

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

Adverse Impact of Diet-Induced Hypercholesterolemia on
Cardiovascular Homeostasis in a New Zealand Rabbit Model

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UNIVERSITY OF DEBRECEN
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DEBRECEN, 2014

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at 11 AM, March 31, 2014

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at 13 PM, March 31, 2014

INTRODUCTION

Cardiovascular diseases remain the major cause of morbidity and mortality in industrialized Western nations. As prevalence of obesity, unhealthy diet, dyslipidemia, diabetes, elevated blood pressure, physical inactivity and smoking, known risk factors of atherosclerosis is increasing mortality and morbidity rates are suspected to increase as well. In these disorders, death most often occurs as a consequence of atherosclerosis, leading to stroke, ischemia, myocardial infarction, heart failure and other syndromes characterized by severely dysregulated inflammatory processes and their resulting degradation of tissue function. Atherosclerosis and its consequences have great impact on National Health Care and National Insurance Companies. There are lots of diagnostic scores, invasive and non-invasive tests to detect early manifestations of this disease. Therapy initiated in these early phases is able to prevent irreversible organ involvement. A hallmark of these syndromes is microvascular damage associated with a progressive decline in healthy cardiovascular tissue homeostasis, which typically is accompanied by diminished arterial flexibility and onset of age-associated pathologies, especially cardiovascular, neurologic and kidney disease. The association of dyslipidemia and ischemic heart diseases are well known. Nevertheless, at the time of this writing, a detailed description of the etiology and molecular pathomechanisms of hypercholesterolemia-induced atherosclerosis and its complications remains to be determined.

Even in the era of modern molecular engineering the results of treatment modalities extracted from traditional and herbal medicine are remarkable. Previous studies have demonstrated the significant cardiovascular benefits of a wide range of plant materials, including commonly used components of the human diet, as well as natural health products and nutraceuticals. Earlier findings at the Department of Pharmacology, Faculty of Pharmacy, University of Debrecen demonstrated that sour cherry seed extract, a natural product that contains a powerful inducer of heme oxygenase-1, strongly inhibits ischemia-reperfusion (IR) injury.

In our experiments we studied the adverse effects of diet-induced hypercholesterolemia on cardiovascular tissue homeostasis in function of time and examined the impact of sour cherry extract on that. A significant finding of time-dependent study is that the ejection fraction also was greatly diminished in animals maintained long-term (40 weeks) on high dose cholesterol diet (2%), with subsequent development of myocardial infarction.

REVIEW OF THE LITERATURE

Under physiological conditions, cholesterol and triglycerides are complexed with protein into configurations that allow these non-polar compounds to move freely in the aqueous environment of the bloodstream. These lipoprotein complexes, ranked in order of increasing size, include high density lipoprotein (HDL), low density lipoprotein (LDL), intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL), and chylomicrons. Enterocytes release nascent chylomicrons into lymphatic vessels in the walls of the villi, called lacteals, and transit into the bloodstream via the thoracic duct and left subclavian vein. Lipids are distributed via the bloodstream to different tissues in the body (skeletal muscle, adipose tissue), where triglycerides are extracted by the activity of lipoprotein lipase. HDL particles, which are the smallest (5-15nm) lipoprotein class, have the highest ratio of protein to lipid and are able to suppress the development of arterial plaques by extracting cholesterol from sites of nascent plaque formation and return it to the liver for recycling. Thus, cholesterol transported in the HDL form promotes cardiovascular health, and individuals with high ratios of cholesterol complexed into HDL chylomicrons experience lower rates of cardiovascular disease than those with low HDL/LDL ratios.

Individuals may favorably affect their HDL/LDL ratios via certain lifestyle changes, including exercise, weight loss, mild alcohol usage, increased dietary fiber, and omega-3 fatty acid consumption. However, other factors related to lifestyle may decrease HDL/LDL, such as smoking and high consumption of saturated fats, thereby increasing the risk of developing cardiovascular disorders and exacerbating existing disease. Cholesterol-rich diets have been observed to decrease HDL/LDL ratios in both humans and rabbits, resulting in similar types of deterioration in cardiovascular health. Consequently, the outcomes of studies using hypercholesterolemic rabbit models are expected to have strong relevance to human medicine.

The activity of heme oxygenase-1 (HO-1) increases in response to a wide variety of stimuli, including oxidative and inflammatory insults, as well as metabolic and hemodynamic factors such as high glucose, elevated blood pressure, and plasma lipids. Nevertheless, in most cases, the pathophysiological activation of HO-1 results only in a transient or marginal increase of HO-1 activity. It falls below the threshold necessary to activate downstream signaling components of the HO system at levels capable of achieving significant remission of serious inflammatory pathologies. Thus, strategies for use of HO-1 as a definitive prophylaxis or

treatment for type 2 diabetes mellitus (T2DM) and other diseases are expected to make increasing use of pharmacological agents that are capable of increasing the activity of the enzyme.

