Protective effect of cyanidin-3-O-glucoside on neonatal porcine islets

Chao Li¹, Bin Yang¹, Zhihao Xu², Eric Boivin², Mazzen Black², Wenlong Huang², Baoyou Xu², Ping Wu², Bo Zhang¹, Xian Li³, Kunsong Chen³, Yulian Wu¹ and Gina R Rayat²

¹Department of Surgery, The Second Affiliated Hospital of Zhejiang University, Hangzhou, Zhejiang, China

²Department of Surgery, Ray Rajotte Surgical-Medical Research Institute, Alberta Diabetes Institute, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada

³Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, Zhejiang, China

Correspondence should be addressed to Y Wu or G R Rayat
Email
yulianwu@zju.edu or grayat@ualberta.ca

Abstract

Oxidative stress is a major cause of islet injury and dysfunction during isolation and transplantation procedures. Cyanidin-3-O-glucoside (C3G), which is present in various fruits and vegetables especially in Chinese bayberry, shows a potent antioxidant property. In this study, we determined whether C3G could protect neonatal porcine islets (NPI) from reactive oxygen species (H₂O₂)-induced injury *in vitro* and promote the function of NPI in diabetic mice. We found that C3G had no deleterious effect on NPI and that C3G protected NPI from damage induced by H₂O₂. Significantly higher hemeoxygenase-1 (HO1) gene expression was detected in C3G-treated NPI compared to untreated islets before and after transplantation ($P < 0.05$). Western blot analysis showed a significant increase in the levels of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K/Akt) proteins in C3G-treated NPI compared to untreated islets. C3G induced the nuclear translocation of nuclear erythroid 2-related factor 2 (NRF2) and the significant elevation of HO1 protein. Recipients of C3G-treated NPI with or without C3G-supplemented drinking water achieved normoglycemia earlier compared to recipients of untreated islets. Mice that received C3G-treated islets with or without C3G-supplemented water displayed significantly lower blood glucose levels at 5–10 weeks post-transplantation compared to mice that received untreated islets. Mice that received C3G-treated NPI and C3G-supplemented drinking water had significantly ($P < 0.05$) lower blood glucose levels at 7 and 8 weeks post-transplantation compared to mice that received C3G-treated islets. These findings suggest that C3G has a beneficial effect on NPI through the activation of ERK1/2- and PI3K/AKT-induced NRF2-mediated HO1 signaling pathway.

Key Words

- ▶ type 1 diabetes mellitus
- ▶ neonatal porcine islets
- ▶ cyanidin-3-O-glucoside
- ▶ hemeoxygenase-1

Journal of Endocrinology
(2017) **235**, 237–249

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that usually occurs in childhood, adolescence or young adulthood and accounts for approximately 10–15% of all diagnosed cases of diabetes (Pociot & Lernmark 2016).

In individuals with T1DM, the β cells, which produce insulin in islets of the pancreas, are destroyed by the immune cells. If not properly treated, T1DM may result in serious secondary complications including cardiovascular

disease, retinopathy, neuropathy and nephropathy, and some may be life threatening even when blood glucose levels are well controlled (Yu *et al.* 2015). Pancreatic islet transplantation offers a promising therapeutic option for T1DM because this therapy may prevent the long-term complications associated with T1DM. During the past two decades, significant improvement has been achieved in clinical islet transplantation, especially after the development of the Edmonton protocol (Shapiro *et al.* 2006, Pepper *et al.* 2013). However, significant obstacles to the widespread implementation of islet transplantation remain unsolved. One of these obstacles is the progressive loss and dysfunction of islets during isolation and transplantation procedures, which consequently requires a large amount of donor islets (Biarnes *et al.* 2002, Kin 2010).

