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Anthocyanins from Chinese Bayberry Extract Protect β Cells from Oxidative Stress-Mediated Injury via HO-1 Upregulation

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Oxidative stress plays a pivotal role during the islet transplantation procedure, and antioxidant supplementation may protect grafts against oxidative injury. Chinese bayberry is one of six *Myrica* species native to China, and we demonstrated here that anthocyanins from Chinese bayberry extract (CBE) protect pancreatic β cells (INS-1) against hydrogen peroxide (H_2O_2)-induced necrosis and apoptosis. Anthocyanins time- and dose-dependently upregulated heme oxygenase-1 (HO-1) gene expression in β cells and primary islets. HO-1 knockdown increased H_2O_2-induced cell death and attenuated the cytoprotective effect of anthocyanins. Anthocyanin treatment activated ERK1/2 and PI3K/Akt signaling, and ERK1/2 and PI3K inhibitors partially attenuated anthocyanin-mediated induction of HO-1. Additionally, β cells pretreated with anthocyanins displayed a decreased extent of apoptosis after transplantation. In summary, these results suggest that anthocyanins in CBE protect β cells from H_2O_2-induced cell injury via ERK1/2- and PI3K/Akt-mediated HO-1 upregulation.

KEYWORDS: Anthocyanins; cyanidin-3-*O*-glucoside; Chinese bayberry; heme oxygenase-1; apoptosis; oxidative stress

INTRODUCTION

Type-1 diabetes mellitus (T1DM) is characterized by the selective destruction of insulin-producing β cells, which leads to the deficiency of insulin secretion and, as a result, hyperglycemia. At present, β-cell replacement through islet transplantation presents the best opportunity to the patients with T1DM and prevents the long-term serious complications associated with this disease. However, early and progressive graft losses contribute to the failure of transplantation therapeutics. Improved islet isolation and better viability preservation before transplantation have been the keys to the evolution of this procedure.

Many researchers excitingly discovered that the addition of some antioxidants in islet culture conditions prior to transplantation was a promising strategy to better preserve their viability and function; i.e., nicotinamide (NA) or glutathione supplementation significantly improved islet yields (*1*), protected β cells from cell death, or reduced inflammatory factor production (*2*). Recently, more attention has been focused on the bioapplications of naturally existed antioxidants, especially from plants (*3*, *4*). Previous studies demonstrated that some flavonoid components (such as epigallocechin, genistein, etc.) could protect pancreatic β cell against cytokine and/or oxidative stress-induced damage through various mechanisms (*5*). Anthocyanins, one type of flavonoids, are naturally occurring polyphenolic compounds in the plant foods. Many studies have shown that anthocyanins exhibit an array of pharmacological properties, such as anti-inflammatory, antitumor, and antioxidative activities. Berry extracts rich in anthocyanins have been linked to protective effects in some disease models (*6*). For diabetes, long-term administration of berry-derived supplements, including anthocyanins, could inhibit the development of the early stages of some diabetic complications without adverse drug reactions (*7*). Jayaprakasam et al. suggested that bioactive anthocyanins from *Cornus* fruits could increase insulin release of INS-1 cells (*8*).

Chinese bayberry, one of six *Myrica* species native to China, is rich in anthocyanins, and cyanidin-3-*O*-glucoside (C3G) was indentified as a major anthocyanin component (*9*, *10*). Our previous studies showed that Chinese bayberry extracts (CBEs) possessed notable radical scavenging activities, which might be served as a source of potent natural antioxidant. However, thus far, few studies have been conducted to investigate its biomedical applications. Especially, the information about its use in the islet/β-cell protection field has not yet been reported. Therefore, we designed the present study to decorticate the potential protective effect of anthocyanins in CBE against oxidative stress-induced cell death in β cells both *in vitro* and in renal subcapsular transplantation model and to characterize the underlying mechanisms.

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MATERIALS AND METHODS

Extracts Preparation, High-Performance Liquid Chromatography (HPLC) Analysis, and Antioxidant Ability Assessment. Chinese bayberry fruits were obtained from Xianju County, Zhejiang Province, China. The extract was prepared initially as described previously (*10*), with slight modification. In this study, the Chinese bayberry fruit was extracted with extract solvent, then dissolved in double-distilled water, and was further cryo-concentrated. HPLC analysis of the extract was carried out, and the content of total flavonoids, anthocyanins, and C3G were determined. The conditions for the determination of C3G are as follows. Waters HPLC was equipped with a 2695 pump and 2996 photodiode array detector. Formic acid (4.5%) in water was used as solvent A, and HPLC-grade methanol (100%) was used as solvent B. The gradient elution was as follows: from 5 to 25 min, 35−50% B; from 25 to 40 min, 50−80% B; then B was maintained for 2 min; from 42 to 45 min, 80−15% B; and B was maintained for 5 min, concluding 1 cycle. A flow rate of 1 mL/min was used. The temperature was 30 °C. Determinations for total flavonoids and total anthocyanins and the free radical scavenging activity of fruit extracts were performed according to the methods by Zhang et al. (*10*).

Culture of the Pancreatic β-Cell Line and Mice Islet Isolation. INS-1 cells, a rat pancreatic β cell line, were grown in monolayer cultures in regular RPMI-1640 medium supplemented with 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L β-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere (5% CO_2 and 95% air). Primary islets were isolated by collagenase digestion from ICR mouse and separated using density gradient centrifugation (*11*). Islets were incubated at 37 °C in 2 mL of RPMI-1640 medium (containing 10 % FCS) at a density of 100−150 islets/well in 6-well plates.

Cell Viability Assay. Cell viability was determined using a 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (Sigma, St. Louis, MO), as previously described (*12*). Cell viability was also measured using the trypan blue dye (Sigma) exclusion method (*13*). The proportion of cells remaining non-viable (unable to exclude trypan blue) was counted and expressed as a percentage of the total number of cells.

Lactate Dehydrogenase (LDH) Release Assay. LDH release was measured using a commercially available assay kit (Cytotox, Promega, Madison, WI). After various treatments, the 96-well plate was centrifuge at 250*g* for 4 min. A total of 50 μL of medium was removed from all wells to be used for the assay. The samples were incubated with substrate mix (containing NAD+, lactate, and tetrazolium) for 30 min. LDH converts lactate to pyruvate, generating NADH. The NADH then reduces tetrazolium to formazan (red), which was detected by absorbance at 490 nm.

Cellular Apoptosis Detection and Free Radical Scavenging Activity Assay. Both detached and attached cells were harvest after 1 mM H_2O_2 stimulation in the presence or absence of pretreating with anthocyanins, and cellular apoptosis was evaluated quantitatively by flow cytometry (FCM), as described previously (*12*). Additionally, cell apoptosis were also observed by Hoechst 33258 staining.