Several well-known plant materials from a broad phylogenetic background contain known inducers of HO-1 activity, including dietary phytochemicals. Examples include epigallocatechin-3-gallate, a polyphenol component of green tea, and curcumin, which is best known as a component of curry. SCSE is a particularly attractive candidate for the therapeutic use of HO-1, based on the capacity of this extract at relatively low dosage to increase the activity of the enzyme to levels that allow protection against ischemia-reperfusion injury. This makes SCSE potentially very valuable in cardiovascular medicine and many other healthcare venues. This advantage is combined with negligible toxicity of the extract at in vivo dosages in excess of 200x the dosage known to be therapeutic.

AIMS

Two separate investigations were conducted as core elements of the research initiative on which the present thesis is based. Each study was conducted with the objective of providing insight into pathomechanisms of hypercholesterolemia and its consequences, and to explore the capacity of sour cherry seed products to improve strategies for management of this disorder. The core hypothesis of the first investigation is that oxidative stress induced in a hypercholesterolemic rabbit model by sustained cholesterol diets promotes pathological changes in ways that may be revealed by changes on the molecular level. The investigators examined the effects of hypercholesterolemia on cardiac systolic and diastolic parameters using echocardiography. Mechanistic experiments were conducted that included measurement of the activity of cytoprotective enzymes, such as heme-oxygenase (HO-1), influences on revascularization of vascular endothelial growth factor (VEGF) protein, antiapoptotic influences and the effects of dietary treatments on mitochondrial function. Corollary studies also included assessment of the role of cytochrome c oxidase (COX) enzymes in regulating ATP metabolism and hosting adaptive, anti-inflammatory processes, including ROS scavenging. This investigation is expected to yield insight into mechanisms by which hypercholesterolemia-related oxidative stress degrades the function of critical macromolecules and promotes disease.

The second investigation evaluated the effectiveness of upregulating the activity of the cytoprotective enzyme heme oxygenase-1 (HO-1) on multiple parameters related to cardiac

health in hypercholesterolaemic rabbits fed cholesterol-enriched diets during a 16-week timeframe. A hypothesis was tested that sour cherry seed extract (SCSE), a known inducer of heme oxygenase-1 (HO-1), mediates changes in cardiac tissue homeostasis that will make this plant material an attractive candidate for the prevention and therapy of cardiac disease.

MATERIALS AND METHODS

Animals and Induction of Hypercholesterolemia

The experiments were carried out with adult male New Zealand rabbits with a body weight range of 2.0-2.5 kg. Animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research, prepared by the National Academy of Sciences (publication No. 86 23, revised 1985). The rabbits were provided with rodent chow ad libitum. 7 groups of animals were examined, in two different studies.

Time-course studies utilized 4 groups of animals, described as follows:

Rabbits were provided with normal laboratory chow 12 and for 40 weeks:

- Control 12 (n=4) and
- Control 40 (n=4) groups

and for 12 and 40 weeks with laboratory chow enriched with 2.0% cholesterol:

- HC 12 (n=4) and
- HC 40 (n=4) groups.

Studies conducted to assess outcomes of treatment with sour cherry seed extract, utilized 3 groups of animals fed for 16 weeks according to the regimens described below:

- Control group, provided with normal laboratory chow (n=6),
- Hypercholesterolemia group (HC), provided with laboratory chow enriched with 2.0% cholesterol (n=6),
- Sour Cherry Seed Extract group (HC+SCSE), provided with cholesterol-supplemented chow containing 30 mg/kg sour cherry seed extract (n=6).

Serum Cholesterol Measurement

Serum cholesterol levels in the venous blood of each animal were measured using a CardioCheck serum cholesterol analyzer at protocol defined time-points following initiation of feeding.

Echocardiography

Echocardiography was conducted under light anesthesia (ketamine 15 mg/kg, xylazine 3 mg/kg, intramuscular (i.m.)). The chest of each rabbit was shaved, and the animal was positioned in a dorsal or in a lateral decubitus position. Images were stored digitally on CD or magneto-optical disks for off-line analysis. Standard views, blood and tissue Doppler measurements were performed recommended by guidelines of echocardiography. 2D cine loops of parasternal long axis view, parasternal short axis view at the level of the papillary muscles, apical 4- and 2 chamber views were recorded. M-mode tracings were performed at the mid-papillary muscle level, either in parasternal long or short axis views using 150 mm/sec sweep speed. Left ventricular in- and outflow spectral Doppler tracings and tissue Doppler tracings at the lateral, septal and tricusidal annulus were recorded respectively. All measurements were averaged over three to five consecutive cardiac cycles.

Septal (IVSTD) and posterior (PWTD) left ventricular (LV) wall thickness in diastole, LV cavity size (end-diastolic (LVEDD) and end-systolic (LVESD) dimensions and aortic root and left atrial anteroposterior diameter were determined. Fractional shortening was computed as follows: $(LVEDD - LVESD)/LVEDD$, and as global systolic function was balanced, the ejection fraction (EF) was derived as $EF = (LVEDD^2 - LVESD^2)/LVIDD^2$. Left ventricular myocardial mass was calculated using $LVMass \text{ (Troy)} = 1.05 \times [(LVEDD + (PWTD) + IVSTD)^3 - (LVEDD)^3]$.

Time-velocity integral at the left ventricular outflow tract, peak aortic flow velocity, early (E) and late (A) mitral flow velocities, deceleration time of E wave (DT), annular systolic (S'), early (E') and late (A') diastolic velocities were determined also.

