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The effect of natural inductors of heme-oxygenase 1 in age-related dementia and Zucker Diabetic Fatty (ZDF) rat models

by Andrea Kurucz MD

Supervisor: Béla Juhász PharmD, PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF NUTRITION AND FOOD SCIENCES

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By Andrea Kurucz, MD.

Supervisor: Béla Juhász, PharmD, PhD .

Doctoral School of Nutrition and Food Sciences, University of Debrecen

Head of the **Examination Committee**: Lajos Gergely, PhD, DSc

Members of the Examination Committee: Csaba Csonka MD., habil, PhD

Sándor Gonda, PharmD. PhD

The Examination took place at Institute of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Debrecen, 19. 06. 2018. 11.00. am.

Head of the **Defense Committee**:	Lajos Gergely, PhD, DSc
Reviewers:	Zsolt Radak, MD, PhD, DSc.
	Bernadett Újhelyi, MD, PhD.
Members of the Defense Committee:	Csaba Csonka MD., habil, PhD
	Sándor Gonda, PharmD. PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 06. 06. 2019. 10:30 am.

1. INTRODUCTION

Changes in demographic patterns, principally increasing life expectancy, with resulting increases in elderly populations, have resulted in a concomitant expansion of age-related chronic diseases, like dementia and diabetes and an increased burden on national healthcare systems.

Type 2 diabetes and Alzheimer-type dementia are two important age-related diseases that share common features, like strong connection with metabolic disorders, vascular problems and the role of oxidative stress. Their consequence is expanded celldeath, therefore severe physical and mental damage. Recent publications refer to diabetes as an important risk factor in the development of Alzheimer's disease.

Diabetic retinopathy, retinal vascular occlusion, and glaucoma are some of the many diseases in which ischemia-reperfusion (I/R) injury or high blood glucose levels and the resulting deterioration of tissue microcirculatory capacity are major contributing factors. Dietary intake of phytonutrients—health-enhancing compounds found in plants—substantially reduces the risk of serious diseases and improves the effectiveness of therapy and outcome, even in the case of ocular disorders. Previously, the authors of this report carried out experiments with the flavonoid-rich extract of *Prunus cerasus* (sour cherry) seed (SCSE), an emerging functional food.

According to the results of a wide spectrum of measurements including GC-MS and HPLC, constituents of SCSE include bio-active compounds such as dihydro-p-coumaric acid, ferrulic acid, caffeic acid, cyanidin, peonidin, squalene, β-tocopherol, γ-sitosterol, and vitamin E, to many of which the sour cherry extract owes its protective, antioxidant capacity.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, with neuropathology characterized by the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles. The sporadic form of the disease is much more frequent (95% of all cases) than the familial form, however neuropathologically, both can be determined by a signaling cascade triggered by amyloid deposits, resulting in neuroinflammation and microtubule-associated tau protein hyperphosphorylation. The triggering factors still need to be cleared up, however the role of metabolic – like obesity, insulin resistance and lack of physical activity – and neuropathological changes are indisputable.

Heme-oxygenase 1 (HO-1), also referred to as heat shock protein (HSP) 32, has a key role in the amelioration of oxidative stress-related pathologies, including cardiovascular, lung, neurological, and kidney disorders. Several studies have demonstrated that increasing the activity of the enzyme by drugs, natural compounds or regular physical activity can mitigate the organ damage caused by oxidative stress. The results of previous works by researchers of the University of Debrecen suggest that sour cherry seed extract may have potential for the prevention and treatment of I/R-associated

pathologies, including diabetes-related ocular disease: previously Bak et al., and later Czompa et al., demonstrated the protective effect of SCSE on ischemic-reperfused rat myocardium; Juhasz et al. confirmed a cardioprotective effect in hypercholesterolemic rabbits; and Szabo et al. proved protection in ischemic-reperfused retina. The extract was further studied toxicologically, which enabled the use of SCSE even in human studies. These results are very promising and make SCSE a potential candidate for future drug development and further studies, such as the present one, to analyze the wide spectrum of effects the extract may exert.

In our study we investigated the effect of SCSE on plasma glucose level and retina function of Zucker Diabetic Fatty rats and the effect of regular physical activity on the cognitive and neuropathological alterations of age-related Alzheimer-type dementia. We also aimed to determine the moelcular background of the processes so we measured the concentration and activity of HO-1.