Intracellular reactive oxygen species (ROS) formation was measured by the ROS-specific fluorescent probe dichlorofluorescein diacetate (DCF-DA, Sigma). After incubation with 1 mM H_2O_2 for 2 h with the presence or abscense of 0.5 and 1 μM anthocyanidins, INS-1 cells were washed with serum-free medium and incubated with 10 μM DCF-DA for 20 min at 37 °C. Then, cells were resuspended in ice-cold phosphate-buffered saline (PBS) and placed on ice in the dark for FCM analysis. Cells were also observed under a fluorescence microscope, and digital images were acquired for analysis (SPOT, Diagnostic Instruments, Inc.).

Immunoblotting Analysis. Protein extraction and immunoblotting were performed as described earlier (*12*). The following primary antibodies were used: Bcl-2, actin, and horseradish-peroxidase-conjugated secondary antibody, which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase 3, caspase 9, ERK1/2, pERK1/2, pAkt, and Akt were obtained from Cell Signaling (Beverly, MA). HO-1 antibody was purchased from Boster (Wuhan, China)

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) for HO-1. Total RNA from the cells was isolated with Trizol reagent (Invitrogen, Carlsbad, CA), and the concentration was detected using a spectrophotometer. Total RNA (2.0 μg) was reverse-transcribed to cDNA in a reaction mixture in a final 20 μL using a Fermentas RNA PCR kit (MMLV) according to the directions of the manufacturer. Quantification of gene expression was measured using the RT-PCR system (Roche Lightcycler 2.0) using SYBR green dye (Takara, Japan) as described previously (*14*). The housekeeping gene GAPDH served as the internal control. The primers were as follows: HO-1, forward, 5′-TCAGGTGTCCAGGGAAGGCTT-3′; reverse, 5′-CTCTAGGGCAGCCCTTCGGT-3′; GAPDH, forward, 5′-GGTGGACCTCATGGCCTACAT-3′; reverse, 5′-GCCTCTCTCTTGCTCTCAGTATCCT-3′ for rat and HO-1, forward, 5′-CTGTGAACTCTGTCCAATG-3′; reverse, 5′-AACTGTGTCAGGTATCTCC-3′; GAPDH, forward, 5′-GGAGAAACCTGCCAAGTATG-3′; reverse, 5′-GGAGTTGCTGTTGAAGTCG-3′ for mice. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method normalized to GAPDH mRNA and expressed relative to the control subjects.

Immunofluorescence for HO-1 in Islets. Primary mouse islets were isolated and incubated in the absence and presence of anthocyanins. After 24 h, islets were fixed in 4% paraformaldehyde (PFA) for 15 min, suspended in 30% sucrose for another 30 min, embedded in OCT compound, and rapidly frozen in liquid nitrogen. Immunocytochemistry was carried out with 6 μm thick sections. The slides were heated for 5 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. After incubation in blocking solution (5% normal goat serum) for 1 h, the slides were exposed to primary antibodies at the dilution of 1:500 (insulin) and 1:200 (HO-1) in 2% BSA at 4 °C overnight and incubated with secondary antibodies linked to Alexor 488 or 594 (1:200 dilution) for 1 h. After PBS wash, the slides were mounted with glycerol mounting medium and examined under a fluorescent microscope.

Retroviral Production and Transfection Assay. A constitutive expression vector, pLNCX2-HO-1, was constructed carrying the coding sequence of the rat HO-1 gene. Retroviral-producing mouse cells, PT67, were transfected with the plasmid using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the instructions of the manufacturer. For infection INS-1 cells, the virus-containing supernatants were collected at 48 h post-transfection, filtered through a 0.45 μm cellulose acetate filter, and supplemented with 4 μg/mL Polybrene (Invitrogen). Target INS-1 cells were incubated in the virus/Polybrene containing supernatants from 4 h to overnight. The next day after the second infection, we added G418 at a final concentration of 600 μg/mL for selection. For control purposes, parallel cultures of INS-1 cells were stably transfected with pLNCX2 alone.

RNA Interference. The target sequence for HO-1-specific small interference RNA (siRNA) was 5′- CCGUGGCAGUGGGAAUUUAUGCCAU-3′, which were synthesized by GenePharma Co. (Shanghai, China), as well as control siRNA (no silencing). Transfection was performed using lipofectamine 2000 reagent according to the manual instructions.

Animal Studies. ICR mice of 8 weeks in age were used as the recipients in the β-cell transplantation experiment. Recipients were rendered diabetic using streptozotocin (STZ; 200 mg/kg, ip). After STZ administration for 5 days, mice with two consecutive blood glucose levels exceeding 350 mg/dL were used as recipients. INS-1 cells ($1-2 \times 10^6$), treated with or without 1 μM anthocyanins for 24 h, were collected and transplanted under left renal capsule. β-Cell grafts including a portion of the kidney were harvested at 3 days post-transplantation and snap frozen in liquid nitrogen or fixed with 4% PFA and embedded in paraffin. Immunohistological staining for insulin and cleaved caspase 3 were performed according to the manual instructions. All experiments were carried out in accordance with the ethical guidelines of the Animal Experimentation Committee at the College of Medicine, Zhejiang University, Hangzhou, Zhejiang Province, China.

Statistical Analysis. When necessary, data were expressed as the mean ± standard deviation (SD). The statistical significance of the difference was calculated using Student's *t* test for single comparison or using an analysis of variance (ANOVA) for multiple comparisons. The criterion employed for statistical significance of the difference was $p < 0.05$.