Rabbit heart isolation

Following the treatment period (12- and 40-weeks for time-dependent, 16 weeks for sour cherry seed extract effect study), heparin (1000 IU/kg) and ketamine/xylazine (40/5 mg/kg) were injected intravenously. Next, thoracotomies were performed on each animal; the hearts were excised and placed into ice-cold perfusion buffer. The aortas were then cannulated, and hearts were perfused according to Langendorff method for a 5-minute washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate buffer: 118 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl₂, 25 mM NaHCO₃, 0.36 mM KH₂PO₄, 1.2 mM MgSO₄ and 10 mM glucose. The left atrium was cannulated, and the Langendorff system was adapted and switched to isolated

working hearts. The revised procedure used a left atrial filling pressure of 17 cm (1.7 kPa) and aortic afterload pressure of 90 cm (9.0 kPa) of buffer. Aortic flow was measured by a calibrated flow meter, and coronary flow rate was measured by a timed collection of the coronary perfusate that dripped from the heart. Heart rate and left ventricular developed pressure values were recorded as well. Heart function parameters were recorded through the whole experiment.

Induction of Global Ischemia and Reperfusion in Isolated Hearts

Following a 10-minute aerobic perfusion of the heart, 30 minutes of global ischemia was initiated by clamping the atrial inflow and aortic outflow lines at a point close to the origin of the aortic cannula, and stopping the peristaltic pump. Reperfusion was initiated by unclamping the atrial inflow and aortic outflow lines and the heart was kept in this condition for 120 minutes. In order to eliminate the incidence of arrhythmias, the initial 10 minutes of reperfusion was carried out in Langendorff mode.

Cardiac function parameters

Time course studies revealed severe resting cardiac function impairment at 40 weeks in the hypercholesterolemic group (HC 40). These conditions developed in both groups of animals without surgical induction of global ischemia. Conversely, animals receiving SCSE-supplemented feed exhibited no significant difference between the resting parameters of the three groups observed, accordingly, the hearts of these animals were subjected to 30 minutes of global ischemia, followed by measurement of cardiac function parameters at 60 and 120 minutes of reperfusion.

Infarct size determination

100 mL of 1% triphenyl tetrazolium chloride (TTC) solution in phosphate buffer (Na₂HPO₄ 88 mM, NaH₂PO₄ 1.8 mM) was administered via the side arm of the aortic cannula and, then, stored at -70 °C for later analysis. TTC (white colour) is enzymatically reduced to TPF (1,3,5-triphenylformazan / red colour) in living tissues due to the activity of various dehydrogenase enzymes. At necrotic areas enzymes do not work, so it remains white. For this reason, TTC has been employed in autopsy pathology to assist post-mortem identification of

myocardial infarctions. Healthy viable heart muscle will stain deep red from the cardiac lactate dehydrogenase; while areas of potential infarctions will be paler.

Freezed hearts were sliced transversely in a plane perpendicular to the apicobasal axis into 2–3 mm thick sections, weighted, blotted dry, placed in between microscope slides and scanned. Using image processing software, each image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity and distinctness. The infarct area of each slice was traced, and the respective areas were calculated by pixel density analysis. Infarct size was expressed as a percentage ratio of the infarct zone to the risk zone (weight of the left ventricle).

Analysis of atherosclerotic lesions

Quantification of fatty streaks was performed with Sudan III stain. Thoracic arteries were harvested, dissected free of excess connective tissue and fat, rinsed with modified Krebs-Henseleit buffer, and fixed in 10 % (vol/vol) buffered formalin. Thoracal arteries were then opened longitudinally, and exposed to 5 mg/mL Sudan III in 70 % (vol/vol) isopropanol for 15 minutes in a water bath at 37 °C, and the stain was differentiated with several rinses of 70 % isopropanol. The arteries were then scanned, and the atherosclerotic plaque was determined.

Cytochrome c oxidase (COX) activity

The activity of cytochrome c oxidase in rabbit myocardium was measured using a colorimetric assay kit for oxidation of cytochrome c by this enzyme. Briefly, mitochondria isolated from freshly harvested heart muscle using the MITOISO1 kit were treated with dithiothreitol to reduce cytochrome c, followed by COX-mediated reoxidation of the molecule. COX activity at room temperature in each sample was measured as a decrease in absorbance of ferrocytochrome c, at an absorbance wavelength of 550 nm in its conversion from a reduced to oxidized state. The COX activity in any particular sample was reported as - $\Delta A_{550}/\text{min}$.

Western blot analysis

Total protein (100 g) in the Clontech Extraction buffer was added to an equal volume of sodium dodecyl sulfate (SDS) buffer and boiled for 10 minutes before being separated on 12 % SDS polyacrylamide gels in a running buffer (25 mM Tris 192 mM glycine, 0.1 % (wt/vol) SDS, pH 8.3) at 120 V. The gel was transferred onto a nitrocellulose membrane at 100 V and left for 1 hour in a transfer buffer. After blocking the membranes for 1 hour in a Tris-buffered saline (TBS-T) containing 0.1 % (vol/vol) Tween-20 and 5% (wt/vol) nonfat dry milk, blots were incubated overnight at 4°C with the primary antibody (HO-1, VEGF, COX III, COX IV, GAPDH). Membranes were washed 3 times in TBS-T before being incubated for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:2,000 in TBS-T and 1 % (wt/vol) nonfat dry milk. Detection was made by autoradiography for variable lengths of time with Medical X-Ray Film. GAPDH (cytoplasm), COX IV (mitochondrial) was used as the loading control (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). Quantitative analysis of scanned Western blots to estimate levels of HO-1, VEGF, COX III, COX IV and GAPDH protein in each sample were calculated using the Scion for Windows Densitometry Image program version Alpha 4.0.3.2. Signal intensity for bands corresponding to each protein of interest was estimated and reported in arbitrary units \pm SEM.