2. AIMS

1. Investigation of the effect of po. sour cherry seed extract (SCSE) treatment on the plasma glucose levels in a type 2 diabetes rat model (ZDF).
2. Examination of the effect of po. SCSE-treatment on retinal function and retinal structure in a type 2 diabetes rat model (ZDF).
3. Evaluation of the influence of regular physical activity on the cognitive and neuropathological changes of age-related Alzheimer-type dementia in an ageing rat model.
4. Assessment of the molecular effects of nutrition and physical activity – measurement of heme-oxygenase 1 (HO-1) concentration and activity.

3. MATERIALS AND METHODS

All of the protocols used in the present study were approved by the Institutional Animal Care Committee of University of Debrecen in Debrecen, Hungary (18/2013/DE MÁB). Rats were housed two per cage at an ambient temperature of 22–24°C under a 12 h : 12 h light : dark cycle with food (Purina 5008) and water ad libitum.

3.1. Effect of SCSE-treatment on plasma glucose and retinal function of ZDF rats

3.1.1. Animals and treatment protocol

We examined 6-week-old male Zucker Diabetic Fatty (ZDF-Leprfa/Crl) rats and their control (lean phenotype). The animals were separated into three groups (n = 6 in each group): a control diabetic group, a treated diabetic group, and a healthy group. The treated group was gavaged with 30 mg/kg SCSE daily, while the control group was gavaged with the vehicle only (methyl-cellulose mucilage).

3.1.2. Oral glucose tolerance test

Oral glucose tolerancy testing was carried out when the animals got involved in the experiment, and at the end of the 50 day treatment period. Twelve hours before the test, food was withdrawn from the animals. During the test, fasting glucose levels were obtained (AccuChek Active blood glucose monitor) first, then 1 g/mL glucose solution was prepared with water, which then—after heating to body temperature—was gavaged into the stomach of each animal in a dose of 3 g/kg. One hour after the glucose-load, the blood glucose levels of the animals were measured again.

3.1.3. Ocular ischemia and reperfusion (I/R)

At the end of the 50-day period, rats were anaesthetized with an intramuscular ketamin/xylazine (50/5 mg/kg) injection. The eyes of the animals were locally anaesthetized with 0.4% oxybuprocain eyedrops as well. Subsequently, the retinal artery of the left eye of each animal was surgically occluded by a silk suture to cut off the blood supply and was maintained for 1 h. Ischemia was verified macroscopically with a 120-D lens. During ischemia the eye of the rats was covered with sterile gauze. To prevent the drying out of the cornea, carbomer-based eye gel was also used. Reperfusion of the retinal tissue was accomplished by release of the occluder.

3.1.4. Electroretinography (ERG)

After 24 h of reperfusion, animals were prepared for electroretinographic measurements by anesthesia with an intramuscular injection of 50/5 mg/kg of ketamin/xylazine. The pupils of each animal were dilated with 0.5% cyclopentolate hydrochloride. Five silver electrodes were used for each measurement as follows. At the corneal surfaces, retinal signals were analyzed using two measuring electrodes (one on each eye), inserted so as to avoid scleral damage or corneal perforation. Reference electrodes were positioned on the earlobes of each animal (one on each earlobe), with the main ground

electrode at the glabella. Effective electrical contacts and protection of eyes from dehydration was provided by carbomer-based eye gel. The ERG measurements were carried out in darkness, after a dark adaptation period (20 min). For stimulation of the retina, the eyes were illuminated with a stroboscope (20 cd/m^2, 0.5 Hz). Electrical signals, i.e., retinal responses to light flashes, passed through an amplifier and an analog–digital converter displayed on a PC monitor, recorded and analyzed using PowerLab Chart software. Waves „a" and „b" were analysed on the elctroretinograms. „a" waves represent the function of photoreceptors, while „b" waves refer to the function of the bipolar cells.