Oxidative stress has been shown to play an important role in the process of islet cell loss and dysfunction during isolation and transplantation procedures (Hennige *et al.* 2000, Bottino *et al.* 2004). Oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses (Schafer & Buettner 2001, Fleury *et al.* 2002). Exposure to high levels of reactive oxygen species (ROS), which are not detoxified by cellular antioxidants, could result in severe oxidative stress and could cause cell death (Lemasters *et al.* 1998, Scherz-Shouval & Elazar 2007). Moderate oxidation could trigger apoptosis, while more intense stresses may cause necrosis (Lennon *et al.* 1991). It is well documented that oxidative stress contributes significantly to cell injury during islet isolation and transplantation procedures (Kajimoto & Kaneto 2004). During these procedures, both immunological and non-immunological events, such as inflammation, mechanical injury, hypoxic microenvironment and ischemia, could produce high levels of oxidative stress, which result in a significant high proportion of islet cell death and/or dysfunction (Evgenov *et al.* 2006, Emamaullee & Shapiro 2007). Since islets are highly susceptible to damage induced by oxidative stress because of their inherently low antioxidant enzyme content (Tiedge *et al.* 1997, Robertson 2006), developing strategies designed to protect islets against oxidative stress is of great importance to the progress of clinical islet transplantation. Therefore, utilization of antioxidants to protect islets from oxidative stress has been one area of intense research in the field of islet transplantation (Mohseni Salehi Monfared *et al.* 2009). For example, vitamins D3 and E, which are known to have antioxidant properties, were used to improve the morphology and function of neonatal porcine islets (NPI) (Luca *et al.* 2000).

Chinese bayberry, one of the six *Myrica* species native to China, is a fruit with high nutrition and health values. This fruit is rich in anthocyanins, and cyanidin-3-O-glucoside (C3G) is identified as the most abundant component of these anthocyanins (Bao *et al.* 2005, Zhang *et al.* 2008). Our previous studies have demonstrated that C3G possesses high antioxidant capacities (Zhang *et al.* 2011, 2013, Sun *et al.* 2012, Cai *et al.* 2015). *In vitro* treatment of pancreatic β cells (INS-1) with C3G protected these cells from hydrogen peroxide (H_2O_2)-induced oxidative injury (Zhang *et al.* 2011). In addition, treatment of these cells with C3G attenuated the oxidative stress-mediated autophagy by activating the transcription factor NRF2 and the subsequent NRF2/HO1 signaling pathway (Zhang *et al.* 2013). *In vivo* study also demonstrated that C3G protected β cells against oxidative stress-mediated injury in streptozotocin-induced diabetic mice (Sun *et al.* 2012). Recently, we showed that C3G enhanced the viability of mouse islets and improved their function after transplantation under the kidney capsule or into the portal vein of syngeneic mouse recipients (Cai *et al.* 2015).

Since the availability of human organs is limited and the demand for islet transplantation remains high, the search for new sources of islets for transplantation is ongoing. By consensus, pigs are identified to be an ideal alternative organ source because they mature rapidly, have organs relatively similar in size and physiologic capacity as those found in humans and their insulin is biologically active in humans (Dufrane & Gianello 2012, Samy *et al.* 2014). NPI, in particular, continue to be a robust option for islet xenotransplantation due to their potential to grow after transplantation and easy to isolate as well as maintain in culture. However, due to the immature nature of NPI, an increased time to return islet recipients to normoglycemia is observed. In this study, we evaluated the potential beneficial effect of C3G on NPI both *in vitro* and in a pig to mouse xenotransplantation model.

Materials and methods

Preparation of C3G extract, high-performance liquid chromatography analysis and evaluation of antioxidant capacity

Chinese bayberries were obtained from Xianju County of Zhejiang Province in China. The C3G extract was prepared as previously described (Zhang *et al.* 2008).

Animals

Three-day-old Duroc/Landrace Large White F1 cross-neonatal pigs (1.5–2.5 kg; University of Alberta Swine Research Centre, Edmonton, Alberta, Canada) of either sex were used as islet donors. Donor pigs were housed under 'High Health' (free of diseases of significant economic impact) condition following the guidelines set by the Canadian Council on Animal Care. Six- to 8-week-old immune-deficient C57BL/6-*rag1*^{tm1/mom} (B6 *rag1*^{-/-}, H-2^b, Jackson Laboratory) male mice were used as recipients of islet transplants. Recipient mice were rendered diabetic by a single intraperitoneal injection of 180 mg/kg streptozotocin (Sigma-Aldrich Canada Co.) 5–7 days before transplantation. Blood samples were collected from the tail vein of mice to measure the glucose levels using a Life Scan One Touch Ultra Mini Meter (Life Scan, Burnaby, British Columbia, Canada). Mice that had two consecutive non-fasting blood glucose levels ≥ 18 mM were considered as diabetic and were used as recipients of an islet transplant. Blood glucose levels of these mice were measured two days before and once a week after receiving an islet transplant. All mice were housed and fed under pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care.

