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

Investigation of the toxicity and cardiovascular effects of materials with natural origin

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DEBRECEN, 2016
Investigation of the toxicity and cardiovascular effects of materials with natural origin

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1. Introduction and objectives of the doctoral thesis

Cardiovascular diseases and malignant tumors are the leading cause of death, worldwide. Based on the hungarian statistical data the most common cardiovascular diseases are the ischemic injuries of the heart and the acute heart tissue necrosis. Main risk factors of these disorders include, inter alia, high blood pressure, dyslipidemia, diabetes mellitus, obesity, smoking and severe non influenced factors, for example age and familiar clustering.

It is very important to emphasize the importance of prevention in relation to any diseases, however not every disorders can be prevented, but the delay is extremly important.

In relation to dietary supplements as part of prevention, we have to talk about the complex healthy lifestyle, which includes healthy diet, exercise and mental balance. People of today have to adapt to the course of time evolved life. I think it is important to insert the exercise into the hectic everyday life, manage the stress and especially replace and compliment the vitamins, minerals, trace elements and fibers. The most efficient means of this are the dietary supplements. In case of these supplements quality, proper fromulation, price and marketing are very important factors.

It can be said, that we live the age of nutrients, which is featured by overuse and selection and taking non proper and harmful preparations. Hugh amount of materials with natural origin can be found in pharmacies, drogeries and on the internet, which are used without appropriate scientific data.
In the first part of our experiments we determined the effects of sour cherry seed kernel extract treatment on hepatic and renal function and toxicity. In further experiment we studied the direct effect on seed kernel oil on the skin and the photoprotective effect of oil containing creams.

In the second part of our experiments we investigated the effects of long term, low and high dose beta-carotene treatment on heart function, infact size, tissue antioxidant capacity and HO-1 level. In further experiments we measured the cytotoxic effect of beta-carotene treatment on H9c2 cell line by MTT assay.
2. Materials and methods

Analysis of sour cherry seed kernel extract toxicity

Seed extracts

The dosage of sour cherry seed extract (SCSE) was used as the primary independent variable in the present study. Sour cherry seeds were dried, and the wall was removed. The kernels were then macerated into a paste with a consistency suitable for administration to animals via gavage. This SCSE was prepared so as to contain both the oil and bioflavone rich solid fractions of the kernel. High pressure compressed seed kernel oil was used for the dermal studies.

Animals

Mice used for organ toxicity and blood biomarker experiments were strain C57BL/6J males with an average weight of 32 ± 4 g. Male Hartley guinea pigs weighing 250–350 g were used for evaluation of dermal effects. The animals were fed regular rodent chow ad libitum with free access to water. The animals were allowed 1 week to acclimatize before the experiments and were kept at 25 ± 2 °C, with a relative humidity of 55 ± 5 % and a 12 h light–dark cycle. All animals included in the present study were handled and received humane care in compliance with the principles of the laboratory animal care formulated by the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (Publication No. NIH 85–23, revised, 1996). All protocols for use in this investigation were approved by the institutional review board (IRB) of University of Debrecen, Hungary.
SCSE dosage groups

The mice were randomly assigned to six test groups based on SCSE dosage, members of which were administered whole kernel meal once daily by gavage for 8 days in dosages in the range 0–3000 mg/kg, defined as follows: Group I: no SCSE, the negative control group, gavaged with normal saline buffer only (n = 7); Group II: 250 mg/kg SCSE (n = 7); Group III: 500 mg/kg SCSE (n = 8); Group IV: 1000 mg/kg SCSE (n = 8); Group V: 3000 mg/kg SCSE (n = 6).

Oral toxicity assessments

Oral administration of SCSE by gavage was conducted to assess hepatic and nephrotoxicity of the seed kernel contents. After completion of the SCSE treatment regimen, the animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and sacrificed under deep anesthesia, followed by measurement of the body weight of each mouse. The livers and kidneys were excised immediately after collection of blood, weighed, washed in ice cold saline and stored in PBS with 10% formalin. The following postmortem evaluations were then conducted.

Macroscopic examination

All mice were given a complete postmortem examination (autopsy). The abdominal, thoracic and cranial cavities were examined for abnormalities, and the organs were removed, examined and fixed in 10% formalin. Weights of kidney and liver were recorded and organ weight ratios were calculated relative to total body weights.
Serum metabolites and enzyme activities

Test kits purchased from Asan Pharmaceutical (Seoul, Korea) were used to determine the occurrence of toxic effects on kidney or liver function by assessment of biomarkers in mouse blood. The blood samples were left to stand at room temperature for 1 hour and then centrifuged at 300 × g for 10 min to obtain the serum. Serum blood urea nitrogen and creatinine levels were assessed as indicators of the kidney function. Serum glutamic oxaloacetic transaminase activity, serum glutamic pyruvic transaminase activity and serum alkaline phosphatase, were measured as indicators of liver function.