Statistics

Results are expressed as mean \pm SD or mean \pm SEM. One-way analysis of variance was first carried out to test any differences between the mean values of different groups. If differences were established, the results between two groups were compared by Tukey test. Results were considered to be significant if $p < 0.05$.

RESULTS

The thesis is based on two, basically quite similar experiments, so the results of these are discussed simultaneously.

Serum cholesterol levels

Serum cholesterol levels in animals provided with feed containing 2% cholesterol were continuously increasing during the entire course of the study, while serum levels of animals provided with normal laboratory chow maintained stable. Total serum cholesterol in animals provided with feed containing 2 % cholesterol became significantly increased after 8 weeks of the time-dependent, and 4 weeks of the SCSE effect study relative to levels observed in control rabbits receiving normal feed ($p < 0.05$).

Total serum cholesterol in hypercholesterolemic animals provided with feed containing 2 % cholesterol became significantly increased at the 16th week of the study relative to levels observed in hypercholesterolemic rabbits receiving cholesterol-supplemented feed containing 30 mg/kg SCSE ($p < 0.05$).

Plaque coverage in thoracic arteries

The extent of plaque coverage in animals maintained on high cholesterol diets (HC 12, HC 40) with or without sour cherry seed extract (HC, HC+SCSE) was significantly greater than in rabbits fed with normal chow (Control) ($p < 0.05$), in both studies. Animals fed high cholesterol diets for longer periods of time (HC 40) exhibited significantly higher plaque coverage than rabbits receiving the 2% cholesterol-supplemented feed for 12-week periods (HC 12) ($p < 0.05$). The extent of plaque coverage in hypercholesterolemic rabbits receiving 30 mg/kg SCSE daily in addition to feed supplemented with 2 % cholesterol was significantly less than the coverage observed in cholesterol-fed animals without SCSE ($p < 0.05$). Negligible atherosclerotic plaque accumulation was noted in rabbits fed with normal chow.

Echocardiography

In the time-dependent study the values of fractional shortening (FS) and ejection fraction (EF) of the left ventricle were significantly reduced in HC 40 animals relative to their values in non-hypercholesterolemia control rabbits ($p < 0.05$) and to animals maintained for shorter

time periods on hypercholesterolemia-inducing chow (HC 12) ($p < 0.05$). No significant differences in other echocardiographic parameters were observed.

In the SCSE effect study there were no significant differences in systolic parameters and anatomic measures between the three groups. Though, it was observed that hypercholesterolemic rabbits fed 30 mg/kg SCSE daily in addition to 2 % cholesterol (HC+SCSE) during a 16-week period exhibited significantly higher E'/A' ratios than hypercholesterolemic animals not administered the extract (HC) ($p < 0.05$).

Hemodynamic parameters

Aortic flow, the magnitude of which is a major indicator of cardiac health, was evaluated in isolated working hearts harvested from non-hypercholesterolemia rabbits maintained on normal chow for 12 weeks (Control 12) or 40 weeks (Control 40). Aortic flow measurements were also made on animals fed high cholesterol chow for 12-week periods (HC 12) or 40 weeks (HC 40). Similar to the echocardiographic findings in vivo, HC 40 rabbits exhibited profoundly significant reduction in resting systolic function of the heart. Aortic flow was reduced relative to both non-hypercholesterolemia control groups and also with respect to rabbits fed 2% cholesterol for 12-week periods (HC 12) ($p < 0.05$). The hearts from hypercholesterolemic rabbits showed very poor heart functions (no recovery), after 40 weeks of treatment many animals failed to produce measurable aortic flow (0 mL/min).

In the SCSE effect study there were no significant differences in the resting hemodynamic parameters between groups, so global ischemia was provoked and post ischemic reperfusion (I/R) cardiac function parameters (heart rate, aortic flow, coronary flow and left ventricular developed pressure) were evaluated. The effect of I/R injury included a trend toward lower values for each of the functional parameters as a result of occurrence of the injury and increasing reperfusion time. Significantly better aortic flow was observed at 60 and 120 min of reperfusion in SCSE-treated hypercholesterolemic animals versus those not receiving the extract ($p < 0.05$), whereas a comparison of these groups revealed coronary flow to be significantly improved only in hearts subjected to a 120-min reperfusion period ($p < 0.05$).

Extent of infarct zones

Infarcted regions failed to develop in control animals. Only small infarcted areas forming in hearts of rabbits receiving high cholesterol chow for 12-week periods (HC 12) were observed. Conversely, infarcted regions were noted in hearts of rabbits fed with the 2% cholesterol-

supplemented diet for 40 weeks (HC 40), which were significantly larger than the infarct size of HC 12 animals ($p < 0.05$).