3.1.5. Measurement of HO-1 concentration

After the ERG measurements, the eyeballs of the animals were removed and the right half was suspended in a homogenization buffer composed of *N*-2-hydroxyethylpiperazine-2-ethanesulfonic acid (HEPES) 10 mM, sucrose 32 mM, dithiotreitol (DTT) 1 mM, ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) 0.1 mM, soybean trypsin inhibitor 10 μg/mL, Leupeptin 10 μg/mL, Aprotinin 2 μg/mL; pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA). The supernatant was collected by a 30 min centrifugation of the homogenate at 20,000× *g* at 4 °C. The HO-1 content was determined by enzyme-linked immunosorbent assays (ELISA). Optical density was measured at 450 nm (Benchmark Microplate reader; Bio-Rad, Hercules, CA, USA) and the values were expressed as ng/mg protein.

3.1.6. Histology

The the vitreum of the left half was fixed in Bouin-solution, alcohol-dehydrated, paraffinized, and were processed into 7 μm sagittal sections, which were then dyed with hematoxylin-eosin (HE) and examined by light microscopy. Average retinal thickness was measured between the inner limiting membrane and the retinal pigment epithel (ILM-RPE) and expressed in micrometers using a manual scale on each glass slide. The number of cells in the ganglion cell layer per unit distance (100 μm) was also counted with the help of the same manual scale on each glass slide. For the quantitative analyses, 6 eyes per group and 6 sections per eye were analyzed.

3.1.7. Statistical analysis

A one-way analysis of variance with a Tukey or Newman–Keuls post-test was used for Gaussian data results from the D'Agostino & Pearson omnibus normality test. Data with non-parametric distribution were analyzed using the Kruskal–Wallis test along with the Dunns post-test. Results are represented with standard error of the mean (SEM).

3.2. Influence of regular physical activity on the cognitive and neuropathological changes of age-related Alzheimer-type dementia in an ageing rat model

3.2.1. Animals and experimental protocol

Animals used in the present study were segregated into six groups, defined as follows: (i) aged sedentary female rats (ASF, n = 8); (ii) aged sedentary male rats (ASM, n = 8); (iii) aged running female rats (ARF, n= 8); (iv) aged running male rats (ARM, n = 8); (v) young (3 months old) control female rats (YCF, n = 8); and (vi) young (3 months old) control males (YCM, n = 8). Rats in the ARF and ARM groups had free availability to a standardized inbuilt running wheel and were able to exercise freely, whereas animals in the ASF and ASM groups were housed without access to running wheels. All groups were maintained in the above described conditions for 3-month periods.

3.2.2. Morris water maze test (MWM)

For assessing the learning and spatial memory of animals, MWM test was used. A circular pool, 150 cm in diameter, 50 cm in height, in a small quiet room, was filled with opaque water at room temperature, to a depth of 30 cm, and divided into four quadrants. An invisible platform, 10 cm in diameter, is submerged 1 cm below the surface, in the middle of one of the four quadrants. The position of the platform is kept unaltered throughout a training session, during which time each animal becomes familiar with structural features and visual cues, memories of which are the basis of the test. Selected visual cues are placed on the inner wall of the pool to indicate the four quadrants and provide navigation reference points, which the rats will remember to varying degrees, depending on their neurological capacity. MWM tests were carried out during three consecutive days, twice a day before the treatment (baseline), and after the 3 months of voluntary running on wheels installed in cages. For each trial, the animals were gently put into the water at one of the four starting points (that differed for each test—the sequences for which were selected randomly). The tests were scored on the basis of ability of the animals to locate the submerged platform. Each animal was allowed a 10-second rest period on the platform. If an animal was unable to locate a platform within a 60-second interval, it was gently guided and placed on it. Escape latency time and swimming patterns were measured by a video tracking system.

3.2.3. Small animal positron emission tomography (PET)

Age associated neuropathological alterations and specific amyloid pathology was evaluated with in vivo PET. Rats were injected with 10.0 ± 0.2 MBq of [^{11}C]PIB – a radioligand, binding specifically to amyloid deposits – via the lateral tail vein. 30 minutes following injection of the radiotracer, the animals were anaesthetized using 3% isoflurane with a dedicated small animal anesthesia device. Next, 15-minute static single-frame PET scans were acquired using a small animal PET scanner (MiniPET-II) to visualize the brain. Radiotracer uptake – that was expressed in terms of standardized uptake values (SUVs) – correlated with the amount of pathological protein aggregates.