Islet isolation

NPI were isolated as previously described (Korbutt *et al.* 1996). Briefly, neonatal pigs were anesthetized with isoflurane and subjected to laparotomy and exsanguination. The pancreases were surgically removed, placed in Hank's balanced salt solution (Sigma-Aldrich Canada Co.), cut into small pieces and digested with 1.0 mg/mL collagenase (Type XI, Sigma-Aldrich Canada Co.). After passing the digest through a 500- μ m nylon screen, the islets were cultured for 7 days at 37°C (5% CO₂, 95% air) in Ham's F10 medium (Sigma-Aldrich Canada Co.), which contained 10 mM glucose, 50 μ M isobutylmethylxanthine (ICN Biomedicals, Montreal, Quebec, Canada), 0.5% bovine serum albumin (BSA) (fraction V, radioimmunoassay grade; Sigma-Aldrich Canada Co.), 2 mM L-glutamine, 3 mM CaCl₂, 10 mM nicotinamide (BDH Inc., Toronto, Ontario, Canada), 100 units/mL penicillin and 100 μ g/mL streptomycin (Lonza, Mississauga, Ontario, Canada). After 7 days of culture, islets from each pig were collected and three aliquots were obtained and then counted using a dissecting light microscope (Wild Leitz Canada, Toronto, Ontario, Canada) with grid lines on the counting glass dish in which

each square represents 50 μ m. The number of islets per sample was calculated in IEQ following published protocol in which one IEQ represents 150 μ m (Rayat *et al.* 2016).

Cytotoxicity assay

The toxicity of C3G on NPI was evaluated using the Trypan blue (Sigma-Aldrich Canada Co.) exclusion dye method and LIVE/DEAD Viability/Cytotoxicity Assay kit (Life Technologies) by the flow cytometry analysis following the manufacturer's instructions. One thousand IEQs were cultured in Ham's F10 medium with 0.1, 0.5, 1.0 and 5.0 μ M concentrations of C3G or without C3G for 24 h at 37°C (5% CO₂, 95% air). After culture, islets were collected and dissociated into single cells by gentle agitation for 5 min in a calcium-free medium containing 10 mL of 0.125% Trypsin/EDTA at 37°C water bath. The cell suspension was filtered through a 70- μ m filter to eliminate cell debris and then the cell suspension that was passed through the filter was centrifuged at 225 g for 5 min. The cell pellet was suspended with 5–10 mL of Ham's F10 medium and cells were stained with Trypan blue or calcein AM and ethidium bromide. The percentage of live cells was determined by counting the live and dead cells using hemocytometer for Trypan blue or by flow cytometry for calcein AM and ethidium bromide.

Total RNA extraction and RT-qPCR analysis

The total RNA was extracted from C3G-treated or untreated NPI and xenografts using Trizol reagent (Invitrogen) to evaluate the expression of *HO1*, *BCL2* and *SURVIVIN* transcripts. One microgram of total RNA was used to construct cDNA using Superscript RNase H-Reverse Transcriptase (Invitrogen). PCR amplifications were performed on a Light Cycle Real-time PCR thermocycler (Roche Diagnostics) using TaqMan gene expression assays. All gene expressions were normalized to the internal control 18s RNA.

Western blot analysis

Ten thousand IEQs were treated with various concentrations (0.1, 0.5, 1.0 and 5.0 μ M) of C3G for 0, 1, 6, 12 and 24 h in which zero represents the time the islets were collected. Untreated islets served as control. After treatment, total islet proteins were extracted using RIPA lysis buffer, and nuclear and cytoplasmic proteins were extracted using NE-PER nuclear and cytoplasmic

