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Protective Effect of Chokeberry Anthocyanin-rich Fraction at Nanomolar Concentrations against Oxidative Stress Induced by High Doses of Glucose in Pancreatic β-cells

Dumitriţa RUGINĂ[1], Zoriţa SCONŢA[1], Adela PINTEA[1], Andrea BUNEA[1], Carmen SOCACIU[1*]

[1]Department of Chemistry and Biochemistry, University of Agricultural Sciences and Veterinary Medicine, Mănăştur 3-5, 400372, Cluj-Napoca, Romania, [*]e-mail: socaciucarmen@gmail.com

Abstract. The purposes of this study was to evaluate the protective action of nanomolar concentrations of an anthocyanin rich fraction separated from chokeberry, against the oxidative stress induced by high doses of glucose (50 and 100 mM) in pancreatic β-cells. Using different concentrations of the anthocyanin fraction (from 0.2 to 3 nM), we evaluated its protective role in pancreatic mouse β-cells (TC-3), by measuring the viability, the intracellular reactive oxygen species (ROS) and the levels of glutathione. Our results indicate the scavenging effect of chokeberry anthocyanins (from 0.2 to 1.5 nM), when oxidative stress was induced by high concentrations of glucose (50 and 100 mM) on the intracellular ROS species, which were quenched, according to the decrease of DCF fluorescence. Also, chokeberry anthocyanins concentrations from 0.2 to 1.0 nM, in the presence of 100 mM glucose proved to restore, proportionally to the dose, the strong decrease of glutathione (induced by high-glucose), as compared to untreated cells.

Keywords: pancreatic cells, diabetes, antocyanins, oxidative stress

INTRODUCTION

Anthocyanins, known as antioxidants, belong to polyphenols class and are responsible for the dark blue color of bilberries, blueberries and chokeberries. Consumption of anthocyanins is associated with a reduced risk of several degenerative diseases such as cardiovascular diseases (Neto 2007), cancer (Lala et al. 2006) and diabetes (Jayaprakasam et al. 2005). The early stages of some diabetic complications without adverse drug reactions could be inhibited by a long-term administration of berry rich anthocyanins-derived supplements (Zafra-Stone et al. 2007). Chronic exposure to hyperglycemia can lead to cellular dysfunction that may become irreversible over time. High levels of glucose have been shown to induce oxidative stress and free radical production in β-cells (Robertson 2004; Robertson et al. 2004) by autoxidation which produces hydroxyl radicals, quenched physiologically by the antioxidant cellular enzymes (Hunt et al. 1988).

Antidiabetic potential of anthocyanidins like pelargonidin has previously been tested against pathological oxidative stress (Roy et al. 2008). Recently a study reported that the Chinese bayberry anthocyanin rich extract protect pancreatic β-cells (INS-1) against hydrogen peroxide (Zhang et al. 2011). The antidiabetic effects were reported for anthocyanins fraction purified from *Cornus mas* fruits on rodent pancreatic β-cells (INS-1) in the presence of 4 and 10 mM glucose concentrations (Jayaprakasam et al. 2005). Administration of Aronia fruit

juice to diabetic rats appeared to attenuate hyperglycemia and hypertriglyceridemia (Valcheva-Kuzmanova et al. 2007). The present study aimed to evaluate the effects and the protective action of an anthocyanin-rich fraction separated from chokeberries, upon pancreatic β-cells treated with high doses of glucose as an inducer of oxidative stress.

MATERIALS AND METHODS

Isolation and purification of anthocyanins from chokeberries. The fruits of *Aronia melanocarpa* were harvested in August 2010 at a plantation near Cluj-Napoca (Romania) and preserved immediately at -20˚C. About 10 g of fresh chokeberry fruits were homogenized with methanol-hydrochloric acid 0.1N, 85:15 (v/v) using an Ultraturax (Miccra D-9 KT Digitronic, Germany). The crude extracts were centrifuged and the supernatants were collected. This procedure was repeated until the sediment was colorless. The extracts were concentrated using a rotatory evaporator Buchi at 30 ˚C. The powder samples were dissolved in 10 ml of methanol-hydrochloric acid 0.1N mixture. Prior to each analysis the samples were centrifuged at 5000 rpm and filtered through 0.45 μm nylon filter (Millipore).