Histopathological/microscopic examination

Blood collected from the jugular veins was utilized for serum preparation. The mice were then sacrificed, followed by removal of liver and kidneys and fixation of these organs in 10% formalin solution, from which 5 μm thick histological sections were prepared. Tissue specimens were hematoxylin–eosin (HE) stained and observed under a light microscope.

Dermal responses to SCSE oil fraction

Experiments were conducted in which the kernel oil fraction was applied topically to shaved skin of animals to evaluate possible adverse skin responses, and also to demonstrate the photoprotective ability of the oil.
Dermal toxicity studies

The capacity of the sour cherry kernel oil to act as an allergen or otherwise damage the skin was evaluated. Guinea pigs were anesthetized with intraperitoneal pentobarbital sodium (30 mg/kg body weight). After anesthesia, the hair on the back was closely shaved followed by epilation of remaining micro hair. Dosages of 0, 1, 2, 3 and 5 µl of the kernel oil was massaged into the epilated skin of five animals. Distilled water was applied to the skin of a control group of guinea pigs (n = 5). Initially, animals were monitored hourly and then, after every 12 h for 21 days.

Photoprotection/phototoxicity studies

The capacity of the oil to protect skin from the effects of ultraviolet radiation was evaluated. Here, five guinea pigs were closely shaved and covered with aluminum foil into which five rectangular slots had been cut. Next, SCSE oil in unguent was applied to the skin under each slot at dosages of 0–5% by weight of the kernel oil. The animals were placed under UVB apparatus and the uncovered skin was irradiated for 40 min. Evaluations of UVB induced skin damage and the protective effect of the SCSE oil were made 6 h after irradiation. Here, the dermal allergic response and phototoxicity experiments were conducted as stand alone studies independently demonstrating aspects of interaction between skin and the kernel oil fraction with relevance to possible human topical use. No correlations between the dermal and oral studies were deemed necessary for validation of outcomes described in the present report, or their potential relevance to the clinical benefits of SCSE.
Statistical methods

SPSS statistical analysis software was used. The data were compared by Student’s t-test in assessment of the significance (p) value for differences between test groups versus negative control animals with respect to enzyme activities and organ weight ratios. For all comparisons, a value of \( p < 0.05 \) was considered the threshold for significant difference between magnitude of a particular outcome variable in a test group versus the corresponding control group value.

The cardiovascular effects of beta-carotene

Animals

The experiments were accomplished using adult male rats (Charles River Laboratories), with a body weight range of 350–400 g. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” (formulated by the U.S. National Society for Medical Research, as described in U.S. National Institutes of Health publication No. 86-23, revised 1996) and the “Guide for the Care and Use of Laboratory Animals”. 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. The animals were housed in wire-bottomed cages (three rats per cage) throughout the study and were maintained on a 12:12-h light–dark cycle; and provided with laboratory rodent chow pellets, and water ad libitum.
Groups and administration of beta-carotene

Rats used in the present study were segregated into 3 groups and gavage-administered the following agents: hydroxyethyl cellulose-water (1:4) vehicle control (C); LD–BC (30 mg/kg/day) and HD–BC (150 mg/kg/day) suspended in hydroxyethyl cellulose-water, respectively. BC was obtained from Sigma–Aldrich Kft.

Ischemia-reperfusion and isolated working hearts

Following 4-week treatment with vehicle or BC, the rats were anesthetized with intraperitoneal injections of ketamine-xylazine (75/10 mg/kg), with heparin anticoagulant administered intraperitoneally (1000 IU/kg). After thoracotomy, the hearts were excised and placed in ice-cold modified Krebs-Henseleit bicarbonate buffer (118.5 NaCl, 4.7 KCl, 2.5 CaCl$_2$ × H$_2$O, 25 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, and 10.0 glucose (in mM)), then cannulated through the aorta and perfused in a Langendorff “non-working” mode (100 cm of water) for 5 min to flush blood out from the hearts. Subsequently, ”working” perfusion was conducted using a cannulated pulmonary vein, during which the isolated heart preparatatum was switched to working mode (at a filling pressure of 17 cm of the buffer). After 10 min of working perfusion, 30 min of global ischemia (ISA) was initiated for each procedure, by clamping the pulmonary inflow and the aortic outflow. At the end of the ischemic period, 120 min of reperfusion (REP) was initiated by unclamping the inflow and outflow lines. The first 10 min of REP was conducted in Langendorff mode to avoid the fatal ventricular arrhythmias.
Cardiac function measurements

