Cellular apoptosis and cardiac dysfunction in STZ-induced diabetic rats attenuated by anthocyanins via activation of IGFI-R/PI3K/Akt survival signaling

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Abstract
Anthocyanins are known cyto-protective agents against various stress conditions. In this study cardio-protective effect of anthocyanins from black rice against diabetic mellitus (DM) was evaluated using a streptozotocin (STZ)-induced DM rat model. Five-week-old male Wistar rats were administered with STZ (55 mg kg⁻¹, IP) to induce DM; rats in the treatment group received 250 mg oral anthocyanin/kg/day during the 4-week treatment period. DM and the control rats received normal saline through oral gavage. The results reveal that STZ-induced DM elevates myocardial apoptosis and associated proapoptotic proteins but down-regulates the proteins of IGF1R mediated survival signaling mechanism. Furthermore, the functional parameters such as the ejection-fraction and fraction-shortening in the DM rat hearts declined considerably. However, the rats treated with anthocyanins significantly reduced apoptosis and the associated proapoptotic proteins and further increased the survival signals to restore the cardiac functions in DM rats. Anthocyanin supplementation enhances cardiomyocyte survival and restores cardiac function.

KEYWORDS
anthocyanin, cardiac apoptosis, diabetes mellitus, IGFI-R, streptozotocin

INTRODUCTION
Diabetes mellitus (DM), characterized by elevated fasting blood glucose levels, is often associated with organ complications. On an estimate a population of 366 million people will be with DM by 2030. Although the prevalence of type-1 DM is below 5% of total diabetic population, the frequency of inflicting severe complications is higher in type-1 DM with shorter patient survival when compared with T2DM. Both environmental and genetic factors serve as a risk factor in the pathogenesis of type 1 diabetes mellitus. Streptozotocin (STZ) is a broad spectrum
antibiotic that specifically recognizes GLUT2 receptor and induces cytotoxic effects in β cells; thereby STZ specifically targets pancreatic β cell and induces DM in rodents.4–8 A single dose of STZ can induce type-1 DM in rodents and such animal models are widely used in studying DM associated cardiovascular complications.9,10

DM associated cardiovascular complications include ischemic heart disease, myocardial infarction and cardiomyopathy and are often associated with cardiac apoptosis which is widely recognized as a predictor of adverse cardiac outcomes.11–17 DM patients with coronary heart disease are also found to exhibit a shortened left ventricular ejection time, and increase of end-diastolic pressure. In addition to controlling the cardiac-apoptosis, promoting the cell survival mechanism such as those involving insulin-like growth factor-1 (IGF-I) signaling pathways, mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) and phosphatidylinositol 3’-kinase (PI3K)-protein kinase B (Akt) is an important strategy in treating DM related cardiac-dysfunction. Various alternative medicines with such potential have been extensively studied and reported.10,18–26

Consumption of anthocyanin-rich dietary plants and fruits is known to provide strong neuro- and hepato-protective effects,27,28 and could potentially reduce the risk of cardiovascular diseases in animals and humans.29,30 Anthocyanin isolates are also known to provide protection from DNA cleavage, estrogenic activity, enzyme inhibition, increased cytokine production, anti-inflammatory activity, lipid peroxidation, decreased capillary permeability and fragility, and membrane strengthening.31 Various studies show that anthocyanins provide effective protection from diabetic associated disorders such as diabetic cataract, impaired insulin secretion, ischemia-reperfusion injury, pathological cardiac remodeling.32–36 Our results show that anthocyanins extracted from a species of black rice (selected black glutinous indica rice cultivated at Asia University, Taiwan) regulate the molecular events associated with DM-induced apoptosis and enhances cardiomyocyte survival and thereby restores cardiac function.

2 | MATERIALS AND METHODS

2.1 | Animal models

Five-weeks-old Wistar rats (LASCO Biotechnology) were randomly divided into three groups-Normal group (n = 10), DM group (n = 10) and DM with anthocyanin treatment group (n = 10). All the animals were allowed to adapt to the environment for 3 weeks before the experiment. The rats were injected with STZ (55 mg kg$^{-1}$ body weight in citrate buffer, pH 4.5) to induced DM. The rats were considered to be diabetic if their fasting glucose levels remained >200 mg dL$^{-1}$ after 48 h after injection of STZ as detected by Accu Soft (Hoffmann-La Roche) test strips. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International, Brentwood, MO) and water ad libitum. The animals were maintained at 22–24°C, and were kept in an artificial 12-hr light-dark cycle. The rats were fed with 250 mg kg$^{-1}$ of anthocyanin for 4 weeks using oral gavage. All protocols were reviewed and approved by the Institutional Review Board (IRB, ethical clearance number 102–127-N), Animal care and use committee of the China Medical University, Taichung, Republic of China, and the study was conducted in accordance with the principles of laboratory animal care.37