The average extent of infarct zones in the different groups of SCSE effect study reveal that relative to non-hypercholesterolemic rabbits, hearts from hypercholesterolemic animals manifested larger infarct zones. Moreover, the average extent of infarcted areas was observed to be significantly lower in hearts from SCSE-treated hypercholesterolemic animals ($p < 0.05$).

Activity of cytochrome c oxidase (COX)

Total COX activity in heart tissues of animals maintained on high cholesterol diets was significantly lower than the activity of their control pairs. Rabbits provided with chow containing 2% cholesterol for 40 weeks (HC 40) had significantly elevated COX activity in heart tissue relative to the activity of the enzyme in hearts of hypercholesterolemia rabbits receiving high cholesterol chow for 12 weeks (HC 12) ($p < 0.05$).

In the SCSE effect study, animals receiving cholesterol rich chow without SCSE exhibited significantly lower COX activity in cardiac tissue compared with rabbits in the HC+SCSE group ($p < 0.05$).

Protein levels of COX III, HO-1 and VEGF in heart tissue

Left ventricular tissue taken from HC 40 animals were found to contain significantly more COXIII protein than hearts from HC 12 rabbits, but significantly less COXIII than control animals receiving normal chow. I/R-injured heart tissue from hypercholesterolemic rabbits administered SCSE (HC+SCSE) was observed to contain significantly greater quantities of COX III than heart tissues from the other two groups (Control and HC).

Hypercholesterolemia engenders processes that greatly increase the level of oxidative stress to which cardiac tissue is subjected. Therefore it is unsurprising that significantly lower levels of HO-1 protein were detected in hearts of animals treated with high cholesterol diets. Tissue level of HO-1 of rabbits receiving cholesterol rich chow for 40 weeks was significantly lower than tissue levels of animals on cholesterol rich diet for 12 weeks. In the SCSE effect study I/R-injured heart tissue from hypercholesterolemic rabbits (HC I/R) had significantly lower HO-1 protein levels. In the SCSE treated group (HC+SCSE I/R) hearts contained significantly greater quantities of HO-1 protein than HC I/R hearts.

There was lack of significant differences in the VEGF content of heart tissue from any of the groups of rabbits included in these studies.

DISCUSSION

In our experiments we studied the adverse effects of diet-induced hypercholesterolemia on cardiovascular tissue homeostasis and examined the impact of sour cherry extract on that.

Serum cholesterol levels

In our studies animals provided with feed containing 2% cholesterol significant hypercholesterolemia could be observed. Level of serum cholesterol increased proportionally with the time course of feeding. Normal levels of serum cholesterol ($2 \pm 0,5$ mmol/l) elevated to $14 \pm 1,3$ mmol/l at 12 weeks of feeding, to $24 \pm 1,6$ mmol/l at 16 weeks and to $37 \pm 3,5$ mmol/l at 40 weeks respectively. This increasing stress put on HC animals by elevated serum cholesterol levels explain those devastating deterioration observed in group of animals receiving cholesterol rich chow for 40 weeks.

Familial hypercholesterolemia

Levels of serum cholesterol measured in our studies can be observed in patient having familial hypercholesterolemia. Familial hypercholesterolemia is a genetic disorder characterized by high cholesterol levels, especially very high levels of low-density lipoprotein (LDL) in the blood and early cardiovascular disease. Familial hyperlipidemias are classified according to the Fredrickson classification which is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation. Accelerated atherosclerosis caused by elevated levels of LDL can be observed in familial hypercholesterolemia (IIa) and in familial combined hyperlipidemia (IIb). Elevated level of LDL provokes formation of atherosclerotic lesions all over the vascular tree by triggering inflammatory processes and excessive plaque formation. The overall prognosis for homozygotic individuals is in the worst case scenario, individuals usually die at the age of 30-40. Treatment modalities of these diseases include combined conventional lipid lowering drug therapy, monoclonal antibodies, gene therapy, LDL apheresis and liver transplantation.

Cardiovascular effects of elevated level of serum cholesterol

Plaque coverage in thoracic arteries

During the lifetime of an individual afflicted with hypercholesterolemia, sustained high levels of serum cholesterol result in arterial stiffening and an accumulation of atheromatous plaques,

causing stenosis (narrowing) of the involved blood vessels. Rupture of plaques may cause clots and thrombus formations, resulting in obstruction of blood vessels, heart attack, and stroke. These processes reduce tissue oxygenation, leading to ischemic injury to many organs, most notably tissues of the heart, kidneys, lungs, and brain. In addition, the presence of a large number of macrophages in plaque matrices results in plaque material becoming a potent source of inflammatory cytokines and other mediators of inflammation, which lead to hyperstimulation of the primary immune response and a diverse range of pathologies.

Rabbits provided with normal laboratory chow showed no macrovascular signs of atherosclerosis. Feeding with cholesterol rich chow resulted in severe atherosclerosis of thoracic artery. The extent of plaque coverage in animals maintained on high cholesterol diets (HC) were $61 \pm 14\%$ at 12 weeks, $75 \pm 11\%$ at 16 weeks and $88 \pm 5\%$ at 40 weeks of feeding respectively.