Baseline assessment of cardiac function for each isolated heart was made following 10 min of working perfusion. To examine the recovery of the left ventricle, these parameters were measured after 30, 60, and 120 min of REP. Cardiac function evaluation for each experiment was conducted described here. Briefly, heart rate (HR) was measured using a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia); coronary flow (CF) values were obtained by timed collection of effluent dripping from each heart; aortic flow (AF) measurements were made using a calibrated flow meter; cardiac output (CO) was generated as the sum of AF and CF. Stroke volume (SV) was calculated as the quotient of CO/HR; and alteration in SV values, as a function of treatments, were calculated as the ratio of SV at reperfusion divided by baseline SV and multiplied by 100.

Infarct size determination

Estimations of infarct size (IS) were conducted using the triphenyl tetrazolium chloride (TTC) staining method. Briefly, following each 30-min ISA/120-min REP period, hearts were perfused with 50 ml 1% (w/v) solution of TTC in phosphate buffer (pH 7.4), 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 and the risk area were measured using planimetry software (Image J, National Institute of Health, Bethesda, Maryland, USA). 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 percentage of the weight of infarcted tissue and the weight of risk zone (whole heart).

**Western blot analysis of heart tissue**

Content of HO-1 protein in the myocardium were obtained by Western blot. Approximately 300 mg of left ventricular myocardial tissue were homogenized on ice using a tissue homogenizer (IKA T10 basic ULTRA-TURRAX®) in isolating buffer (25 mM Tris–HCl, 25 mM NaCl, 1 mM ortho-vanadate, 10 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1 × protease inhibitor cocktail) and centrifuged at 2000 rpm at 4 °C for 10 min. The supernatants were transferred to fresh tubes and centrifuged at 10,000 rpm at 4 °C for 20 min, after which the resulting supernatant was used as cytosolic fraction. The protein concentration was measured by ND-1000 Nano drop spectrophotometer with BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Thirty µg of protein in each sample were loaded in 10% polyacrylamide gel and resolved using SDS-PAGE electrophoresis and then transferred to 0.45 µm pore size nitrocellulose membrane to concentrate the samples. After blocking the membranes with 7% nonfat dry milk in TBST, membranes were incubated overnight with primary antibody solution in 1% of nonfat dry milk in TBST (GAPDH 1/40000, antibody was obtained from Cell Signaling Technology, Boston, MA; and HO-1 1/50 was ordered from Sigma–Aldrich Kft. Budapest, Hungary) at 4 °C. Then, the membranes were washed 3 times, each for 10 min, in TBST and incubated with horseradish peroxidase-conjugated secondary antibody solution (Cell Signaling Technology) containing 1% of nonfat dry milk in TBST, for 2 h at room temperature. 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, Mortsel, Belgium). The films were then digitalized by flat-bed scanner (Epson J232D) and analyzed using ImageJ program and normalized the HO-1 band intensities to GAPDH.

**MTT cell viability assays for beta-carotene cytotoxicity**

Evaluation of BC cytotoxicity on cellular survival was accomplished using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). H9c2 cells (ATCC, CRL-1446, LGC Standards GmbH, Wesel Germany) dissociated by trituration in medium (Dulbecco’s modified eagle’s medium from Sigma with 10% FBS, 1% penicillin-streptomycin). Cells were seeded into 96 well plates at a density of 3000 cells/well and cultured for 1 day to establish adhesion of the wells. Cells were treated with 0, 2.5, 5, 10 and 20 µM BC for 4 h, respectively. Next, following a 30-min incubation period, half of the wells were treated with 125 µM H2O2. Four hours later, addition of 20 µl MTT solution (5 mg/ml in PBS) to each well and an additional 3 h incubation at 37 °C to allow mitochondrial uptake, the medium was removed and cells were lysed by addition of 150 µl of isopropanol, incubated for 15 min followed by measurement of absorbance at 570 and 690 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech). Within each experiment, absorbance values were averaged across 4 replicate wells and repeated 3 times. BC cytotoxic effect assessments were estimated based on linear correlation of absorbance values with MTT-associated H9c2 viability and reported as percentage of cells surviving 4 h of BC exposure relative to control cells not exposed to BC.
Tissue antioxidant capacity (TAC)