RESULTS

Identification and Quantification of Chinese Bayberry Extracts. Flavonoids, including anthocyanins, flavonols, etc., are important phenolic compounds in fruits. In the present study, flavonoid contents, determined by a spectrophotometer, occupied the greatest proportion of total phenolics, in which anthocyanins were

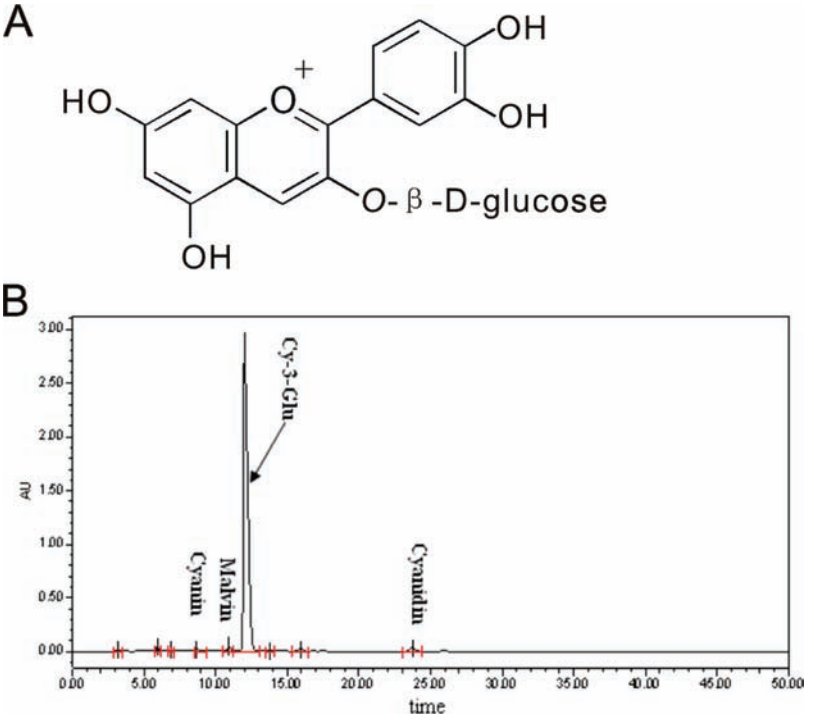

Figure 1. (A) Chemical structure of C3G. (B) HPLC analysis showed that C3G accounted for the major portion of anthocyanins in CBE.

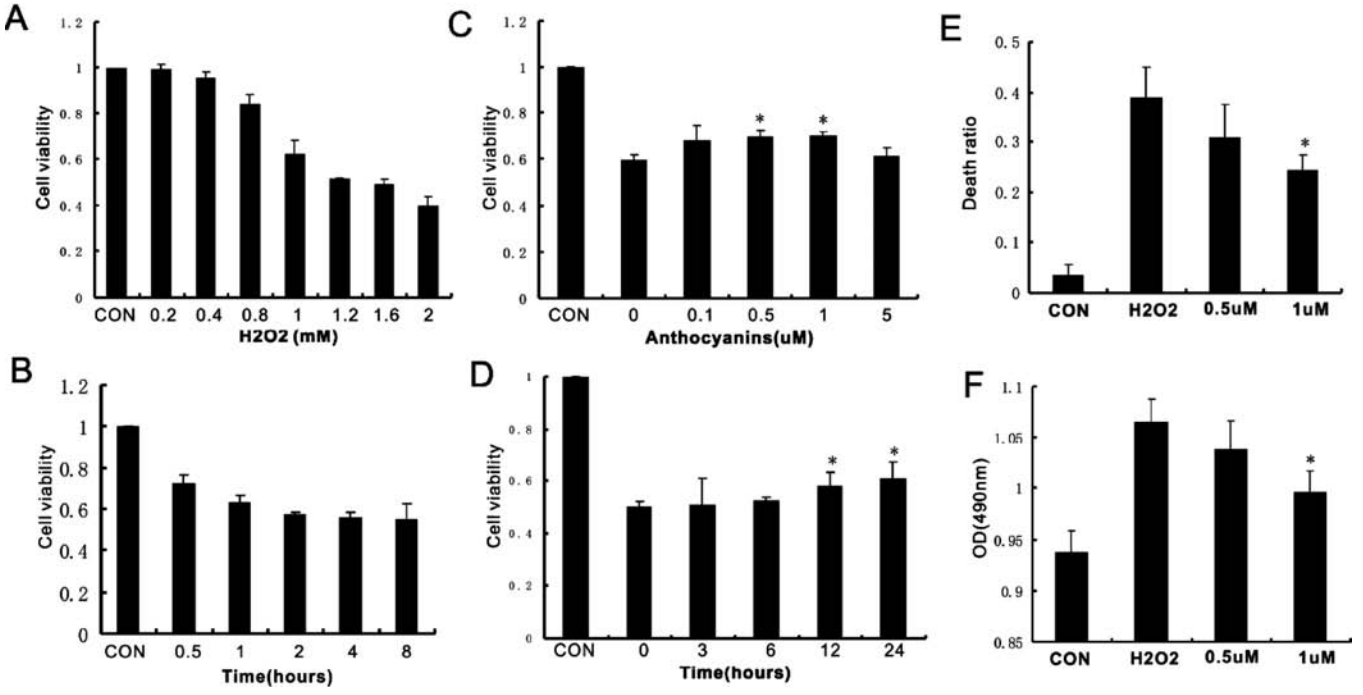

Figure 2. Anthocyanins attenuated H_2O_2-induced cell injury. (A) Dose-dependent reduction of cell viability by H_2O_2. (B) Time course of H_2O_2-induced cell damage. (C) Dose−effect study of the protective role of anthocyanins against H_2O_2-induced cell damage. (D) Time course of the protective effects of anthocyanins against H_2O_2-induced cell damage. (E) Inhibition by anthocyanin of H_2O_2-induced trypan blue uptake. The proportion of dead cells occupying total cells was calculated. (F) LDH release assay. Cells were treated with 0.5 and 1 μM anthocyanins for 24 h followed by 1 mM H_2O_2 for a further 4 h. The released LDH was detected at a 490 nm wavelength ($n = 6$). (*) $p < 0.01$, significant difference from the H_2O_2-treated group (C−F).

the major part followed by rutin and myricetin. Further HPLC analysis of anthocyanins demonstrated that C3G occupied about 95% (**Figure 1A**), followed by cyanins. The DPPH assay indicated the strong antioxidant capacity of CBE, and C3G is the dominant contributor. Because C3G is the major component in CBE, the usage dose of the extract was standardized by the concentration of C3G.

