and n=14 in the SCSE treated group, * p □ 0.05.

Figure 2: Effects of sour cherry seed extract treatment on infarct size. Following I/R, TTC-perfused hearts were frozen, sectioned and scanned as described. Infarcted and risk areas were assessed by planimetry software ImageJ. Extent of infarct zones were expressed as a ratio of infarcted zone and risk zone as a percentage of the weight of the heart shown below as the dependent variable in the indicated histograms. Results are expressed as mean ± SEM; n=4 in both groups, * p □ 0.05.

Figure 3 : Western blot analysis for major biomarker of cardiomyocyte survival. Expression of Bcl-2 (Panel A) protein and ratio of p-Akt/Akt levels (Panel B) in rat myocardium were measured in homogenized cardiac tissue samples drawn from SCSE or vehicle treated hearts in the presence or absence of I/R. **C. BL** (control baseline): Rats receiving no SCSE and no I/R-injury to hearts; **C. I/R** (control I/R): Rats receiving no SCSE, but subjected to I/R; **SCSE. BL** (SCSE-treated baseline): Rats receiving SCSE, without I/R; **SCSE. I/R** (SCSE-treated I/R,): Rats receiving SCSE and subjected to I/R. GAPDH

expression level was measured as a reference protein. Data are expressed as mean ± SEM of 5-6 different blots. * p □ 0.05

Figure 4: Western blot analysis for heme oxygenase-1 protein expression.

A. Expression of HO-1 protein levels in rat myocardium was measured in homogenized cardiac tissue samples drawn from SCSE or vehicle treated hearts in the presence or absence of I/R. Abbreviations are described in Fig 3. GAPDH expression level was measured as a reference protein. Data are expressed as mean ± SEM of 5-6 different blots. * p □ 0.05

B. Immunohistochemical staining for HO-1 of formalin-fixed 5 µm slices of left ventricular tissue from control I/R and SCSE-treated I/R-injured hearts, visualized by light microscopy at 400x, demonstrating relative HO-1 content in each class of tissue. The brown staining indicates the HO-1, a relative lower level was detected in the control I/R samples (panel B left side), and an intense brown staining, indicating a higher HO-1 level, was found in the SCSE-treated I/R samples (panel B, right side) Both image include the negative control in the bottom right corner.

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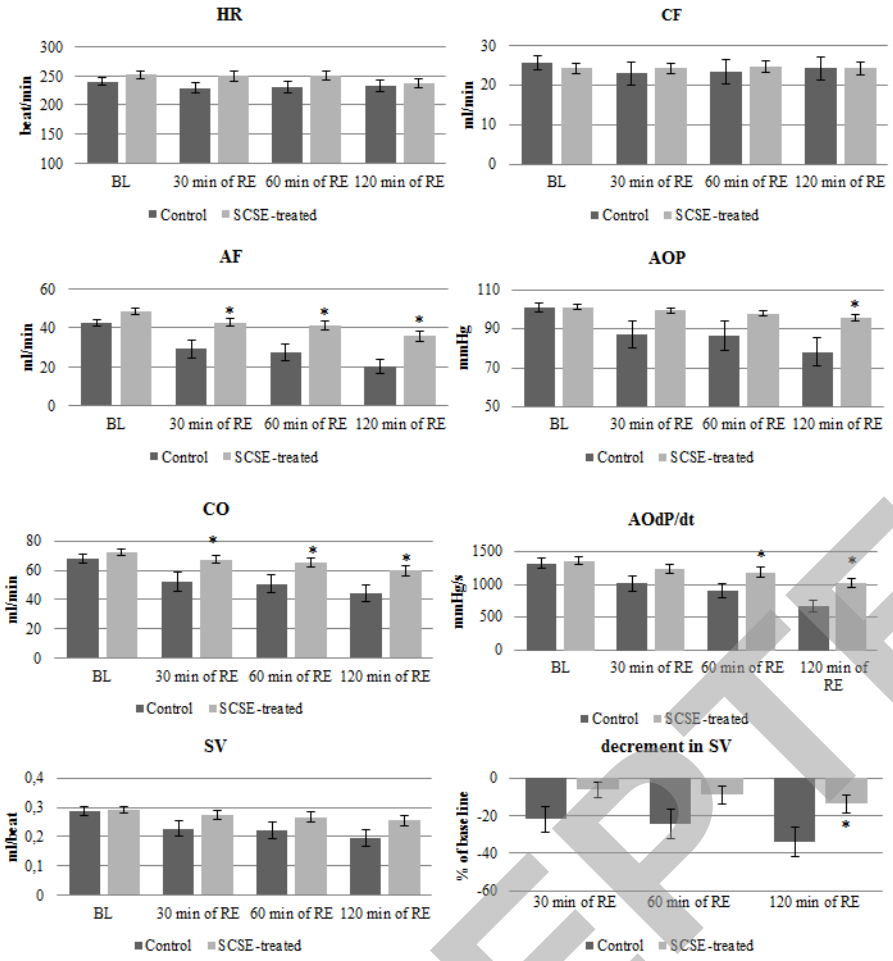