TAC of heart tissue was measured using the CS0790-1KT antioxidant assay kit (Sigma–Aldrich Kft., BP., Hungary). Briefly, at the end of reperfusion, the hearts were frozen and stored at −70 °C until analysis. Approximately, 100 mg of left ventricular myocardial tissue from each heart were homogenized in 0.5 ml of 1 × assay buffer, centrifuged at 12,000 rpm, for 15 min at 4 °C and the supernatants collected for assay. Following sample preparation according to manufacturer’s instructions, the absorbance was measured at 405 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech). TAC values for each heart were derived from absorbance values and expressed as Trolox equivalent (µM).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. The data are expressed as mean ± SEM. One-way ANOVA followed by Bonferroni post-test was carried out for heart function data analysis. MTT data were compared by t-test. For Western blot and TAC data outcomes, repeated measures of one-way ANOVA followed by Tukey’s post-test was conducted. Differences were considered significant at values of p<0.05.
3. New scientific results of the thesis

Results of sour cherry seed kernel extract experiments

Macrosopic observations

Macrosopic evaluations of the animals failed to demonstrate gross anatomical abnormalities or other visible pathological changes. Examination of brain, eyes, heart, lung, liver, pancreas, spleen, gastrointestinal tract, kidney, urinary tract, genital tract, skeletal muscle and skin in test animals revealed no differences from the corresponding organs in the negative control group.

Results of serum metabolite and enzyme activity experiments

For each variable evaluated, no significant differences in outcome were observed when the SCSE-treated animals were compared with the normal saline-treated negative control group. Moreover no significant differences were noted when outcomes were compared between dosage groups.

Results of tissue pathology experiments

Histopathological observation of kidney

Hematoxylin-eosin (HE)-stained, 40 μm tissue sections taken from the medullar region of each kidney were examined for changes in tissue ultrastructure that may indicate toxic effects. Urinary and blood vessels, and malpighian corpuscles were visible and seen to be normal in the representative image selected from kidneys taken from the control animals gavaged with normal saline and those treated with 250, 500 and 1000 and mg/kg SCSE. Conversely, images of kidneys taken from animals gavaged
with 3000 mg/kg SCSE show congestion of blood and urinary vessels and changes in tissue microstructure suggestive of inflammatory damage.

**Histopathological observation of liver**

Structural features examined included hepatocytes, hepatic cords, sinusoids and the structure as a whole. No abnormalities were observed in any of the dosage groups and liver tissue appeared morphologically intact throughout the dosage range.

**Results of sour cherry seed kernel oil experiments**

**Dermal toxicity studies**

Comparison of test and control animals revealed that the skin of guinea pigs receiving dermal applications of various doses of seed kernel oil was not different from the skin of animals administered distilled water. After 21 days, none of the test animals exhibited adverse changes to the skin suggestive of an allergic or otherwise toxic reaction to contact with the oil.

**Effect of sour cherry seed kernel oil effect on UV radiation induced skin damage**

The result of experiments assessing the capacity of the kernel oil to inhibit UV phototoxicity. Unprotected skin irradiated with UVB for 40 min exhibited significant erythema 2 h after irradiation was halted, with maximal skin effects observed after 6 h. Creams containing kernel oil at a dosage of 3 % or greater provided significant protection against UV damage.
Results of beta-carotene experiments

Effects of beta-carotene treatment on cardiac function in isolated hearts subjected to I/R

No BC-mediated effects on AF were observed in hearts not subjected to REP, however, relative to hearts from vehicle-treated control animals, hearts from animals subjected to 30 min of ISA followed by 30 and 60 min of REP exhibited significantly increased AF when taken from rats treated with LD–BC (p<0.05); but interestingly, not which were subjected to 120 min of REP or from those fed with HD-BC. No significant BC dosage effects on CF were observed in non-reperfused hearts, or in hearts from animals subjected to 30 min of I/R. Furthermore, HD–BC treatment resulted in a significant increase on CF values after 60 and 120 min of REP relative to organs from non BC-treated controls (p<0.05). Likewise CO in non-ISA/REP hearts and those subjected to 120 min of REP did not vary significantly as a result of BC treatment, where as significant CO increases in these hearts relative to organs from non-BC-treated controls were observed in those sustaining reperfusion periods of 30 min (p<0.05) and 60 min (p<0.05) from rats receiving 30 mg/kg/day BC, but not 150 mg/kg/day of the agent. Treatment of animals with 150 mg/kg/day BC resulted in significant increase in HR for I/R hearts (p<0.05). Evaluation of the effects of BC on heart SV, revealed no significant BC effect on this variable in non I/R hearts, or organs subjected to I/R. It was further noted that SV values in hearts receiving 30 and 60 min of REP from animals treated with 150 mg/kg/day BC were reduced; however it was not significant, relative to those from rats receiving low BC dose. This apparent loss of cardio protection at elevated BC dosage was further confirmed by measurement of stroke volume decline (dSV). These results demonstrated that relative to
Effects of beta-carotene treatment on I/R-induced infarct size and tissue antioxidant capacity (TAC)