Cytotoxicity and Protective Effect of Anthocyanins against H_2O_2-Induced Oxidative Stress. We first analyzed the cytotoxicity of anthocyanins in CBE on β cells. The results of the MTT assay showed that there was no significant change in cell viability after 12, 24, and 48 h treatment with 0.01−5 μM anthocyanins (data not shown). H_2O_2 injured INS-1 cells in a dose- and time-dependent manner (panels **A** and **B** of **Figure 2**). The 2 h time point and

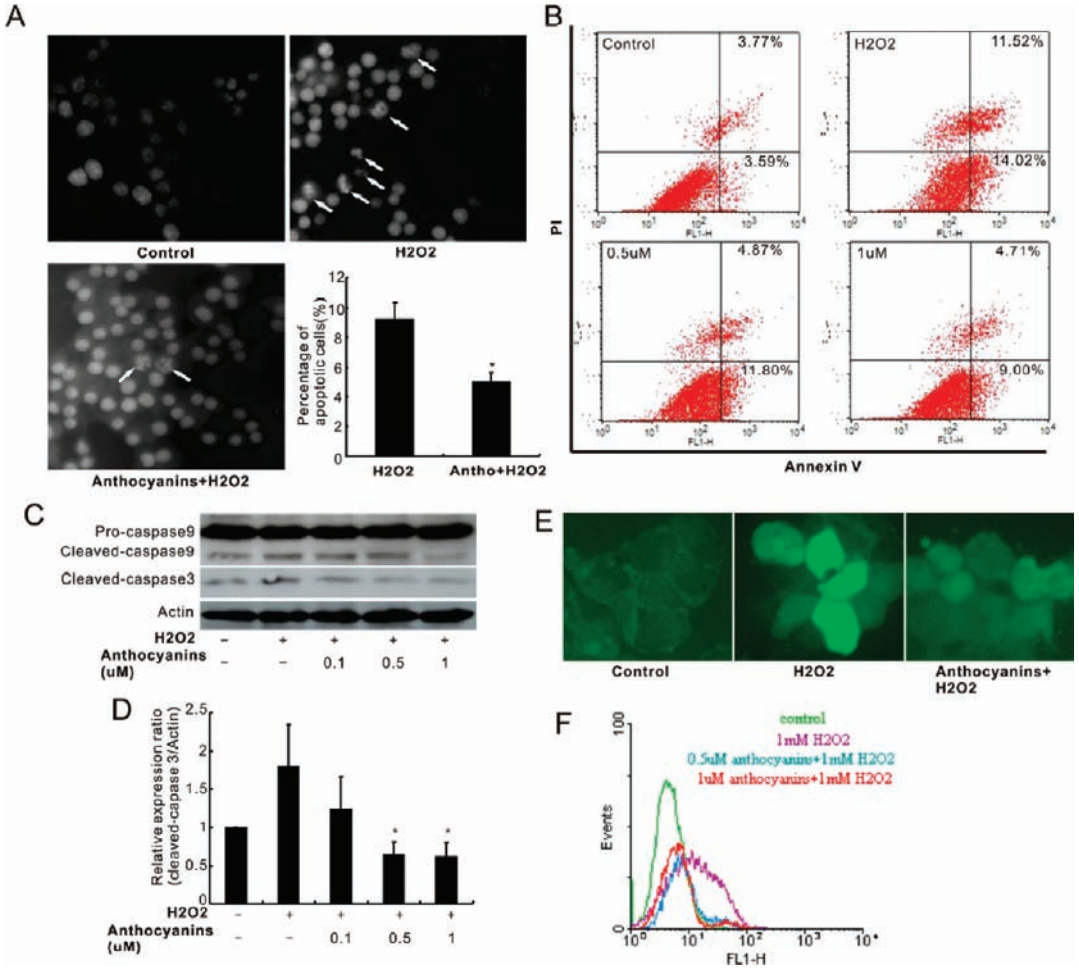

Figure 3. Anthocyanins reduced H_2O_2-induced apoptosis. (A) Apoptotic cells with condensed or fragmented chromatin nuclei (arrow) in different groups were detected by staining the nuclei with H33258. The representative images were from at least three independent experiments. The percentages of apoptotic cells in H_2O_2-stimulated and anthocyanin-pretreated groups were quantified and shown as the mean ± SD (n = 3). (*) $p < 0.01$, versus H_2O_2-treated group. (B) Apoptosis and necrosis of β cells in different groups were observed by FCM analysis. (C) Anthocyanins decreased H_2O_2-induced activation of caspase 3 and caspase 9. (D) Band intensity analysis of cleaved caspase 3 in different groups (n = 3). (*) $p < 0.05$, versus H_2O_2-treated group. (E and F) Intracellular ROS production. After various treatment, β cells were stained with DCF-DA and analyzed (E) under a fluorescence microscope or (F) by FCM.

1 mM concentration of H_2O_2 were chosen for the subsequent studies. We next explored the protective effect of anthocyanins against H_2O_2-induced oxidative injury. In comparison to the control, 1 mM H_2O_2 caused a significant reduction in cell viability to 59.9 ± 2.0% (**Figure 2C**). However, pretreatment of INS-1 cells with 0.5 and 1 μM anthocyanins resulted in the prevention of cell death by H_2O_2. To evaluate the time-dependent effects of anthocyanin-mediated protection, anthocyanin pretreatment was performed for 3, 6, 12, or 24 h before 2 h of 1 mM H_2O_2 stimulation. A significant increase in cell viability was achieved with 12 and 24 h of anthocyanin pretreatment (**Figure 2D**). Similar results were obtained when cell viability was measured using the trypan blue exclusion method and LDH release assay (panels **E** and **F** of **Figure 2**). The trypan blue uptake and LDH release were significantly reduced in the 1 μM anthocyanin-treated group. These results indicate that anthocyanin incubation prevented H_2O_2-induced injury in INS-1 cells.

Anthocyanins Reduced ROS-Mediated Apoptosis of β Cells. Because the ROS-mediated apoptotic pathway plays an important role in H_2O_2-induced cell injury, we then explore whether such a protective effect of anthocyanins is partially attributed to decreased apoptosis. H33258 staining revealed that treating with 1 mM H_2O_2 for 2 h caused significant cellular apoptosis (**Figure 3A**), and the proportion of apoptotic cells was decreased by anthocyanin pretreatment (1 μM; **Figure 3A**). The statistical differences existed on the percentage of apoptotic cells between these two groups (9.26 ± 1.09% in the H_2O_2-treated group versus 4.99 ± 0.6% in the anthocyanin-pretreated group; $p < 0.01$). Such observations were further supported by flow cytometry (FCM) analysis, and notably, FCM demonstrated that anthocyanin incubation reduced the extent of both cellular apoptosis and necrosis (**Figure 3B**). Treatment of INS-1 cells with 1 mM H_2O_2 for 2 h caused the activation of caspase 9 and caspase 3, which were mitigated by pretreatment with anthocyanins for 24 h (panels **C** and **D** of **Figure 3**). Anthocyanin pre-incubation also decreased oxidative stress-induced intracellular ROS generation (panels **E** and **F** of **Figure 3**).

Anthocyanins Increased HO-1 Expression in β-Cell Lines and Islets, Which Contributed to Its Protective Effect. Because HO-1 is an important component of the cellular defense system against oxidative stress, we then examined whether anthocyanins could induce HO-1 expression in β cells. INS-1 cells treated with anthocyanins resulted in a dose- and time-dependent increment in HO-1 mRNA and protein expression (**A**−**D** of **Figure 4**). However, *N*-acetyl-cysteine (NAC), a free radical scavenger, did not increase HO-1 expression at the dose of 10 and 50 mM in INS-1 cells (**Figure 4E**).