2.2 | Anthocyanin extraction and purification

Anthocyanin extraction and characterization was performed following methods mentioned in a previous report.28 Up to 72.753% of the content of the extract was found to be Cyanidin-3-glucoside and the data was verified as mentioned in our previous reports.

2.3 | Echocardiography examination

Heart functions were examined by echocardiography based measurement of Left ventricular internal diameter end diastole (LVIDd), Left ventricular internal diameter end systole (LVIDs), Left ventricular posterior wall end diastole (LVPWd), Left ventricular posterior wall end systole (LVPWs), fractional shortening (FS) and ejection fraction (EF). EF% was calculated according to the following equation: $EF\% = \frac{[(LVIDd - LVIDs)/LVIDd] \times 100}{1}$, and EF% was calculated according to the following equation: $EF = \frac{[(LVIDd - LVIDs)/LVIDd] \times 100}{1}$.

2.4 | Terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL)

The sections were incubated with proteinase K, washed in phosphate-buffered saline, incubated with permeabilization solution, blocking buffer, and then washed twice with PBS. The terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP (Roche Applied Science, Indianapolis, IN, USA) was applied on the tissue sections for 60 min at 37°C to stain the apoptotic nuclei. Then the tissue sections were treated with DAPI (4,6-diamidino-2-phenylindole) for 5 min, the nucleus were illuminated under blue light at 340/380 nm and the TUNEL-positive nucleus (fragmented DNA) was illuminated under bright green light at 450–500 nm. The mean numbers of TUNEL-positive cells were counted and all counts were performed on at least three independent tissue sections in a blinded manner.

2.5 | Tissue extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer in a ratio of 100 mg tissue/mL buffer. The homogenates were placed on ice and then centrifuged at 12,000g for 40 min. The supernatant was collected and stored at −80°C for further experiments.

2.6 | Electrophoresis and western blotting analysis

Protein concentration of cardiac tissue extracts was determined by the Lowry protein assay. Protein samples (40 μg/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to
polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) in a transfer apparatus (Bio-red). PVDF membranes were incubated in 5% skimmed milk in TBS buffer. Primary antibodies against Fas/L, Fas receptor, FADD, caspase-8, caspase-3, Bid, Bad, Bak, cytochrome c, Caspase-9, IGF-1, pIGF1R, p-Akt, Akt, Bcl-xL, Bcl-2, p-Bad, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted to 1:500 in antibody binding buffer and applied onto respective blots and incubated overnight at 4°C. The immunoblots were washed three times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 h and diluted 500-fold in TBS buffer. The immunoblots were then washed three times in TBS buffer for 10 min. The immunoblotted proteins were visualized using an enhanced ECL western Blotting Luminal Reagent (Santa Cruz, CA) and recorded using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

**FIGURE 1** Effect of anthocyanin on cardiac apoptosis. Representative photomicrographs show TUNEL-positive apoptotic cells in hearts of normal (n = 3), DM (n = 3), and DM rats treated with (DM + ANT, n = 3). The nuclei of cardiomyocytes were stained by DAPI and the apoptotic nuclei were specifically stained by TUNEL reagent. **P < 0.01 represents significant difference as compared to Normal group. ***P < 0.01 represents significant difference as compared to DM group [Color figure can be viewed at wileyonlinelibrary.com]
2.7 | Statistical analyses

The results shown are the means ± SD of three independent experiments. Statistical analysis was performed by one way ANOVA analysis followed by Tukey’s post hoc test for intergroup comparison. (P values) < 0.05 were considered to exhibit significant differences.

3 | RESULTS

3.1 | Effect of anthocyanin on cardiomyocyte apoptosis

Effect of anthocyanin on apoptosis-positive cardiac cells was identified in Normal, DM and DM + anthocyanin groups by using TUNEL assay. We found that TUNEL-positive cardiac cells in the left ventricle increased in the DM group compared to the Normal group (Figure 1A). However, anthocyanin treatment significantly reduced TUNEL-positive cardiac cells (Figure 1B).