3.2.4. Histology

We tried to demonstrate neurodegenerative alterations, amyloid pathology and amelioration of these phenomena by traditional hematoxylin-eosin and Congo red staining and by special antibodies against amyloid β. At the end of the three-month experiments, the rats were deeply anesthetised and transcardially perfused with 10 ml of 4°C PBS, followed by 30 ml of 4°C paraformaldehyde solution (4% in phosphate buffer, pH 7.4). Rat brains were removed and the right hippocampal, frontal, and temporal lobes. The samples were fixed in 4% formalin, then embedded into paraffin, then microtomed into 7 μm thick sections, stained with hematoxylin-eosin (H&E) and Congo red (Sigma-Aldrich) and analyzed by light microscopy, using polarized light for the examination of Congo red-stained tissues. Semiquantitative analysis of amyloid pathology was accomplished by first counting the number of amyloid plaques and Congo red-positive vessels as a fraction of the total number of vessels observed in 10 fields, using a 10x objective (100x magnification). The resulting percentage of Congo red-positive vessels within the total number of vessels (number of positive vessels/total vessel number × 100) yielded quantitative data that allowed assignment of tissues into 4 different categories, described as follows: (i) tissue with no (0%) positive vessels (-); (ii) tissue with 1–29% (low number of positive vessels) (+); (iii) tissue with 30–69% (moderate number of positive vessels) (++); and (iv) tissue with more than 70% (numerous positive vessels) (+++). For immunohistochemical examination (IHC) sections were stained for anti-beta amyloid 1–42 antibody (Abcam, 1 : 200) according to the manufacturer's protocol and were analyzed by light microscopy. Semiquantitative analysis of amyloid pathology was accomplished by a similar method to that of the Congo red staining. We counted the number of anti-beta amyloid 1–42 antibody-positive vessels as a fraction of the total number of vessels observed in 10 fields, using a 10x objective (100x magnification). Based on the results we categorized the sections into 4 groups, similarly to Congo red stained sections.

3.2.5. Measurement of HO-1 activity

The HO-1 activity of the animals was evaluated by measuring the reduction of biliverdin into bilirubin. Following the sacrifice of each animal, the frontal cortex, temporal cortex, and hippocampus of harvested brains were isolated and homogenised (Ultraturrax T25; 13,500/s; 2 × 20 s) in the following buffer: 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), 32 mM sucrose, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin, pH 7.4. The supernatant was collected by centrifugation for 30 minutes at 20,000 ×g at 4°C. Each reaction mixture contained the following in a final volume of 1.5 ml : 2 mM glucose 6-phosphate, 0.14 U/ml glucose 6-phosphate dehydrogenase, 15 μM heme, and 150 μM β-nicotinamide adenine dinucleotide phosphate (NADPH). 120 μg/ml rat liver cytosol was used as a source of biliverdin reductase, with 2 mM $MgCl_2$, 100 mM potassium phosphate buffer, and 150 μl of supernatant. Incubation was carried out in the dark at 37°C for 60 minutes. The reaction was stopped by putting samples on ice. The bilirubin formed was calculated from the difference between

optical densities obtained at 460 nm and 530 nm. One unit of heme oxygenase activity was defined as the amount of bilirubin (nmol) produced per hour per mg of protein.

3.2.6. Statistical analysis

Two data sets were compared with unpaired Student *t*-test, otherwise statistical analysis was carried out according to section 3.1.7.

4. RESULTS

4.1. Effect of SCSE-treatment on plasma glucose and retinal function of ZDF rats

4.1.1. Fasting blood glucose analysis and oral glucose tolerance test (OGTT) results

The fasting and OGTT blood glucose levels of the healthy animal group (5.137 mmol/L and 7.571 mmol/L, respectively) did not differ significantly from each other, as occurred in the other two diabetic animal groups. The fasting blood glucose level of the control group (9.626 mmol/L) was significantly different from the appropriate value of the healthy group, and apparently differed also from the fasting value of the treated group, however this latter difference was statistically not significant. The fasting blood glucose level of the treated group (7.510 mmol/L) was statistically not different from the fasting results of the healthy animals. In the case of OGTT blood glucose values, both diabetic groups differed significantly from healthy group values: the mean value of the control group was 17.41 mmol/L, while that of the treated group was 14.330 mmol/L. The difference between the control and treated groups was also significant ($p < 0.05$).