To determine whether this induction took place in islets, islets were isolated and treated with 2 μM anthocyanins for 6 h. RT-PCR

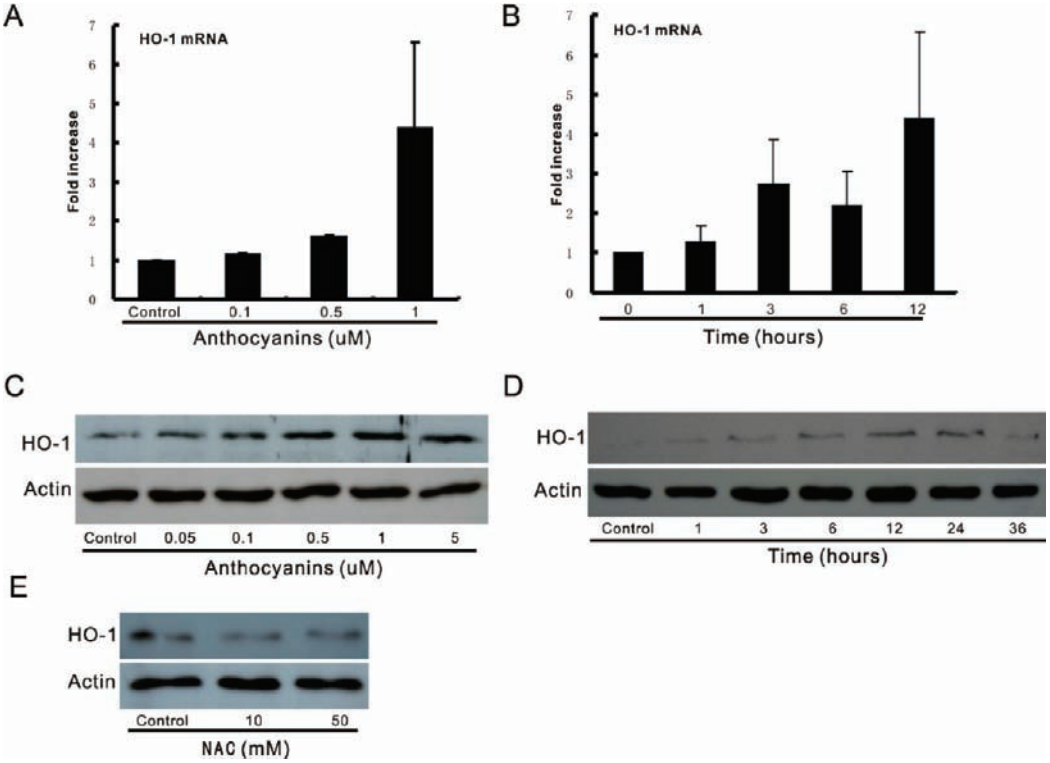

Figure 4. Anthocyanin-mediated HO-1 induction. (A and B) Cells were treated with various concentrations of anthocyanins for 12 h or with 1 μM anthocyanins for various times as indicated. HO-1 mRNA expression was assessed by quantitative RT-PCR with GAPDH as the internal control. (C and D) INS-1 cells were treated with various concentrations of anthocyanins for 24 h or with 1 μM anthocyanins for various times as indicated. Immunoblotting for HO-1 protein expression was performed. (E) INS-1 cells were treated with 10 and 50 mM NAC, and HO-1 protein expression was analyzed.

analysis demonstrated that the expression of HO-1 was significantly upregulated (4.7−8.4-fold increase compared to the control; $p < 0.05$; panels **A** and **B** of **Figure 5**). Immunocytochemical analysis of HO-1 in islets further supported this observation (**Figure 5C**). These results suggest that anthocyanins induce HO-1 in both β-cell lines and mouse primary islets.

To investigate whether HO-1 induction was involved in the protective mechanism of anthocyanins against H_2O_2-induced cytotoxicity, we first knockdown HO-1 by transfection of siRNA against HO-1 in INS-1 cells. INS-1 cells were first transfected with HO-1 siRNA or control siRNA. After 24−48 h, cells were further treated with or without 1 μM anthocyanins for another 24 h. Protein from cell lysates was analyzed for HO-1 expression by western blot. **Figure 6A** showed that HO-1 protein expression induced by anthocyanins was significantly reduced after siRNA−HO-1 transfection, which attenuated the protective effect of anthocyanins against H_2O_2-induced cytotoxicity (panels **B** and **C** of **Figure 6**). HO-1 knockdown also increased the extent of H_2O_2-induced cellular apoptosis (**Figure 6D**). We then established INS-1 cells constitutively expressing HO-1 protein by retrovirus infection (**Figure 6E**). Overexpression of HO-1 reduced H_2O_2-induced injury (**Figure 6F**). These results imply that anthocyanidins suppress H_2O_2-induced cytotoxicity (at least partially) through induction of HO-1 gene expression.

Anthocyanins Upregulated HO-1 Expression via PI3K/Akt and ERK1/2 Activation. To elucidate the possible signal transduction pathways involved in the anthocyanin-induced HO-1 expression, we examined the phosphorylation of several upstream kinases. The results showed that anthocyanins dose- and time-dependently induced the activation of Akt and ERK1/2 via induction of phosphorylation (panels **A** and **B** of **Figure 7**) but not of JNK activation (data not shown). Inhibition of PI3K/Akt and ERK1/2 signaling with LY294002 and PD98059 attenuated the anthocyanin-induced HO-1 expression (panels **C** and **D** of **Figure 7**). The effect was more obvious when cells were pretreated with a higher dose of inhibitor, further supporting the participation of these two signaling pathways. Pre-incubation with PD98059 or LY294002 compromised the protective effect of anthocyanins compared to anthocyanins treated alone (**Figure 7E**).

Anthocyanin Pretreatment Decreased the Occurrence of the Apoptosis of Grafts in the β-Cell Transplantation Model. It is well-established that a large majority of β cells/islets will be lost immediately after transplantation (mainly because of apoptosis), and oxidative stress plays a critical role in the death of grafts during this procedure. We therefore investigate whether anthocyanins have a protective effect at the early phase of transplantation. We found that grafts pretreated with anthocyanins displayed less positive for active caspase 3 (panels **A**−**C** of **Figure 8**), suggesting that treating grafts with anthocyanins prior to transplantation could decrease cellular death at the early phase of post-transplantation through downregulating the apoptosis process.