Myocardial ischemia and infarction

Hypercholesterolemia degrades homeostatic processes in cardiovascular tissue in ways that engender epicardial and microvascular coronary artery disease. Elevated levels of serum cholesterol promote formation of plaque deposits in epicardial arteries, which progressively narrow vessel lumen, depriving tissues they supply of oxygenated blood. As myocardial oxygen demand exceeds supply, infarct zones may develop in regions of myocardial tissue supplied by occluded blood vessels. In humans, the clinical presentation of such processes is termed “non-ST (connects the QRS complex and the T wave) elevation myocardial infarction”. The pathogenesis of this syndrome does not involve occlusion of the infarct-related coronary artery; therefore, no ST segment elevation—a sign of total coronary occlusion—is typically observed in electrocardiography (ECG) evaluations of afflicted individuals. For non-ST elevation myocardial infarction, the extent of necrotized tissue is dependent on the size of the myocardial region supplied by the coronary artery distal to the stenotic lesion and the time interval of the insufficient oxygen supply. Having severely stenotic coronary arteries and stained myocardial necrotic regions, the pathophysiology of myocardial infarction in HC 40 animals for the present study may be similar to non-ST segment myocardial infarction observed in humans. Furthermore, since the total infarcted region or myocardial mass increases, the systolic function of the heart is attenuated, causing a decrease in fractional shortening and the ejection fraction. Experimental ligation of the coronary artery creates disruption of tissue homeostasis that resembles ST segment elevation

myocardial infarction observed in humans when the coronary artery is totally occluded by thrombus formation at the time of rupture of an atherosclerotic plaque. This infarcted region is clearly distinguishable from normal myocardial tissue and plain necrotic areas—and may, thus, be identified with little ambiguity, unlike in non-ST elevation cases. When the myocardial tissue is damaged (typically by oxidative stress), its contractile function is attenuated and emptying capability decreased.

In the present study, hypercholesterolemia adversely affected the vasculature to result in severe atherosclerotic lesions of coronary arteries. The resulting reduction in the availability of blood and oxygen resulted in micro- and macro-myocardial infarctions (TTC perfusion sections—total infarcted region). These multiple necrotic events (total infarcted region of $32 \pm 8,6\%$) caused global left ventricular contractility impairment that was detectable by significantly decreased fractional shortening (FS: $20 \pm 3\%$) and ejection fraction (EF: $38 \pm 5\%$) parameters in vivo and by decreased aortic flow (AF: $4,6 \pm 11,4$ ml/min) in isolated working heart specimens of HC 40 animals compared to Control 12 (FS: $35 \pm 5\%$, EF: $60 \pm 7\%$, AF: 146 ± 53 ml/min) Control 40 (FS: $33 \pm 4\%$, EF: $58 \pm 6\%$, AF: 120 ± 18 ml/min) and HC 12 (FS: $32 \pm 6\%$, EF: $55 \pm 9\%$, AF: 72 ± 18 ml/min) values respectively.

16 weeks of feeding with cholesterol rich chow had no effect on systolic parameters of left ventricle.

Diastolic dysfunction

Diastole is energy dependent process mediated by active dissociation of actin and myosin filaments. This process is initiated by the changes in intracellular calcium concentration. The myofilament is composed of a thick filament “myosin” and thin filament “actin” proteins. Several regulatory proteins including tropomyosin and troponin T, C, and I are bound to actin. Before the closure of aortic valve, calcium is transported from the cytosol into the sarcoplasmic reticulum (SR) causing reduction in intracellular calcium concentration which leads to the dissociation of actin and myosin cross bridges. This transport of calcium is an active process requiring energy.

The mechanisms that cause abnormalities in diastolic function that lead to the development of diastolic heart failure can be divided into factors intrinsic to the myocardium itself (myocardial) and factors that are extrinsic to the myocardium. Myocardial factors can be divided into structures and processes within the cardiac muscle cell (cardiomyocyte), within

the extracellular matrix that surrounds the cardiac muscle cell, and that activate the autocrine or paracrine production of neurohormones.

Hearts with normal diastolic function represent higher early diastolic tissue velocity (E') of mitral annulus than late diastolic velocity (A'), therefore E'/A' ratio is above 1.

Hearts of animals provided with cholesterol-rich chow for 16 weeks showed signs of diastolic dysfunction on echocardiographic examination.

Effect of sour cherry seed extract on cardiovascular parameters

Animals that were fed 30 mg/kg SCSE daily added cholesterol exhibited significantly lower serum cholesterol ($19,3 \pm 1$ mmol/l) than those not receiving SCSE ($24 \pm 1,6$ mmol/l) ($p < 0.05$). These outcomes are the first direct demonstration that an inducer of HO-1 (SCSE) may act to decrease serum cholesterol. Interestingly, previous studies demonstrated that statins, a class of cholesterol-lowering drugs that are also HO-1 inducers, do not mediate anti-inflammatory and anti-oxidant effects through HO-1 induction in hypercholesterolemic humans.

The extent of plaque coverage in hypercholesterolemic rabbits receiving 30 mg/kg SCSE daily in addition to feed supplemented with 2 % cholesterol ($58 \pm 6\%$) was significantly less than the coverage observed in cholesterol-fed animals without SCSE ($75 \pm 11\%$) ($p < 0.05$).