4.1.2. Electroretinography

By comparing the non-IR data, it can be seen that the SCSE treatment significantly increased the mean amplitudes of a- and b-waves relative to the control group (88.11 μV vs. 68.61 μV for the a-waves and 233.9 μV vs. 178.7 μV for the b-waves; $p < 0.001$ for both comparisons); furthermore, the mean amplitude of b-waves of the treated group did not differ significantly from the same values of the healthy group (233.9 μV vs. 236.2 μV). In the case of a-waves, the former mentioned comparison results in a significant difference between treated and healthy non-I/R groups, with the treated group being higher (88.11 μV vs. 69.85 μV).

Regarding the IR values, it can be concluded that a significant difference can be seen between the b-wave mean amplitudes of the control group and that of the healthy animals (96.83 μV vs. 149.9 μV; control vs. healthy; $p < 0.001$), while the treatment increased these values significantly (176.2 μV; $p < 0.001$ vs. control). It should be highlighted that the IR b-wave results of the treated group also proved to be significantly better than the IR b-wave values of the healthy group (treated vs. healthy; $p < 0.001$). Similarly, sour cherry seed extract provided better a-wave amplitudes after ischemia-reperfusion as compared either with healthy or control groups (76.28 μV vs. 47.15 μV or 45.98 μV; treated vs. healthy or control; $p < 0.001$ in both comparisons).

4.1.3. Retinal histology results

Retinas of the control non-IR group were significantly thicker than in healthy animals (121.3 μm vs. 102.4 μm, $p < 0.001$), while the retinal thickness of the treated group (108.7 μm) did not differ from the healthy value, but did from control non-I/R ($p < 0.01$). Similar comparisons could be seen after ischemia-reperfusion (172.3 μm vs. 185.0 μm vs. 165.0 μm). In the case of average ganglion cell

numbers per unit distance (100 μm), no significant differences were seen between the different groups either before (15.50 vs. 17.00 vs. 16.33, respectively), or after ischemia-reperfusion (12.83 vs. 13.67 vs. 11.35).

4.1.4. HO-1 concentration

Tissue concentration of the enzyme was statistically the same in the healthy and control groups, both pre-ischemically and after ischemia (1.170 vs. 1.115 for non-I/R and 1.717 vs. 1.469 for I/R, respectively). In the case of non-IR eyes, a significant boost in expression is seen in the SCSE-treated group compared to both the healthy and the control groups (2.963; $p < 0.05$ vs. control and $p < 0.001$ vs. healthy). The elevation in ischemia protein expression in the healthy group is the reason why the healthy vs. treated comparison lost its former strength, nevertheless, the mean concentration value of the treated group was still significantly higher compared with the healthy group (2.934; $p < 0.05$ vs. healthy/control).

4.2. Influence of regular physical activity on the cognitive and neuropathological changes of age-related Alzheimer-type dementia in an ageing rat model

4.2.1. Results of MWM test

Animals within the aged sedentary group (including both male and female rats) required significantly more time to find the platform and were not able to improve their learning ability ($p < 0.001$). However, animals in the aged running groups (both males and females) were more likely to find the platform within decreasing time compared to the sedentary aging groups (mean differences: Day 1: 10.15 seconds, $p = 0.038$, and 14.60, $p< 0.01$; Day 2: 28.45, $p < 0.001$, and 25.45, $p < 0.001$; Day 3: 30.25, $p < 0.001$, and 33.55, $p < 0.001$ for males and females, respectively.

4.2.2. Brain alterations demonstrated by in vivo PET imaging

PIB radionuclide retention was significantly higher among aged sedentary animals than among young control rats (SUV mean of PIB for aging females: 0.87 ± 0.029 and for young control females: 0.43 ± 0.02). Data for aged males: 0.97 ± 0.024 and for young control males: 0.41 ± 0.01 ($p < 0.001$ for both males and females). Analysis of data from animals in the aged running groups revealed significantly lower PIB retention ($p = 0.0119$ and $p < 0.001$, for males and females, resp.) than in the young groups.