DISCUSSION

It has been well-documented that oxidative stress produced oxygen free radicals, such as superoxide anion, hydroxyl radical, and H_2O_2, which are the key mediators that contribute to cell injury during islet isolation and transplantation procedures (*15*). Because of the relatively low expression of antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase, pancreatic β cells are rather sensitive to ROS attack when they exposed to oxidative stress (*16*). Non-immunological (mechanical injury, ischemia, non-specific inflammation, hypoxic microenvironment, etc.) and immunological (immune rejection) events, which take place during the islet transplantation procedure, initiate a cascade of biochemical reactions, which results in the production of ROS causing necrosis and apoptosis, leading to

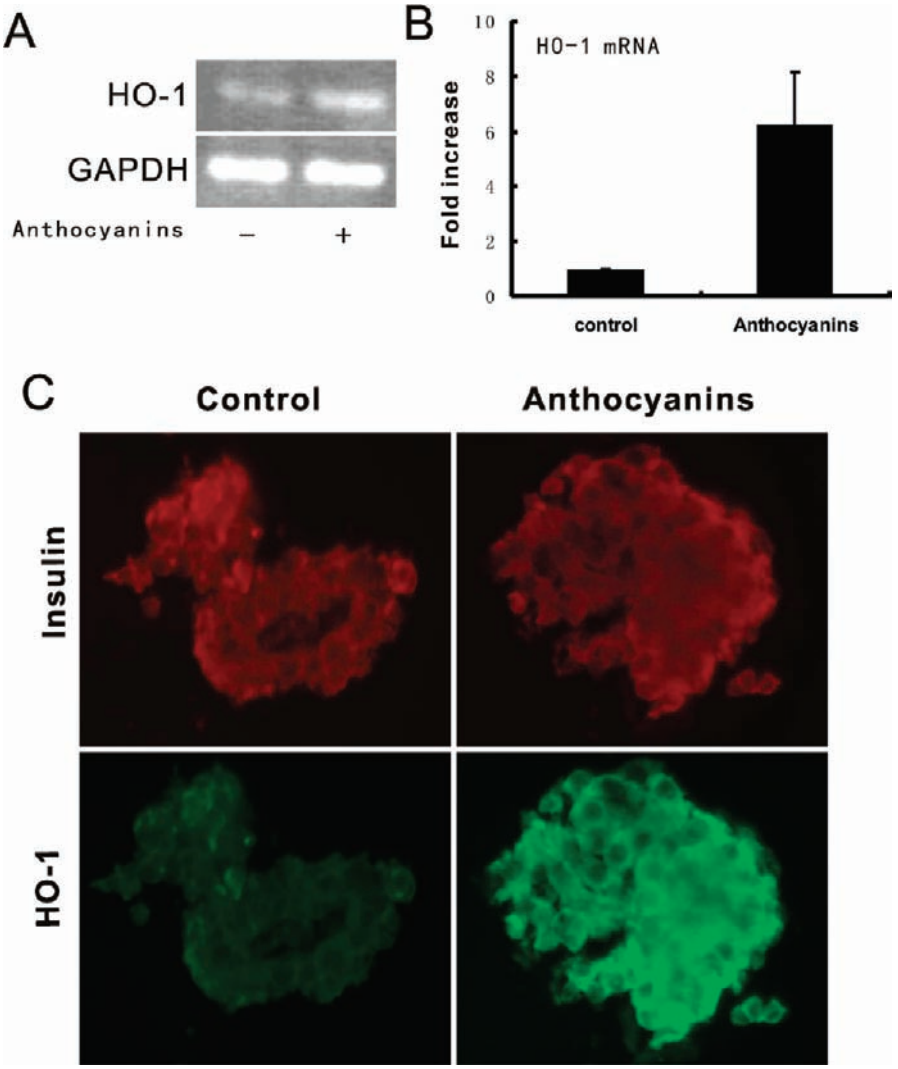

Figure 5. Induction of HO-1 by anthocyanins in mouse islets. (A) Primary isolated islets were incubated with or without anthocyanins for 6 h. (B) mRNA levels of HO-1 were determined by quantitative RT-PCR with GAPDH as the internal control. Results are means ± SD of three independent observations. (∗) $p < 0.05$. (C) Immunocytochemistry for insulin and HO-1 in islets were carried out with 6 μm thick sections and examined by fluorescent microscopy. Representative images from three independent experiments are provided.

significant graft loss (*17*−*19*). In addition, excessive oxidative stress also suppresses the cytoplasm−nuclei translocation of pancreatic and duodenal homeobox factor-1 (PDX-1), an important transcription factor for insulin biosynthesis, and decreases its binding activity, which subsequently cause decrements of insulin mRNA, insulin content, and glucose-induced insulin secretion (*20*). Many researchers have observed the reversal of oxidative stress-induced islet death and dysfunction by antioxidant treatment or intra-islet overexpression of antioxidant enzymes. In many studies, plant-derived antioxidants have already been shown having benefical effects for islets/β cells during transplantation procedures (*21*), as well as in a diabetic state (*3*).

Anthocyanins, the major component in CBE, displayed strong radical scavenger ability, as indicated by the DPPH assay. In this study, we used H_2O_2 supplementation as the *in vitro* oxidative stress model, subrenal capsular transplantation as *in vivo* stress stimulation model, and anthocyainin pre-incubation as the possible protective regimen. We were excited to find that pretreating β cells with CBE could notably decrease excessive ROS production and protected against oxidative stress-induced cell necrosis and apoptosis *in vitro*. Furthermore, anthocyanin pre-incubation improved the tolerance of β-cell grafts at an early phase after subcapsular transplantation. These observations, for the first time, indicated that anthocyanins might serve as a potent protective agent for islets.

In our experimental system, β cells were pretreated with anthocyanins and the culture medium was discarded prior to H_2O_2 stimulation. This excluded the possibility of direct neutralization of oxidants by anthocyanins but possibly via upregulating the cellular defense system. Furthermore, *in vitro* studies displayed that pretreatment with anthocyanins for less than 6−12 h did not have an obvious protective effect on β cells, indicating that such positive effects might depend upon the production of some protective genes/proteins. Indeed, we found that anthocyanins could time- and dose-dependently induce HO-1 expression in β cells/islets, which has emerged as an important mediator of cellular defense against wide-ranging tissue injuries via free radical scavenging and apoptosis prevention (*22*). Previous studies have demonstrated the cytoprotective effects of HO-1 in pancreatic β cells (*23*, *24*). Rodent islets overexpressing HO-1 induced by either gene transfection or chemical stimuli possess better function and improved survival rate after transplantation (*25*, *26*).