Ischemia-reperfusion

The effect of I/R injury included a trend toward lower values for each of the functional parameters as a result of occurrence of the injury and increasing reperfusion time. Significantly better aortic flow was observed at 60 and 120 min of reperfusion in SCSE-treated hypercholesterolemic animals versus those not receiving the extract ($p < 0.05$), whereas a comparison of these groups revealed coronary flow to be significantly improved only in hearts subjected to a 120-min reperfusion period ($p < 0.05$).

Deterioration of cardiac function, myocardial infarction, and diastolic dysfunction are major indicators of hypercholesterolemia-associated heart disease, the ability of SCSE to significantly improve each of these measures relative to rabbits receiving no SCSE extract ($p < 0.05$) is additional evidence for the preventive and therapeutic value of this extract.

Molecular consequences of hypercholesterolemia

Studies were conducted to evaluate the effect of hypercholesterolemia on the expression of COXIII, VEGF and HO-1. As in previous experiments, animals exposed for the longest time periods (40 weeks) to high cholesterol diets exhibited the most significant changes.

Left ventricular tissue taken from HC 40 animals were found to contain significantly more COXIII protein than hearts from HC 12 rabbits, but significantly less COXIII than control animals receiving normal chow. The physiologic significance of this observation cannot be fully defined based on the data presented here; however, this intriguing finding may be related to the effect of elevated cholesterol on mitochondrial metabolism. A report published in 2010 demonstrates that exposure of porcine cardiac endothelium responding to interaction with oxidized LDL (which is a known feature of the hypercholesterolemic state) exhibited significant dysregulation of mitochondrial enzyme function, including COXIII. Thus, hypercholesterolemic animals are more likely to experience disruption in COXIII activity; however, the specific effects will need further analysis in order to place them in a mechanistic picture of hypercholesterolemic disruption of mitochondrial function.

The lack of significant differences in the VEGF content of heart tissue from any of the groups of rabbits included in the two studies is puzzling given the adverse effect on neovascularization of hypercholesterolemia observed in rats and the pivotal role played by VEGF in this process.

The outcome of these studies also merits further exploration in future research. Hypercholesterolemia engenders processes that greatly increase the level of oxidative stress to which cardiac tissue is subjected. Therefore it is unsurprising that significantly lower levels of HO-1 protein were detected in hearts of animals treated for 40 weeks with high cholesterol diets. HO-1 expression is an adaptive response, which is upregulated by elevation in oxidative stress. Nevertheless, the protective effect of pathophysiologic increase of HO-1 activity is often overwhelmed by the intensity of a particular type of trauma to which the tissue is subject; in this case, the hypercholesterolemic condition imposed by diet.

These results are intriguing in the context of observations made in SCSE effect study, which demonstrated that serum cholesterol levels in hypercholesterolemic rabbits administered sour cherry extract in addition to 2% cholesterol-enriched feed was significantly lower than in hypercholesterolemic animals not receiving the extract. Moreover, the extract-treated rabbits exhibited greatly improved cardiac function, reduced arterial atherosclerotic plaque and lower infarct size than untreated controls. These effects, which positively correlated with HO-1

expression in heart tissue, raise the possibility that statin drugs, which are also HO-1 inducers, may share common mechanisms contributing to cholesterol reduction. This possibility is nevertheless complicated by findings presented in 2011 that statins do not act primarily through HO-1 induction. It is therefore likely that investigations of the relationship between statin drugs and processes in which HO-1 is a participant will yield novel strategies for prevention and management of cardiovascular disease.

Total activity of cytochrome c oxidase (COX) was evaluated in the cardiac tissue of test animals. The patterns of COX enzyme activity paralleled expression of COXIII protein with respect to the status of the animals from which cardiac tissue was harvested to assay for COX protein using Western blot or COX-mediated conversion of reduced ferrocytochrome c to its oxidized form. Left ventricular tissue taken from HC 40 animals exhibited significantly greater ability to convert COX substrate than heart tissue from HC 12 rabbits, but significantly less than control animals receiving normal chow. Although data presented here does not permit an evidence-based interpretation of the mechanistic basis for these observations, here, too, it is likely that high levels of oxidized LDL resulting from the hypercholesterolemic condition of the animals has impaired both function and expression of critical mitochondrial enzymes, including COX.

HO-1 protein expression in I/R-injured hearts of SCSE-treated hypercholesterolemic animals was significantly higher than rabbits not receiving the extract ($p < 0.05$). This outcome is expected based on the known properties of SCSE. In recent years, HO-1 has emerged as a particularly attractive tool for the prevention and management of a broad range of human and animal diseases characterized by elevated levels of reactive oxygen-containing molecules. A major mechanism by which the enzyme contributes to antioxidant defense is through suppression by bilirubin (a heme degradation metabolite) of NADPH oxidase, a major physiologic source of reactive oxygen. This inhibition has been shown to be a potent contributor to protection against oxidative tissue damage in cardiovascular disease and many other serious chronic diseases.