4.2.3. Histology results

H&E staining revealed remarkable degenerative abnormalities in the AS groups, compared to the young control animals. Disintegration of the pyramidal layer structure, neuronal loss, and severe pericellular edema was observed in the dentate gyrus. However, following the 3 months of voluntary exercise, these changes were attenuated to some extent. The accumulation of amyloid in rat brains demonstrated by Congo red staining failed to demonstrate amyloid plaques. However, age-related cerebral amyloid angiopathy (CAA) was detectable. The number of Congo red-positive vessels and the

intensity of staining was significantly higher in the aged sedentary animals —both females and males— (32.4 ± 2.237% in males and 27 ± 0.89% in females) than in the aging running groups (13.60 ± 0.77% in males and 7.8 ± 0.57% in females) ($p < 0.001$ in the case of males and females as well). Age-related cerebral amyloid angiopathy (CAA) was also detectable by immunohistochemistry. Results were similar to those of Congo red staining. The aging sedentary animals—both females and males—exhibited a significantly higher number of Congo red-positive vessels (31.6 ± 1,78% in males and 27,1 ± 0.9% in females) than the animals of aging running groups (14.70 ± 0.47% in males and 8 ± 0.55% in females). The difference was statistically significant irrespective of the gender.

4.2.4. HO-1 activity

Relatively low activity of the heme oxygenase-1 enzyme was observed in the frontal cortex and hippocampus of aged sedentary rats both in females (1.3 ± 0.08 and 0.95 ± 0.02 nmol/h/mg protein, resp.) and in males (0.8 ± 0.03 and 0.72 ± 0.04 nmol/h/mg protein, resp.). Interestingly, three months of voluntary exercise increased the activity of this enzyme, both in females (2.49 ± 0.08, $p < 0.001$, and 1.53 ± 0.06 nmol/h/mg protein, $p < 0.001$, resp.) and in males (1.35 ± 0.1, $p < 0.001$, and 0.99 ± 0.04 nmol/h/mg protein $p < 0.001$, resp.).

5. DISCUSSION

5.1. Effect of SCSE-treatment on plasma glucose and retinal function of ZDF rats

Diabetes mellitus is a metabolic disease with an impaired carbo-hydrate turnover, characterized by a decreased rate of insulin secretion and reduced insulin sensitivity of cells expressing insulin receptors. It is well known that, among diabetic patients, ophthalmologic complications are very common. In our study, the effect of the flavonoid-rich extract of the *Prunus cerasus* (sour cherry) seed on the blood glucose levels of the Zucker diabetic fatty rat—a type 2 diabetes model—was examined.

According to our OGTT measurements, sour cherry seed extract showed a modest, but significant blood glucose lowering effect: blood glucose levels of the diabetic animal groups were higher than the values of the healthy animals, however, values of the treated group after oral glucose load were improved compared with control.

Several studies have shown that phytocompounds found in fruits, vegetables and herbals have beneficial health effects like prevention of cancer, cardiovascular diseases, and obesity. Most recently, Lachin et al. published a paper on the effect of different cherries on diabetes with many promising results: e.g., yellow cherry, sweet cherry, or tart/sour cherry fruit showed significant blood glucose lowering effects. The data of our experiments contribute to this finding with a novel result, that an extract made of sour cherry seed may also be modestly antidiabetic.

Data from the electroretinographical measurements provide insight into the damaging effects of diabetes mellitus on retinal function (when comparing the non-IR results of healthy and control animals), and allow easy comparison of functioning of IR-injured and non-IR retina in the animal groups. A major finding of this current report is the significant improvement in the electrophysiological functions of photoreceptors and retinal cells post-synaptic to photoreceptors (i.e., cells of the inner nuclear layer (INL)) as seen on non-IR electroretinograms of the SCSE-administered group compared with control. Despite the high blood glucose levels, retinal function of non-IR eyes of animals in the treated group was at least similar to that of the healthy group, which is also a major finding. This means that SCSE treatment was able to prevent the retina-damaging effect of diabetes mellitus on the functional level: both photoreceptors and post-receptoral pathway cells remain highly active despite the deteriorative effect of hyperglycaemia. Such retinoprotective effects were demonstrated by other authors using treatment with resveratrol, an active agent also of herbal origin, or with peptides such as GLP-1 analogue exenatide or liraglutide. Upon investigating the IR values on our electroretinograms, it can be concluded that, while a significant deterioration in retinal function can be seen in the control group compared with healthy animals, significant functional improvement was demonstrated in the treated group. Furthermore, the SCSE-treated group showed significantly better retinal function compared with the healthy animal group. This novel result supports the

hypothesis that SCSE may have capacity in preventing IR-induced retinal damage at the functional level even in a diabetic setting.