Many electrophilic antioxidant compounds, including plant-derived polyphenolic substances, might be the inducer of HO-1, such as enterolactone (*27*), demethoxy curcuminoids (*28*), quercetin (*29*), etc. Especially, it is reported that anthocyanins in

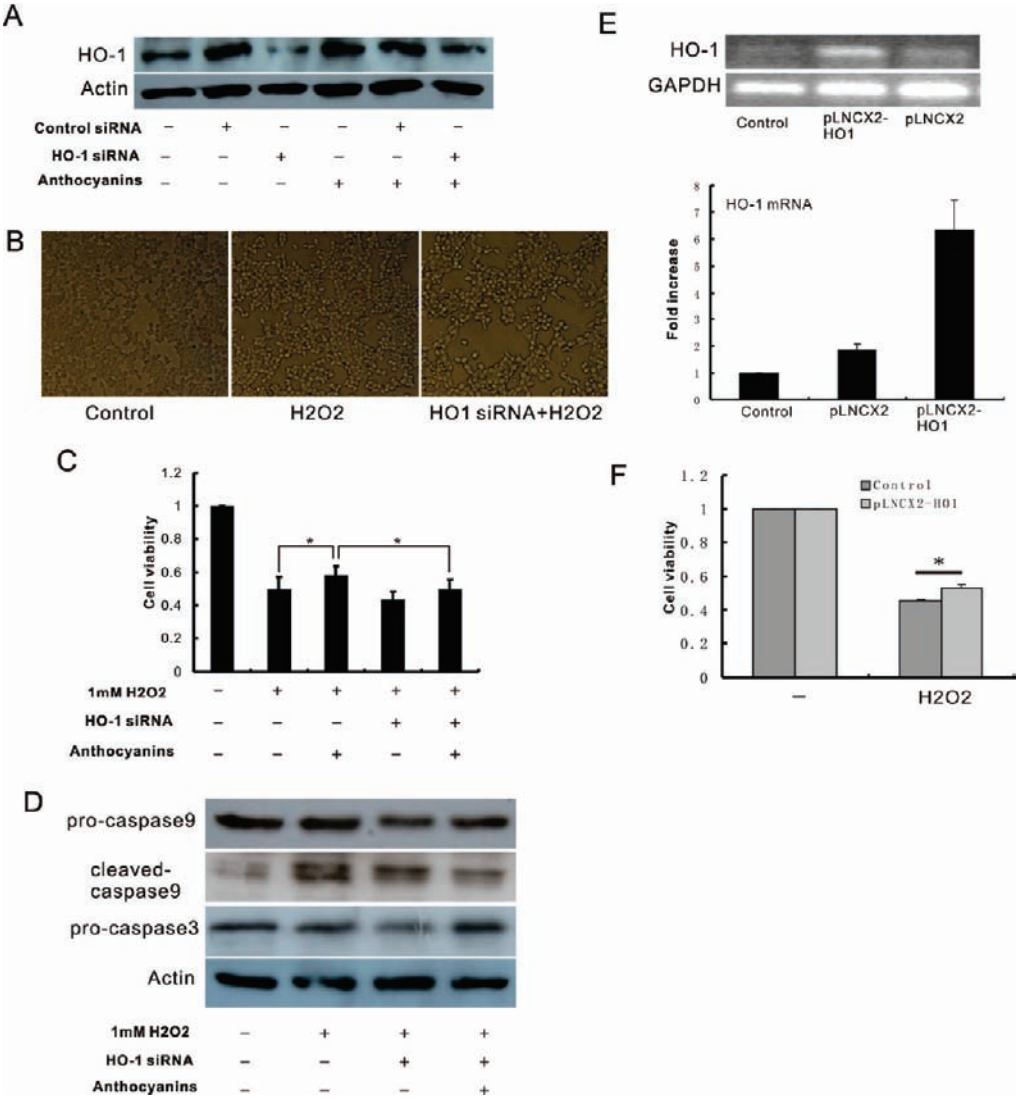

Figure 6. HO-1 participated in the cytoprotection of anthocyanins against H_2O_2-induced cytotoxity. (A) Western blot analysis of HO-1 expression in INS-1 cells transfected with HO-1 or control siRNA in the presence or absence of anthocyanins. (B) Control/HO-1 siRNA-transfected INS-1 cells were treated with 1 mM H_2O_2 for 3 h and then examined by an inverted microscope. (C) Protective effect of anthocyanins against H_2O_2-induced cytotoxicity was attenuated by the addition of HO-1 siRNA. INS-1 cells were transfected with HO-1/control siRNA. After 24−48 h, transfected cells were then incubated with or without anthocyanins for another 24 h, followed by 1 mM H_2O_2 for another 2 h. Cell viability was determined ($n = 5$). (∗) $p < 0.05$ versus anthocyanin-pretreated group. (D) INS-1 cells were treated as described in panel C, and immunoblottings for caspase 3 and caspase 9 were carried out. (E) After retrovirus infection, the HO-1 expression level was determined by RT-PCR and quantitative RT-PCR in INS-1 cells carrying different plasmids. (F) INS-1 cells carrying different vectors were treated with 1 mM H_2O_2 for 2 h ($n = 4$). (∗) $p < 0.05$.

bilberry and C3G could upregulate HO-1 expression in ARPE-19 and HIAE-101 cells, respectively (*30*, *31*). In the present study, HPLC analysis confirmed that CBE as-prepared here was very rich in anthocyanins, and C3G accounting for the greatest part. We therefore hypothesized that anthocyanins in CBE might modulate HO-1 expression in β cells and islets. In the following experiments, knockdown HO-1 expression decreased the protective ability of anthocyanins compared to anthocyanins treated alone, suggesting that such a protective effect, at least partially, attributed to the HO-1 gene upregulation.

Our further exploration revealed that the PI3K/Akt and ERK1/2 signaling pathways might mediate anthocyanin-induced HO-1 regulation, because LY294002 and PD98059 partially blocked such upregulation and also decreased its cytoprotective effects. Generally, there are two major transcription regulators of HO-1: nuclear factor erythroid 2-related factor-2 (Nrf2) and Bach1. Nrf2 contains a transcription activation domain and positively regulates HO-1 transcription after dissociating from its docking protein Keap1 (*32*, *33*), whereas Bach1 competes with Nrf2 and represses transcription (*34*). In our study, we also observed that anthocyanins in CBE induced nuclear translocation of Nrf2 (unpublished data), and it is reported that ERK and PI3K could phosphorylate Nrf2, which may facilitate the release of Nrf2 from the Keap1−Nrf2 complex, allowing for activated Nrf2 to translocate into the nucleus and form a heterodimer with the small Maf protein (*35*, *36*).