The anthocyanin-rich fraction was separated to be used in cell culture assays, following the procedure described previously (Rodriguez-Saona and Wrolstad 2001). Shortly, 500 μl of the aqueous extract was filtered through a C-18 Sep-Pak cartridge (sorbent mass = 50 mg) (Waters Corp., Milford, MA). Anthocyanins and other polyphenolics were adsorbed onto the Sep-Pak column while sugars, acids, and other water-soluble compounds were removed by washing it with 2 volumes of 0.01% aqueous HCl. The second fraction, containing polyphenols (other than anthocyanins), was subsequently eluted with 2 volumes of ethyl acetate and finally anthocyanins were eluted with 4 volumes of methanol containing 0.01% HCl. This fraction was evaporated to dryness under vacuum, at 35˚C and the anthocyanin content was measured by HPLC analysis. According to HPLC-DAD analysis the purified anthocyanin extract contained: 68.7% cyanidin 3-galactoside, 1.1% cyanidin 3 - glucoside, 26.7% cyanidin 3-arabinoside, 3.4% cyanidin 3-xyloside. Their concentration was calculated to be 345 μg/ml expressed as cyanidin 3-galactoside.

Cell culture and viability assay. Pancreatic mouse β-cells TC-3 (kindly provided by Dr. Ştefana Petrescu, Institute of Biochemistry, Bucharest, RO) were routinely cultured in 5% CO2/air at 37 °C in RPMI-1640 medium (Sigma) containing 5 mM glucose and supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM glutamine. Cells were passed weekly after trypsin-EDTA detachment. Cells were plated on 96 well plates at a density of 10^4 cells per well and grown for 24 h. After 24h from the seeding the cells were treated with different concentrations of anthocyanins and/or glucose (100 and 50 mM) as oxidative stress inducer.

MTT assay was used to asses the cell viability. This method uses the property of viable cells to reduce MTT reagent into a colored formazan, product which is detected by reading the absorbance at 550 nm. Briefly, the cells were washed with PBS and 200 μl MTT solution in HBSS buffer were added to each well. After 2 h of incubation the MTT reagent was removed and the formazan particles were solubilized with 200 μl DMSO. The absorbance was read at 550 nm, respectively at 630 nm (for background) with a microplate plate reader HT BioTek Synergy (BioTek Instruments, USA). Cell viability was expressed as a percentage of control (cells incubated in normal medium only, containing 5 mM glucose).

Intracellular reactive species assay. Mouse pancreatic β-cells were exposed to oxidative stress via high glucose concentrations (50 and 100mM) and oxidative stress was measured by oxidation of 2',7'-dichlorofluorescein diacetate. The determination of intracellular reactive oxygen species is based on the oxidation of 2',7'-dichlorodihydrofluorescein (DCHF) by intracellular peroxides, forming the fluorescent compound 2',7'-dichlorofluorescein (DCF). Cells were cultivated in 96 well black plates and treated with purified anthocyanin fraction incubated with 20 μM dichlorofluorescein diacetate (DCFDA). Fluorescence was monitored for 3h at 37 ºC at excitation 485/20 nm and emission 528/20 nm (LeBel et al. 1992) using a microplate reader.

Glutathione assay. The glutathione assay was performed using an optimized enzymatic recycling method with glutathione reductase (Cayman Chemical Company, Michigan, USA). Standard curve was made with GSSG standard, having the equivalent GSH concentration between 0-16 μM. Results are expressed as μmol GSH/mg protein in cell pellet. The concentration of GSH in pancreatic β-cells was calculated using a calibration curve, made from eight concentrations of GSSG standard. The calibration curve was expressed in equivalents of total GSH, because under the assay conditions GSSG is reduced to produced 2 mole equivalents of GSH. Protein concentrations for each sample were determined using the bicinchoninic acid assay (Sigma).

Statistical analysis was done using One-way analysis of variance ANOVA, Tukey's Multiple Comparison Test of Graph Pad Prism version 5.00.