Sour cherry seed extract was able to counteract the edema-inducing effect of diabetes as seen from the comparison of treated non-IR and control non-IR retinal thickness values. Ischemia-reperfusion injury may also cause retinal edema, an effect compensated by SCSE treatment based on retinal thickness values of ischemic-reperfused retina tissue samples. These novel results are not individual among herbal agents: such a protective effect was demonstrated with other herbal treatments, such as *Lycium barbarum*, ginsenosides or Flos puerariae. Ganglion cell numbers did not change significantly according to our results. The reason behind this may be the relatively short time interval between the ischemia and tissue sampling: the time course of ischemia-reperfusion injury starts with initial retinal edema, followed by structural degenerative changes which need more time to develop, i.e., a consequential cell death, e.g., a decrease in number of ganglion cells.

Based on our results we can draw a conclusion that HO-1 may play an important role in the retinoprotective effect of sour cherry seed extract in a diabetic setting. Elevated blood glucose levels may imply a stress for the retinal tissue, as the level of this well-known stress protein—although not significantly, but still—increases in the control group as compared with healthy, low blood glucose animals. The sour cherry seed extract further elevated the level of this protective enzyme, which may contribute to the hypoglycemic activity of the extract. Similar elevated heme oxygenase enzyme concentration after sour cherry seed extract treatment was seen formerly in conventional, i.e., non-diabetic animal models in other tissues and even in retina as well. A novel finding of this current report is that the capacity of SCSE in elevating HO-1 levels was confirmed in a diabetic setting. Similar mechanisms of effect are not unheard of among herbal medicines: retinoprotective effects of blueberry anthocyanins are also mediated through the Nrf2/heme oxygenase signaling pathway

All things considered, according to our data presented here, SCSE may have the capacity to alleviate the damaging effect of high blood glucose and protect the retina from I/R injuries in diabetic conditions.

5.2. Influence of regular physical activity on the cognitive and neuropathological changes of age-related Alzheimer-type dementia in an ageing rat model

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is the most common cause of dementia in the elderly, and it is clinically characterized by progressive loss of cognitive ability, including memory, communication, judgement, and reasoning. In the present study we have chosen an aging rat model, in which the animals underwent deterioration of cognitive functions that have significant commonality with AD but occur naturally and thus have better relevance to clinical aspects of spontaneously occurring human AD than drug-induced and transgenic models. A major result of the

present study was an observation that elderly animals manifest typical features of dementia. It was observed that aged rats (both males and females) exhibit Morris Water Maze (MWM) test performance scores significantly worse than those of young rats. PET scans revealed significantly high accumulation of amyloid beta in the brains of elderly rats, and histopathological examination showed neurodegenerative lesions. This included disintegration of the pyramidal layer structure, neuronal loss, and severe pericellular edema in the dentate gyrus, as well as cerebral amyloid angiopathy (CAA). The present study additionally revealed age-related decrease in HO-1 activity within the frontal and parietal cortex and hippocampus.

Physical exercise ameliorated the cognitive decline in the aged rats. The animals in the aged running (AR) group required significantly shorter escape latency time to remember locations of hidden platforms in the Morris Water Maze test than the aged sedentary (AS) rats, irrespective of their gender. This finding is supported by other recent studies. For example, Yosefi et al. demonstrated that 4-week treadmill running improved the spatial learning and memory of rats with icv, STZ-induced cognitive impairment.

Experiments conducted in this study showed that recreational exercise significantly decreased PIB retention in the brain of aged running animals, in comparison to aged sedentary animals. This outcome suggests that brains of animals that were allowed to exercise accumulated lower amounts of insoluble amyloid-β than did sedentary animals.