Figure 1

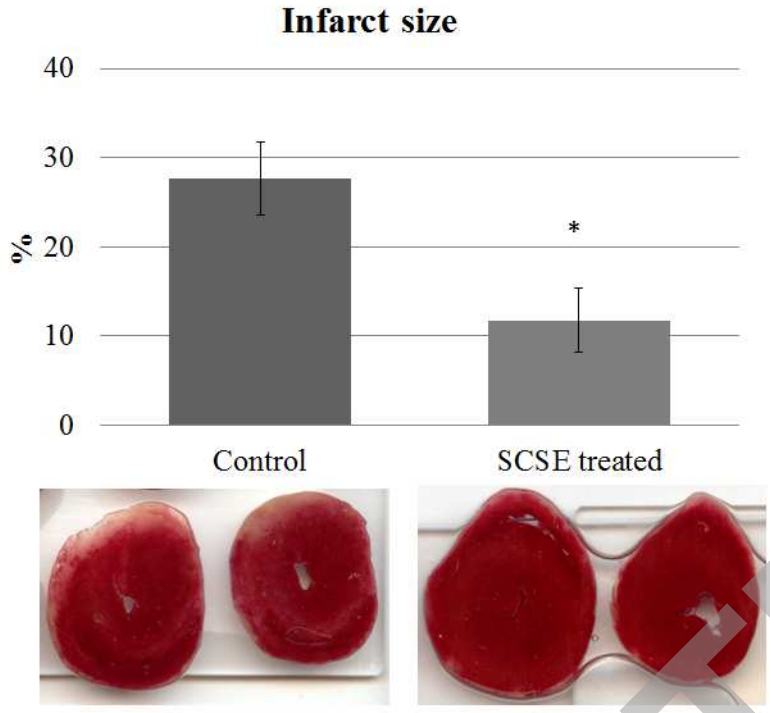

Figure 2

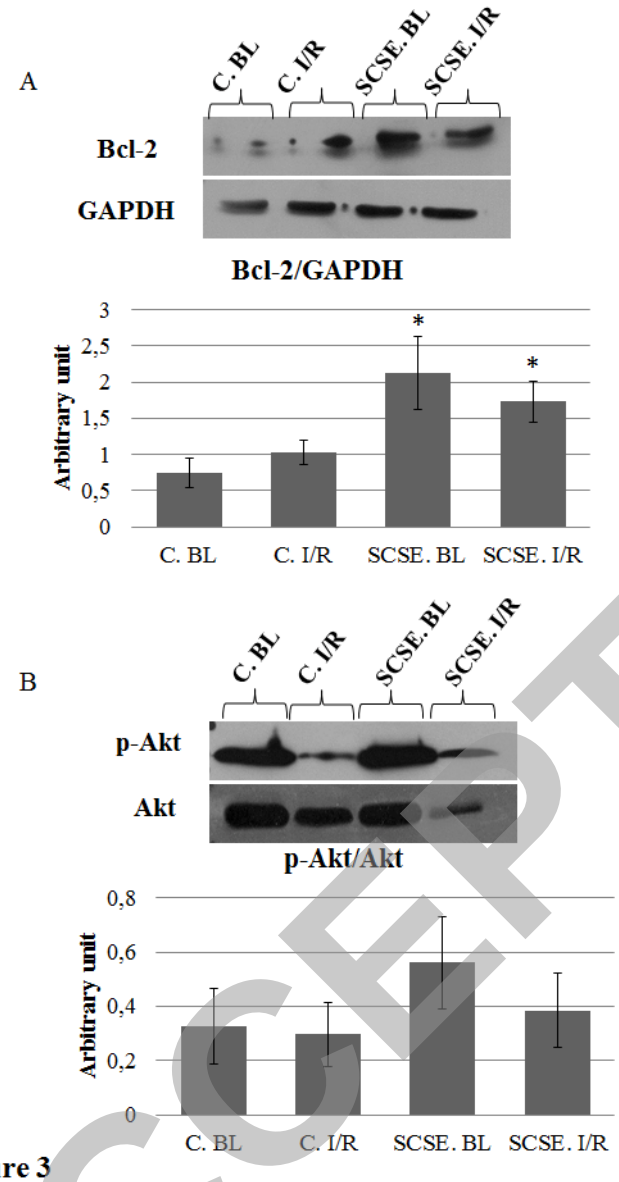

Figure 3

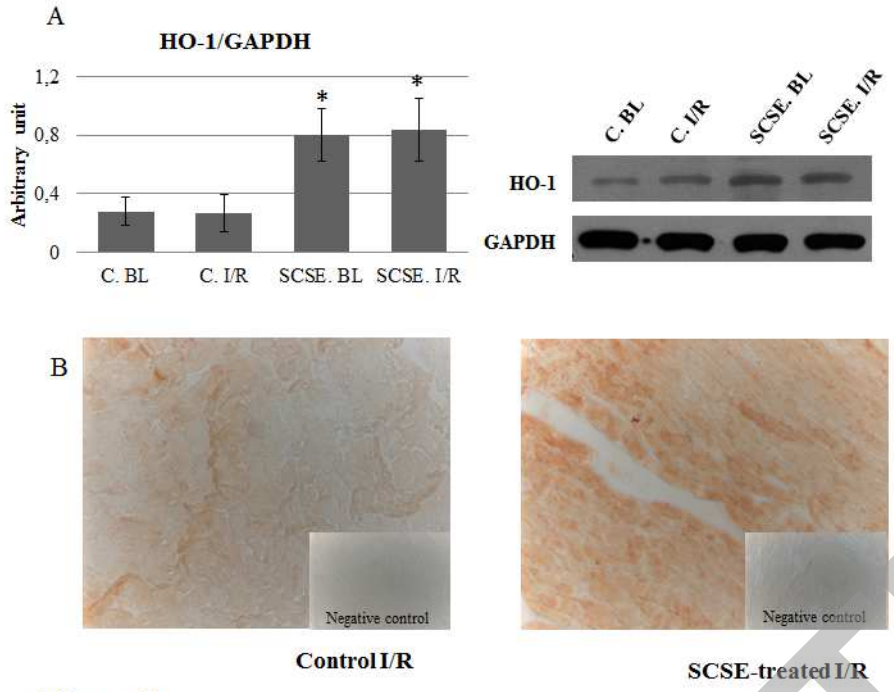

Figure 4