3.2 | Effect of anthocyanin on DM induced extrinsic apoptosis

To further understand the changes in the Fas-dependent activation of apoptotic pathway proteins in DM rat hearts and in anthocyanin treated DM rat hearts, western blotting analysis was performed on proteins from the left ventricles of the excised hearts. The protein levels of Fas ligand, Fas receptor, Fas-associated death domain (FADD) and also the activated Caspase-8 in the DM group rat were significantly higher than Normal group, but the increase in the apoptotic protein levels was totally attenuated in DM + anthocyanin group (Figure 2A).

3.3 | Effect of anthocyanin on DM induced intrinsic apoptosis

The changes in the mitochondria-dependent apoptotic pathways in DM rats and in DM rats treated with anthocyanin were also analyzed by western blotting assay. Data shows that the protein levels of the pro-apoptotic proteins, Bcl-2-associated death promoter (Bad) and Bcl-2 homologous antagonist/killer (Bak) were upregulated, the total cytochrome c accumulation increased (Figure 3) and the levels of active Caspase-9 was elevated in DM group. However, anthocyanin reduced the expression of pro-apoptotic proteins Bad and Bak, cytochrome c accumulation, Caspase-9 activation. Further, apoptosis in DM rat hearts was confirmed by molecular events involving increase in cleavage forms of caspase-3 and PARP. The results show that the increase in the levels of cleaved caspase-3 and PARP appeared in the DM group rat hearts were significantly suppressed in the DM + anthocyanin group (Figure 4).

3.4 | Effect of anthocyanin on survival pathway

To identify the effect of anthocyanin on cardiac IGF-I receptor (IGFIR) dependent survival pathway, we examined the protein levels of IGF-1, IGF1 receptor, p-AKT in hearts by western blotting. The protein levels of IGF-1, IGF1 receptor, and phosphorylated AKT were significantly reduced in the left ventricles of DM group compared to Normal group. However, anthocyanin reversed the effect of DM on the levels of IGF1, IGF1 receptor, and phosphorylated AKT in the excised left ventricles of DM + anthocyanin group as showed in Figure 5A. In addition, the protein levels of the anti-apoptotic proteins such as Bcl-2,
Bcl-xL, and p-Bad also remained high in the anthocyanin treatment group (Figure 5B).

3.5 | Effect of anthocyanin on cardiac function

To analyze the cardiac function, the cardiac functional parameters such as LVIDd, LVIDs, LVPWd, LVPWs, EF, and FS were measured in Normal, DM, and DM + anthocyanin treatment group rats. Significant variations in the cardiac functional parameters were observed in the DM rats however treatment with anthocyanin showed positive modulation in the cardiac parameters of DM rats. Particularly the ejection fraction and fraction shortening that were deteriorated in the DM rats were restored in the rats treated with anthocyanin (Table 1). Therefore the results show that anthocyanin efficiently maintains the cardiac contractile function.

4 | DISCUSSION

Diabetes represents a major threat to human health, with global incidence projected to reach 300 million by 2025.\textsuperscript{39} DM is associated with several pathological events including insulin resistance, endothelial dysfunction, dyslipidemia, chronic inflammation, procoagulability, and impaired fibrinolysis\textsuperscript{40} and a high incidence of cardiovascular disease in DM is a major cause of morbidity and mortality.\textsuperscript{41} Various traditional and alternative medicines have been used in several parts of the world for DM treatment and numerous herbs have been accepted in different traditional and alternative medicine practices to control DM.

Anthocyanins, a class of naturally occurring water soluble polyphenols, have been proven to exhibit beneficial effects such as antioxidant potential and cytoprotection. Anthocyanins from various sources have
been shown to possess antidiabetic properties\textsuperscript{42–45} and potential cardioprotective properties.\textsuperscript{46–48} However, scarcely available evidences on the cardioprotective effects of anthocyanins are usually restricted to ischemia/reperfusion-induced injury and the effects of anthocyanins on DM associated cardiac injury in general are limited or unavailable.\textsuperscript{47–49}

The antioxidant properties of anthocyanins are generally considered to constitute their cardioprotective effects. Cyanidin-3-glucoside, an anthocyanin with strong antioxidant property, exhibits high cytochrome c-reducing property and provide protection against ischemia/reperfusion-induced apoptosis and necrosis. Apoptosis and necrosis often leads to unrecoverable loss of cardiomyocytes, a critical factor causing left ventricle dysfunction and chronic heart failure.\textsuperscript{50}