Macroscopic analysis of TTC solution perfused heart sections from rats subjected to 30 min of global ISA followed by 120 min of REP in a “working-heart” apparatus revealed that LD–BC treatment, correlated with significant reduction of the extent of infarcted myocardium relative to the control values (p<0.05). This protective effect is abolished in hearts taken from animals treated with high-dose BC (p<0.05). The phenomenon of diminished BC-mediated protective effect on cardiac physiology was also dramatically illustrated in outcomes of experiments to assess contribution of the agent to tissue antioxidant capacity (TAC), a process that has evolved as a homeostatic countermeasure to oxidative stress. The isolated, I/R injured working hearts taken from rats administered LD–BC, exhibited TAC values significantly in excess of vehicle-treated controls (p<0.05) and hearts isolated from HD–BC treated animals (p<0.05). Nevertheless, TAC values measured in hearts from animals received HD–BC were not significantly higher, that hearts from control rats received vehicle.

Effects of beta-carotene on heme oxygenase-1 (HO-1) protein expression

Hearts excised from the animals were either sham treated (BL-baseline), or I/R injured (I/R) by 30 min of global ISA and 120 min of REP. The results show that in control hearts which were not subjected to I/R
(C-BL) HO-1 expression was not significantly different from non injured hearts removed from rats receiving LD–BC (LD–BL), while high dose treatment (HD–BL) resulted in significantly increased HO-1 level (p<0.05). Production of HO-1 protein in I/R injured hearts from rats fed with either low dose (LD-I/R) or high dose BC (HD-I/R), was significantly higher compared to non treated non injured (C-BL) group (p<0.05). Moreover, HO-1 expression in hearts removed from vehicle treated animals and subjected to I/R (C-I/R) was not significantly different from hearts excised from rats receiving LD–BC and subjected to I/R (LD-I/R); however, hearts from high-dose treated animals expressed significantly elevated levels of HO-1 protein relative to corresponding levels measured in vehicle treated controls (C-I/R) (p<0.05).

**MTT assay-based evaluation of beta-carotene cytotoxicity**

Significant increases in H9c2 cell viability challenged by H\textsubscript{2}O\textsubscript{2} was observed in cultures of these cells grown 24 h in Dulbecco’s medium supplemented with 2.5, 5 and 10 \mu M BC, relative to cells grown with no BC added (p<0.05). Furthermore, this protective effect was not observed in cells treated with 20 \mu M of BC.
4. Summary

The devastating health statistics of Hungary and the World are sufficient reason to humans to change their lifestyle. Cardiovascular diseases are leading cause of death worldwide and obesity is one of its main risk factor. In these conditions body suffers from continuously elevated oxidative stress. Nowadays, there is a growing demand for prevention and supplementary therapy besides to the medical treatment of an existing disease. Substances with natural origin are able to fulfill the requirements due to their favorable toxicological and side effect profile.

We have studied the toxicological profile of sour cherry seed in a mouse model. No sign of liver and kidney toxicity were found during the experiments. Moreover, we have examined the photo protective properties of creams containing sour cherry seed oil in different concentration in Guinea pig model. Our results demonstrated that sour cherry seed oil possesses photo protective effect.

We have investigated the effect of low versus high dose beta-carotene treatment using rat model. The low-dose pretreatment resulted in cardioprotection evidenced by recovery of postischemic heart functions, decreased infarct size, not significantly but increased HO-1 enzyme expression and elevated tissue antioxidant capacity. In high dose treated we failed to observe any protection on the heart.

Outcomes of our research may contribute to use in prevention both materials with natural origin, as well as to extend the scientific knowledge related to them.
5. List of publications

List of publications related to the dissertation


   DOI: http://dx.doi.org/10.1097/FJC.0000000000000132 
   IF: 2.135

   IF: 3.288

   DOI: http://dx.doi.org/10.1007/s12272-013-0958-z 
   IF: 1.751

**Total IF of journals (all publications): 14.076** 
**Total IF of journals (publications related to the dissertation): 6,902**

The Candidate’s publication data submitted to the iDEa Tudóstár have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

13 July, 2016