In our transplantation model, we only focused on the protective effect of anthocyanins at an early stage (72 h), and its long-term effect is not determined here. In fact, it is published that HO-1 upregulation could ameliorate islet rejection, facilitate donor immune tolerance, and alleviate chronic allograft changes (*24*, *37*). It would thus be reasonable to speculate that anthocyanin administration might also possess a positive effect on β-cell grafts during a longer time. Also, whether anthocyanins have any effect on other detoxifying enzymes or antioxidant proteins in β cells should be further investigated.

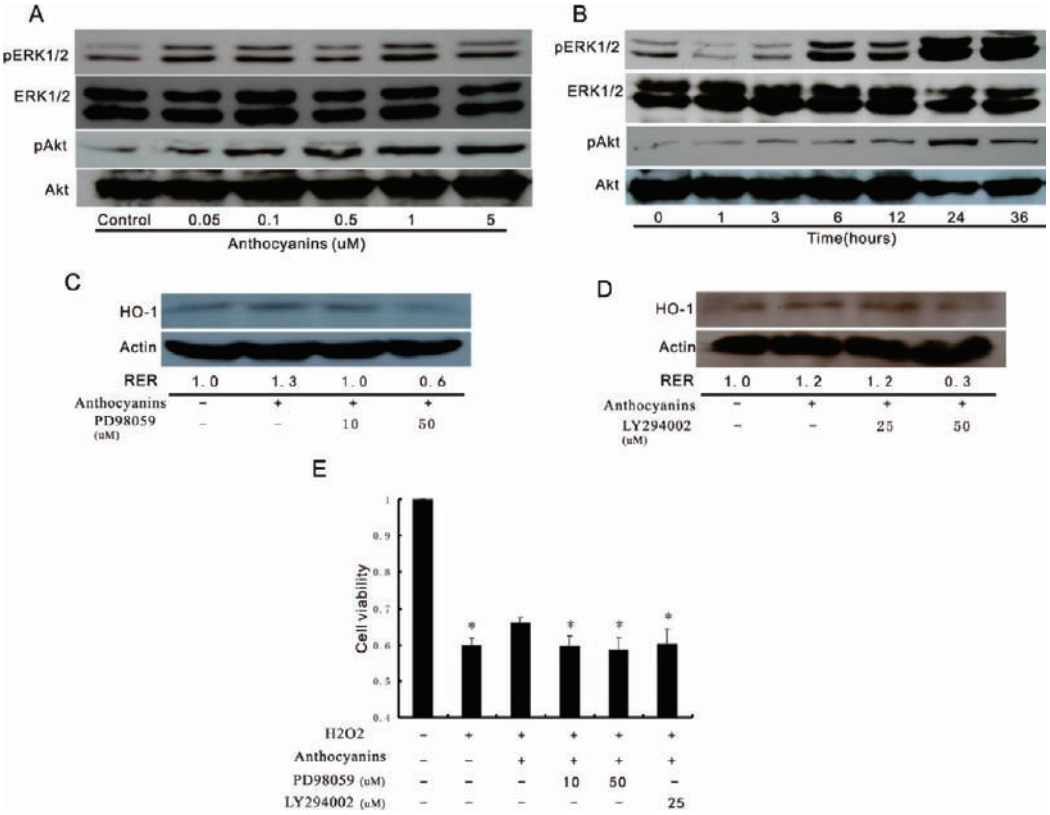

Figure 7. Anthocyanin-induced HO-1 expression via phosphorylation of ERK1/2 and Akt in INS-1 cells. (A and B) Levels of phosphorylated as well as total ERK1/2 and Akt were measured by western blot. (C and D) INS-1 cells were pretreated with a pharmacological inhibitor of ERK1/2 (PD98059, 10 and 50 μM) or inhibitor of PI3K (LY294002, 25 and 50 μM) for 1 h, further co-incubated with anthocyanins for an additional 24 h. The expression ratio value (HO-1/actin, with the control as the basal level) was calculated. (E) Effect of PD98059 and LY294002 on anthocyanin-mediated cytoprotection against H_2O_2 toxicity. Cells were pre-incubated with 1 μM anthocyanins and PD98059/LY294002 for 24 h and then exposed to 1 mM H_2O_2 for another 2 h ($n = 3$). (*) $p < 0.05$ versus anthocyanin-treated group.

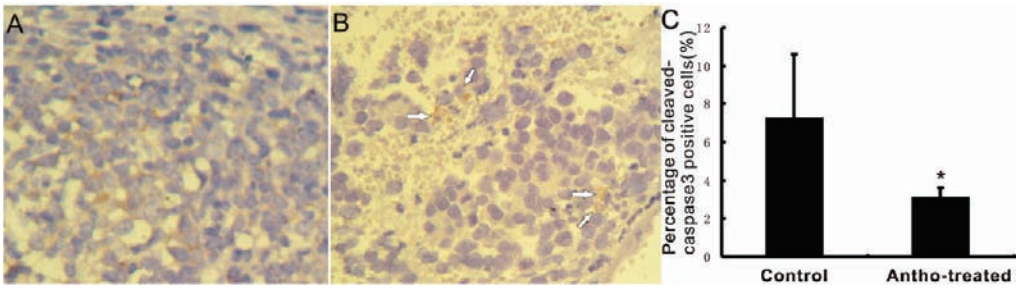

Figure 8. Immunohistochemical staining for cleaved caspase 3 in β-cell grafts in the (A) control and (B) anthocyanin-pretreatment groups. (C) Percentages of cleaved caspase 3 positive cells in both groups were calculated and shown as the mean $\pm$ SD ($n = 4$). (*) $p = 0.048$ versus the control group.

In summary, we demonstrated here that anthocyanins in CBE protect β cells against ROS-induced cell injury (both necrosis and apoptosis) *in vitro*. Such a protective effect was partially attributed to the HO-1 gene upregulation, which was modulated via ERK1/2 and PI3K/Akt pathways. We also observed that INS-1 cells pretreated with anthocyanins displayed decreased apoptotic activity during the early phase of transplantation. Taken together, these findings highlight the potential utility of anthocyanins in islet protection and transplantation fields.

ABBREVIATIONS USED

C3G, cyanidin-3-*O*-glucoside; LDH, lactate dehydrogenase; ROS, reactive oxygen species; HO-1, heme oxygenase-1; CBE, Chinese bayberry extract; siRNA, small interference RNA; FCM, flow cytometry; NAC, *N*-acetyl-cysteine.

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