Daily running also attenuated deleterious neuropathological alterations found in the brain tissues of aged rats. Disintegration of the pyramidal layer structure, neuronal loss, and severe pericellular edema in the dentate gyrus of the hippocampus, as well as cerebral amyloid angiopathy (CAA)—represented by Congo red staining and immunohistochemistry—were significantly mitigated by physical activity. The effect of delaying neurodegeneration or restoring synaptic function and neuronal integrity has previously been attributed to physical exercise by numerous studies. For example, Lin et al. reported that regular physical activity restores hippocampal- and amygdala-associated memory and dendritic arbor, along with reducing the severity of edema and neuronal loss.

Oxidative stress, mitochondrial dysfunction, and hyperinflammatory processes constitute a positive feedback process that may be described as a vicious circle, which increases the extent of amyloidogenesis and tau-hyperphosphorylation, leading to neuronal impairment, cell death, and neurodegeneration. This phenomenon has been described by several previous studies to be a major feature of Alzheimer's disease. Recent investigations also suggest that HO-1 activity is one of the most important adaptive physiologic countermeasures to AD-associated oxidative stress. Many recent studies support this broad objective. For example, Bhardwaj et al. showed that pharmacological induction of HO-1 by hemin decreased oxidative stress and restored cognitive function in an intracerebroventricular streptozotocin-infused rat model of Alzheimer's disease. Our research group

conducted the study on which it is based, in part to further demonstrate the future potential of HO-1-based strategies for dealing with the technical challenges posed by AD. We demonstrated significantly increased HO-1 enzyme activity levels in the frontal cortex and hippocampus of aged running rats. Together with the attenuation of cognitive decline, imaging, and neuropathological alterations, the results of these experiments suggest that the severity of age-related Alzheimer's-type dementia may be mitigated by augmentation of naturally occurring HO-1 activity, with outcomes expected to include significantly reduced oxidative stress and reduced severity of AD symptoms.

It has long been known that physical exercise confers many beneficial effects on health. Studies demonstrate that exercise improves cardiovascular function, cognitive ability, and insulin resistance and symptoms of metabolic syndrome and decreases the severity of neuropsychiatric problems, including depression. Di Loreto et al. demonstrated that regular physical activity potently augmented neuroprotective functions as a correlate of age-related amyloidogenesis and additionally preserved synaptic function. The present investigation uses the above-mentioned reports as a framework for a comprehensive effort by the authors of this report to develop HO-1 inducers as a mainstay of prevention and therapy of serious chronic disease. Moreover, at the time of this writing, this investigation is the first ever to examine mechanisms of association between physical activity and HO-1 effects. The outcomes shown here demonstrate that HO-1 activity is significantly increased as a result of voluntary running in aging rats and thus has potential for future strategies to treat cognitive decline in AD and related disorders.

6. MAIN RESULTS AND CONCLUSIONS

1. We demonstrated that sour cherry seed extract has a plasma glucose-lowering effect, as it significantly reduced the glucose level of T2DM rats following oral glucose tolerance test.
2. We proved that SCSE is retinoprotective, moreover mitigates the deleterious effects of IR that often occurs in diabetes. The electrophysiologic function of photoreceptors and the cells of the inner nuclear layer of diabetic animals significantly improved – following the treatment – in non-IR and in IR conditions as well.
3. It was shown, that the level of the well-known stress protein, heme-oxygenase 1 – that is increased in diabetic conditions – further increased due to treatment with SCSE. Based on this finding we concluded that the enzyme might have a role in the mediation of SCSE's retinoprotective and glucose-lowering effect in non-IR and also in IR conditions.
4. Our outstanding result is that regular physical exercise improved the cognitive function of aged rats suffering from dementia that was demonstrated with Morris water maze test.
5. With the help of histological (H&E, Congo red, immunohistochemical method) and imaging diagnostic technique (PET) we also proved that recreational physical activity decreased the neuropathological alterations of Alzheimer's disease – hippocampal atrophy, amyloid accumulation and angiopathy.
6. We measured elevated HO-1 activity in the hippocampus and frontal cortex of the animals, therefore we thought that regular exercise improves neurodegeneration through increasing the activity of the antioxidant HO-1 enzyme.

The results of our study suggest that the natural activation of HO-1 enzyme (e.g. with herbal drugs or physical activity) may have an important role in the amelioration of age - and life style - related diseases.

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

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A PhD értekezés alapjául szolgáló közlemények

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DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

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