RESULTS AND DISCUSSION

Effect of chokeberry anthocyanins on pancreatic β-cells viability. Mouse pancreatic β-cells (TC-3) were treated with chokeberry anthocyanins at concentrations between 0 and 3 nM, expressed as cyanidin 3-galactoside. HPLC-DAD analysis confirmed that the purified anthocyanin extract contained 68.7% cyanidin-3 galactoside, 1.1% cyanidin-3 glucoside, 26.7% cyanidin-3 arabinoside, 3.4% cyanidin-3 xyloside, similar data being reported by other studies (Oszmiański and Wojdyło 2005; Wu et al. 2004). Fig. 1 A shows that the viability and proliferation of TC-3 cells is stimulated by all tested anthocyanin concentrations. We decided to use three small chokeberry anthocyanin concentrations 0.2; 0.5; and 1.0 nM for further experiments in order to evaluate their potential to reduce oxidative injury after exposure to high glucose concentrations. Viability of pancreatic β-cells (TC-3) was also evaluated using glucose alone (Fig. 1B). Glucose was administrated to medium with or without anthocyanins. In the case of treatment only with glucose, concentrations from 5 to 100 mM in culture medium were used. The pancreatic β-cells (TC-3) viability decreased according to the dose administrated up to 25 % for 100 mM glucose, comparing to standard conditions cultivated cells (5 mM glucose). In cells cultivated with 50 mM glucose the addition of anthocyanins 0.5 and 1 nM stimulates the viability. For cells cultivated in 100 mM glucose, a small decrease of viability (10%) can be observed for 0.2 nM anthocyanin. However, a recovering effect was recorded for higher anthocyanin concentration. The cells viability is close to the control for 0.5 nM and higher but not statistically significant for 1.0 nM anthocyanin (Fig. 1C). All this result indicates the protective effect of chokeberry anthocyanins on pancreatic β-cells (TC-3) in oxidative stress condition.

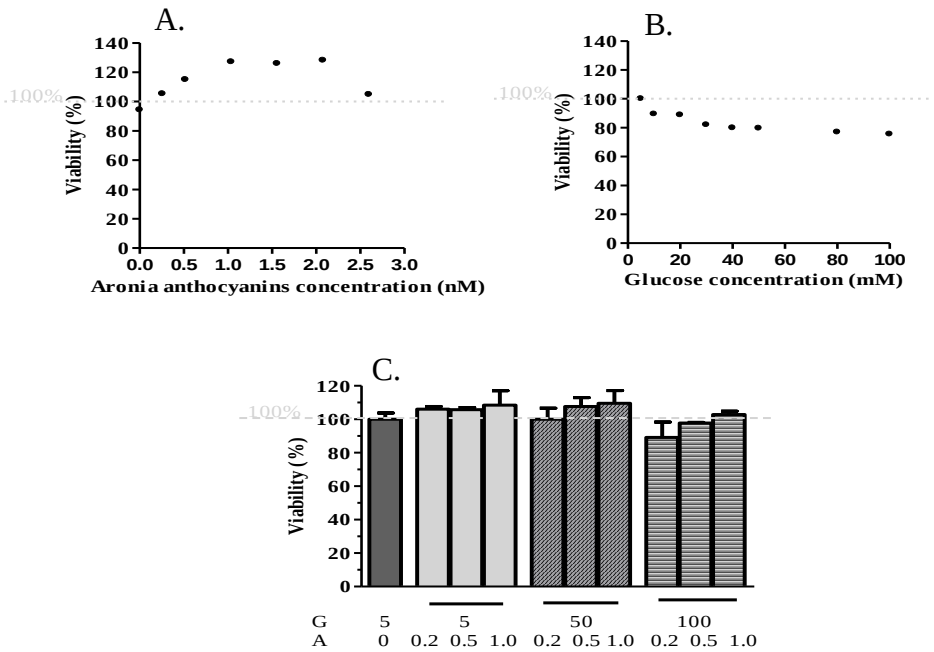

Fig.1. The viability of pancreatic β-cells TC-3 exposed to different concentrations (from 0.2 to 3 nM) of chokeberry anthocyanins (A) and glucose (from 5 to 100 mM), respectively (B). The viability of pancreatic β-cells TC-3 after incubation with anthocyanins (0.2, 0.5 and 1.0 nM) in the presence of glucose at physiologic (5 mM) and high concentrations (50 and 100 mM) in culture media for 48h compared with untreated cells (C).