extraction reagents (Thermo Scientific) according to the manufacturer's protocols. Western blot was performed as previously described with little modifications (Li *et al.* 2015). Briefly, 20 µg proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), and subsequently transferred to polyvinylidene fluoride membranes (Millipore). Following a 1-h blocking in phosphate buffer solution containing 1% Tween-20 (Sigma-Aldrich Canada Co.) and 2% BSA, the blots were incubated overnight at 4°C with the primary antibodies. The following primary antibodies and their respective dilutions were used: p-ERK1/2 (1:2000), ERK1/2 (1:1000), p-AKT (1:1000), AKT (1:1000) and β-actin (1:1000), which were all purchased from Cell Signaling Technology, HO1 (1:1000, Millipore), NRF2 (1:1000, Abcam) and Lamin B1 (1:5000, Abcam). The membranes were further incubated for 1 h with IRDye goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody (1:5000, LI-COR, Lincoln, NE, USA). Finally, the immunoreactive bands were visualized using the Odyssey CLx infrared imaging system (LI-COR). The protein concentration was normalized to the internal control total ERK, total AKT, Lamin B1 or β-actin and the values were expressed as normalized data relative to the control.

Exposure of islets to H₂O₂ and hypoxia

To determine the effect of C3G on islet injury induced by ROS, 1000 IEQs were cultured with or without 1.0 µM C3G in Ham's F10 medium for 24 h at 37°C (5% CO₂, 95% air). Then, the medium was changed to fresh medium with or without 1.0 mM H₂O₂ for another 2 h. After the incubation, islets were collected and their gross morphology was determined using dithizone (0.5 mg/mL, Sigma-Aldrich Canada Co.). In addition, the number of islets per NPI sample was counted and results from six samples were added and then divided by 6 to obtain the mean total number of islets for each condition. The numbers of non-disaggregated and disaggregated islets from six NPI samples were also counted and then added. The results were divided by the number of NPI samples to obtain the mean number of non-disaggregated and disaggregated islets under each condition. The mean percentage of non-disaggregated islets was then calculated by dividing the mean number of non-disaggregated islets by the mean total number of islets (disaggregated plus non-disaggregated) counted under each condition and multiplying the result by 100. Similarly, the mean percentage of disaggregated islets was calculated by

dividing the mean number of disaggregated islets by the mean total number of islets counted under each condition and multiplying the result by 100. Non-disaggregated islets are islets that maintained their spherical structure, which remained aggregated and appeared to be surrounded with a membrane. Disaggregated islets are clusters of cells that no longer have a spherical structure surrounded with a membrane.

To determine the effect of C3G on islets exposed to hypoxia, 1000 IEQs were cultured in Hams' F10 medium with or without 1.0 µM C3G at 37°C, 5% CO₂, with 20% (HERACELL 150i CO₂ incubator, Thermo Scientific), 3% or 1% (Xvivo System X3, BioSpherix, New York, USA) O₂ for 24 h. All incubators used in these experiments had CO₂ controlled to 5%. The hypoxia incubators had O₂ controlled to 1% or 3% and the hypoxia O₂ sensors were calibrated the day before each experiment. The normoxia incubator does not control O₂, so we infer that it was about 20% given that air is ~21% O₂ and 5% of the air is displaced by CO₂. The 1% and 20% groups were humidified to 95%, while the 3% group had less (at least 30%) due to the nature of the incubation chamber. Since the culture time was short, we expected that any humidity-related effects on the experiment, such as evaporative losses, would be marginal. After incubation, the islets were collected and assayed for islet viability using Annexin V-Cy3 Apoptosis detection kit (Sigma-Aldrich Canada Co.) following the manufacturer's protocol. This kit includes annexin V conjugated to Cy3.18 as the fluorochrome and the non-fluorescent compound 6-carboxyfluorescein diacetate (6-CFDA) that enters the cell is hydrolyzed by the esterases in live cells to the fluorescent compound 6-carboxyfluorescein. As such, the kit allows the differentiation among early apoptotic cells (annexin V positive, 6-CFDA positive), necrotic cells (annexin V positive, 6-CFDA negative) and viable cells (annexin V negative, 6-CFDA positive) (Sigma-Aldrich Canada Co.). In addition, islet preparations were stained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen), which stains the nucleus of a cell blue, for further identification of the islet cells. Images of islets were obtained using the Zeiss fluorescent microscope (Carl Zeiss) and analyzed using ImageJ software. The number of live, necrotic or apoptotic cells per NPI sample (*n* = 4) under each condition was counted and the number of these cells in four NPI samples was added and then divided by 4 to obtain the mean number of live, necrotic or apoptotic cells under each condition. The mean percentage of live, necrotic or apoptotic cells was calculated by dividing

the mean number of live, necrotic or apoptotic cells by the mean total number of cells (live plus necrotic plus apoptotic) counted under each condition and multiplying the result by 100.