SCSE has potential to emerge as a major contributor to prevention and management of cardiovascular disease, and many other disorders characterized by dysregulated inflammation. Other than low cost and broad availability, the benefit to patients of using SCSE-based therapies is the negligible toxicity of this plant material. This is contrasted with the risk posed to patients by the anti-hypercholesterolemic drugs that are currently in use. Examples include simvastatin, lovastatin, atorvastatin, rosuvastatin, and others which may have adverse side

effects, such as rhabdomyolysis. Therefore, SCSE is potentially a very valuable adjuvant to statins. Many other healthcare applications are possible for this fascinating and valuable plant material.

SUMMARY

In our experiments we studied the adverse effects of diet-induced hypercholesterolemia on cardiovascular tissue homeostasis and examined the impact of sour cherry extract on that.

Our experiments confirmed, that hypercholesterolaemia can be induced by chow supplemented with 2% cholesterol in rabbits. Moreover, a linear correlation could be observed between the serum cholesterol levels and the feeding time. Based on the serum cholesterol levels and pathophysiological alterations an animal model of familial hypercholesterolemia can be generated following an exposition time of 40 weeks. The degree of atherosclerosis observed in the thoracic aorta correlated with the duration of the high cholesterol diet.

In animals fed with high cholesterol diet echocardiography showed left ventricular diastolic dysfunction after 16 weeks. However, high cholesterol diet for 40 weeks induced severe coronary atherosclerosis and consequent myocardial necrosis leading to severe systolic dysfunction. The impairment in the left ventricular function could be both assessed by echocardiography *in vivo* and measured in isolated myocardial preparations *ex vivo*.

The expression level and activity of cytochrome c oxidase (COX) decreased in animals fed with cholesterol-free rabbit chow. In contrast, expression level and activity of COX in the high cholesterol diet group was very low after 12 weeks and increased significantly after 40 weeks to the levels observed in the animals fed with cholesterol-free diet for 40 weeks. Despite similar COX expression hypercholesterolemia led to reduced COX activity. The expression of COX was not influenced by ischemia-reperfusion. Significantly lower levels of HO-1 protein were detected in heart tissue derived from rabbits with high cholesterol diet compared to hearts from the non-hypercholesterolemia control animals. No apparent significant differences could be observed in the VEGF protein expression levels between non-hypercholesterolemia control rabbits and those experiencing diet-induced hypercholesterolemia.

Addition of sour cherry seed extract (SCSE) to high cholesterol diet for 16 weeks significantly decreased serum cholesterol level, the degree of atherosclerosis in the thoracic aorta and improved left ventricular diastolic dysfunction. SCSE-treated animals exhibited significantly reduced infarct size, improved cardiac function and improved coronary flow following global ischemia-reperfusion. Increased myocardial HO-1 and COX protein expression were also observed in hearts from SCSE-treated rabbits with no major changes in the VEGF levels.

Using an animal model of hypercholesterolemia our studies confirmed the time-dependent atherogen effects of hypercholesterolemia, the cholesterol-lowering and cardioprotective effects of the sour cherry seed extract. Based on these observations the sour cherry seed extract could serve as a potential supplementary therapeutical option in conditions associated with elevated cholesterol levels (e.g. familial hypercholesterolemia).

ACKNOWLEDGEMENT

I would like to express my gratitude to Professor Árpád Tósaki, Director of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, and to Professor István Édes, Director of Department of Cardiology, Faculty of Medicine, University of Debrecen for allowing the accomplishment of our investigations in their institutes.

I am thankful for Béla Juhász, my Mentor, who provided lot of help and support during my Ph.D. studies, and made it possible for me to get familiar with exciting and honoring feeling of scientific learning, and also I would like to thank all the team members of the Department of Pharmacology for their constant friendly help during this scientific work.

I would like to offer my special thanks to my parents, who supported me through my life, my children, Gergely and Csongor who give me endless energy and power to overcome any obstacle.

This study was supported by a grant of the Hungarian Scientific Research Fund Programs, by the European Union, by the University of Debrecen and by the Hungarian Academy of Sciences: OTKA (78223, 104017, K-83478, K-75883); TAMOP-4.2.1/B-09/1/KONV-2010-0007; TAMOP-4.2.2.A-11/1/KONV-2012-0045; TAMOP 4.2.4. A/2-11-1-2012-0001; TAMOP-4.2.2./B-10/1-2010-0024; (MTA-DE Vasc. Biol. 11003). This research was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TAMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program' (providing personal support to Arpad Tosaki).



Register Number: DEENKÉTK/401/2013.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Attila Béla Kertész

Neptun ID: S5APBH

Doctoral School: Doctoral School of Pharmaceutical Sciences

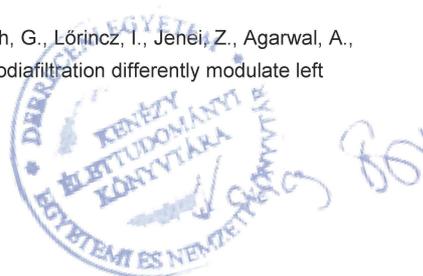
Mtmt ID: 10037250

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DOI: <http://dx.doi.org/10.1556/OH.2010.28798>

Total IF of journals (all publications): 20.622

Total IF of journals (publications related to the dissertation): 5.775

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

17 December, 2013