Intracellular reactive oxygen species level determination. DCF assay provides information about the amount of intracellular reactive oxygen species generation, which is directly correlated with the increase of DCF fluorescence. Mouse cells were exposed to oxidative stress via cultivation in medium containing high doses of glucose (50 and 100 mM) and the oxidative stress was measured by oxidation of 2',7'-dichlorofluorescein diacetate. In stress conditions the level of reactive oxygen species was increased, but after the anthocyanin fraction treatment for 48h a dose-response attenuation of oxidative stress was observed. The most effective results were recorded for the experiment in which the glucose 100 mM oxidative stress inducer and the long-term anthocyanin treatment 48h were used. Exposure to 100 mM glucose (HG) concentration (Fig. 2A) increased the oxidative stress in pancreatic β-cells comparing to untreated cells, but the highest dose of chokeberry anthocyanins (1.5 nM) administrated was able to decrease the reactive oxygen species level with 64%. The same dose of chokeberry anthocyanin (1.5 nM), in cell cultivated in 50 mM glucose (LG) medium reduced the level of intracellular ROS with 15% (Fig. 2B). Recently the attenuation of oxidative stress was observed also in MIN-6 cells after a chronic incubation with the American ginseng berries extract for 48 hours (Lin et al. 2008).

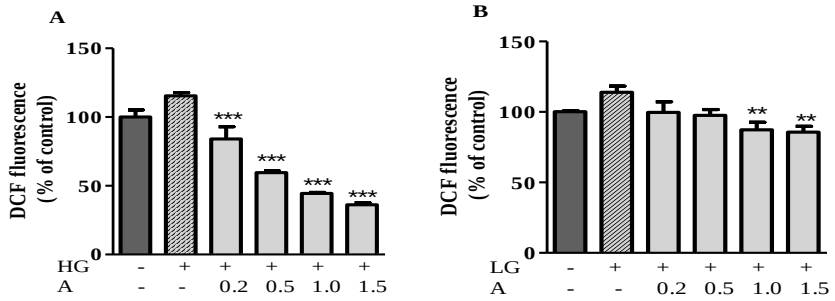

Fig.2. The effects of high glucose exposure conditions of pancreatic β-cells TC-3 on the intracellular peroxide level. A) Cells were exposed to 100 mM glucose (HG) for 48h and/ or different concentrations (0.2-1.5 nM) of the anthocyanin (A) fraction B) Pancreatic mouse β-cells incubated with 50 mM glucose (LG) and/ or the chokeberry anthocyanin (A) fraction (0.2-1.5 nM). The bars represent the mean ± SD, calculated from three experimental values, with $p<0.05$, *** extremely significant, ** very significant, * significant ($p<0.05$).

Glutathione level. Glutathione, a tripeptide is an important antioxidant. Reduced glutathione normally plays the role of an intracellular radical scavenger. As GSH is an important antioxidant molecule, its depletion leads to the increase of oxidative stress. Our data shows that the oxidative stress induces by 100 mM glucose determined a significant decrease (50%) of GSH levels. The intracellular GSH level increased significantly (25%) in cells preincubated with 0.2 nM chokeberry anthocyanins compared to glucose (100 mM) treated cells, but remain under the normal GSH level in untreated pancreatic β-cells. The GSH level in cells treated with the highest concentration of chokeberry anthocyanins (1.0 nM) and 100 mM glucose was restored, being even higher than for untreated cells (Fig. 3). These results demonstrate that GSH protect the cellular system against high glucose induced stress. A recent study showed similar results, by observing that the depletion of GSH content in streptozotocin induced diabetic rats compared to the normal control rats was restored after administration of *Punica granatum* aqueous extract (Bagri et al. 2009).

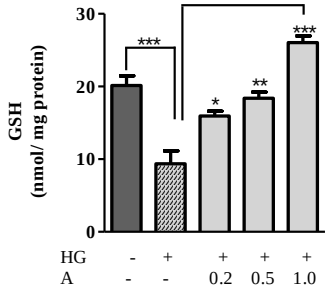

Fig.3. The effects of high-glucose exposure (HG, 100 mM) to pancreatic β-cells TC-3 on the glutathione level (expressed as nmol/mg protein)

CONCLUSIONS

This is the first report about antioxidative potential of chokeberry anthocyanins administrated in nanomolar concentrations to pancreatic β-cells exposed to oxidative stress conditions. Our results suggest that chokeberry anthocyanins have a scavenging effect on the intracellular ROS species which were quenched according to the decrease of DCF fluorescence in oxidative stress-induced pancreatic β-cells (TC-3) by high concentrations of

glucose. Also, chokeberry anthocyanins in the presence of a high glucose concentration proved to restore, proportionally to the dose, the strong decrease of glutathione in pancreatic β-cells (TC-3) compared to untreated cells.

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