Islet transplantation

After 7 days of culture, the islets were incubated in Ham's F10 medium (untreated control) or with 1.0 μ M C3G for 24 h at 37°C (5% CO₂, 95% air), and then aliquoted for transplantation. Two thousand IEQs of NPI were transplanted under the left kidney capsule of diabetic mice as previously described (Korbutt *et al.* 1996). After transplantation, some mice were provided with C3G (1.0 μ M) in their drinking water for 6–8 h daily throughout the 15 week follow-up period. After 6–8 h, drinking water was replaced with normal water and on average, each mouse received 2–4 mL of C3G-supplemented drinking water every day. Blood glucose value of less than 10 mM was considered as a successful islet engraftment.

Two weeks after the mice achieved stable normal blood glucose levels (≤ 8.4 mmol/L for two consecutive weeks), an intraperitoneal glucose tolerance test (IPGTT) was performed on each mouse. After a 2-h fasting, mice were injected intraperitoneally with 50% glucose solution (3 mg/kg), and blood glucose levels were measured at 0, 15, 30, 60 and 120 min post-glucose injection. At the end of the study (>15 weeks post-transplantation), all recipients underwent a survival nephrectomy of the graft-bearing kidney to confirm that the islet transplant was responsible for their normoglycemic state.

Immunohistochemistry analysis

To determine the presence of insulin-producing β cells and glucagon-producing α -cells, islet xenografts were collected and fixed with zinc fixative overnight and then washed thrice with 70% ethanol and embedded in paraffin. Sections of 5- μ m thickness were stained with guinea pig anti-porcine insulin (1:1000, DAKO) and mouse anti-porcine glucagon (1:5000, Sigma-Aldrich Canada Co.) primary antibodies. Biotinylated goat anti-guinea pig IgG (1:200, Vector Laboratories) and biotinylated goat anti-mouse IgG (1:200, Jackson ImmunoResearch Laboratories) were used as secondary antibodies, respectively. The avidin–biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (DAB, BioGenex, Fremont, CA, USA) were used to produce a

brown color. All sections were counter-stained with Harris' hematoxylin (Electron Microscopy Sciences, Hatfield, PA, USA) and eosin (Sigma-Aldrich Canada Co.).

Statistical analysis

All experiments were performed at least in triplicate and the data were presented as means $\pm$ standard error of the mean (S.E.M.). Statistical analysis was performed with the SPSS 19.0 and Stata/IC 13.0 softwares (Stata Corp LLC, College Station, TX, USA). Differences were considered statistically significant when *P* value was 0.05 or less using Student's *t*-test analysis and non-parametric analysis of variance.

Results

C3G was not toxic to NPI

To determine the cytotoxicity of C3G on islets, NPI were treated with various concentrations of C3G and were subjected to Trypan blue exclusion dye staining analysis and flow cytometry analysis. The Trypan blue staining analysis showed that islets treated with C3G had slightly higher viability compared to the untreated islets, but no statistical significance was found except in islets treated with 1.0 μ M C3G, which also showed the highest cell viability (Fig. 1A). Flow cytometry analysis showed the same trend as with Trypan blue staining; however, there was no statistically significant difference found between the groups compared (Fig. 1B).

Figure 1

Cell viability of islets treated with or without various concentrations of C3G. The islets were incubated in Ham's F10 medium with or without various concentrations of C3G at physiological conditions (37°C, 5% CO₂, and 95% air) for 24 h, and cell viability was assessed by Trypan blue exclusion dye staining (A) and flow cytometry (B). Results are presented as means $\pm$ S.E.M. from four independent experiments. **P* < 0.05 vs the untreated islets.