Our previous study demonstrated that anthocyanins from purple rice effectively protect rats from DM associated cardiac hypertrophy.\textsuperscript{51} This study shows that these anthocyanins with high levels of Cyanidin-3-glucoside content, efficiently protects the hearts of STZ-induced diabetic rats from apoptosis. The anthocyanin treatment not only reduced cytochrome c but also suppressed caspase activation to ameliorate the apoptotic effects. While in mitochondria-dependent apoptotic pathway the activated pro-apoptotic proteins such as Bad and Bak bind to mitochondria causing instability in the mitochondrial membrane potential causing the release of cytochrome c and activation of caspase-9 and caspase-3, the extrinsic apoptosis pathway involves the recruitment of FADD and pro-caspase 8 by Fas receptor oligomerization\textsuperscript{52} and activation of caspase-8 that leads to the cleavage of caspase-3.\textsuperscript{18} STZ induced DM elevated both the extrinsic as well as the intrinsic pathway proteins. However, anthocyanin treatment proved to be highly effective in regulating the effects of diabetics on cardiac health as seen from the reduced apoptosis levels and the down-regulation in the expression of pro-apoptotic proteins. Suppression of caspase activation and reduction of cytochrome c has been evidently correlated as a crucial event in the suppression of cardiac apoptosis associated with cardiac damages induced by DM, obesity and as well as drug overdose.\textsuperscript{10,53–55} Our present results also correlate with our previous reports on the cardio-protective effects of other agents such as probiotics, co-enzyme Q10, deep sea minerals, diallyl trisulfide and extracts of \textit{Alpinata oxyphyllae MIQ} and \textit{Andrographis paniculata}.\textsuperscript{10,53–56}

Moreover, our previous studies show that STZ-induced type 1 DM could down regulate the IGFI-R/Pi3K/Akt survival signaling and the Bcl-2 family associated pro-survival mechanisms.\textsuperscript{57} Similarly in this study the levels of cardiac IGFIIR, Pi3K and p-Akt in STZ-induced DM hearts were significantly decreased compared to normal rat hearts however; the impaired IGFIIR/Pi3K/Akt survival pathway was regulated after anthocyanin treatment. The anti-apoptotic proteins Bcl-2, p-BAD and Bcl-xL were significantly decreased in STZ-induced DM hearts but the levels remained comparatively high in the anthocyanin treatment group. Enhancement of IGFI-R associated survival factors has been viewed as a hallmark for the effect of efficient cardioprotective agents and has been also observed in our previous studies.\textsuperscript{10,53–55} The enhancement of IGFI-I and its associated proteins including p-Pi3K, p-Akt, Bcl-2 and Bcl-xL in the STZ-induced DM rat hearts, could be possibly the result of a compensative survival mechanism activated to
counter the elevated cardiac apoptosis.\textsuperscript{58,59} The effect of anthocyanins in ameliorating diabetics induced cardiac apoptosis also resulted in the overall improvement in cardiac function as seen from the LVIDd, LVIDs, LVPWd, LVPWs, ES% and FS% as determined by echocardiographic analysis.

Previous studies show that anthocyanin such as delphinidin or other flavonoids may protect the heart against ischemia-reperfusion injury by mechanisms not related to their antioxidant activities.\textsuperscript{60} Our current findings correlate with the positive effects of anthocyanin and more than being effective against diabetics associated apoptosis it also

\begin{figure}
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\includegraphics[width=\textwidth]{figure5}
\caption{Anthocyanin enhanced cardiac cell survival pathway in diabetic rat hearts. Western blot show the modulation in the levels of cardiac IGF-I receptor (IGFIR) dependent survival pathway proteins (A) such as IGF-1, p-IGF1 receptor (p-IGF1R) and p-Akt and in the levels of associated anti-apoptotic proteins (B) such as Bcl-xL, Bcl-2 and p-Bad in the hearts of normal Wistar rats \((n = 6)\), DM rats \((n = 6)\) and in anthocyanin treated DM rats \((DM + ANT, n = 6)\). *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) represent significant difference as compared to Normal group. #\(P < 0.05\), ##\(P < 0.01\) and ###\(P < 0.001\) represent significant difference as compared to DM group.}
\end{figure}
There is no conflict of interest to be stated.

**CONFLICTS OF INTEREST**

There is no conflict of interest to be stated.

**REFERENCES**