Figure 2

Representative images of NPI stained with dithizone. Aliquots of islets were left untreated or treated with C3G with or without additional exposure to H_2O_2 . (A) Untreated islets, (B) islets treated with $1.0 \mu M$ C3G for 24 h, (C) islets treated with $1.0 mM$ H_2O_2 for 2 h and (D) islets pre-treated with $1.0 \mu M$ C3G for 24 h before treatment with $1.0 mM$ H_2O_2 for 2 h. Scale bar represents $100 \mu m$. White asterisk represents islets measuring $\leq 50 \mu m$. Six different NPI preparations were examined.

C3G protected NPI from H_2O_2 -induced injury

Dithizone staining of islets showed that ROS in the form of H_2O_2 is harmful to NPI (Fig. 2 and Table 1). There were numerous single cells observed in islets treated with H_2O_2 and the remaining islets (non-disaggregated and aggregated) showed less intense staining with dithizone (Fig. 2C and D) compared to untreated and C3G-treated islets (Fig. 2A and B, respectively). However, islets that were treated with H_2O_2 with C3G (Fig. 2D) appeared to have slightly intense staining with dithizone compared to islets treated with H_2O_2 without C3G (Fig. 2C). There were also fewer small islets ($<50 \mu m$) observed in H_2O_2 -treated islets (Fig. 2C and D) compared to untreated and C3G-treated islets (Fig. 2A and B, respectively). The amounts of untreated (126 ± 21) and C3G-treated (135 ± 22) islets

were significantly ($P \leq 0.01$) higher than those observed in H_2O_2 -treated islets with (60 ± 8) or without C3G (49 ± 7 , $n=6$, Table 1). The proportions of disaggregated islets in untreated ($3.5\% \pm 0.9\%$) and C3G-treated ($1.5\% \pm 0.5\%$) islets were significantly ($P \leq 0.01$) lower compared to those observed in H_2O_2 -treated islets with or without C3G ($29.3\% \pm 5.4\%$ and $45.7\% \pm 10.7\%$, respectively, Table 1). The proportions of non-aggregated islets in untreated ($96.5\% \pm 0.9\%$) and C3G-treated ($98.5\% \pm 0.5\%$) islets were significantly ($P \leq 0.01$) higher compared to those observed in H_2O_2 -treated islets with ($70.7\% \pm 5.4\%$) or without ($54.3\% \pm 10.7\%$) C3G. Although there were less disaggregated islets and more non-disaggregated islets observed in H_2O_2 -treated islets with C3G compared to islets treated with H_2O_2 without C3G,

Table 1 Effect of H_2O_2 on the NPI with or without C3G.

Conditions	Mean total number of islets counted ($\pm$ S.E.M.)	Mean percentage of disaggregated islets ($\pm$ S.E.M.)	Mean percentage of non-disaggregated islets ($\pm$ S.E.M.)
Untreated	126 ± 21	3.5 ± 0.9	96.5 ± 0.9
C3G	135 ± 22	1.5 ± 0.5	98.5 ± 0.5
H_2O_2	$49 \pm 7^{*,§}$	$45.7 \pm 10.7^{*,§}$	$54.3 \pm 10.7^{*,§}$
$H_2O_2 + C3G$	$60 \pm 8^{**,§§}$	$29.3 \pm 5.4^{**,¶}$	$70.7 \pm 5.4^{**,¶}$

Data shown in the table are averages of six NPI samples obtained from six isolations. NPI samples were stained with dithizone and the total number of untreated NPI counted from six NPI samples was 753, NPI treated with C3G was 811, NPI treated with H_2O_2 was 291 and NPI treated with H_2O_2 and C3G was 362. The total number of islets was then divided by 6 to obtain the mean total number of islets counted shown in the table. The mean percentage of disaggregated islets under each condition was calculated by dividing the mean number of disaggregated islets by the mean total number of islets counted (i.e., disaggregated plus non-disaggregated) under each condition and multiplying the result by 100. Similarly, the mean percentage of non-disaggregated islets was calculated by dividing the mean number of non-disaggregated islets with the mean total number of islets counted and multiplying the result by 100. No significant differences were found between untreated and C3G-treated NPI under all conditions.

* $P < 0.01$ H_2O_2 vs untreated; ** $P < 0.01$ $H_2O_2 + C3G$ vs untreated; § $P < 0.01$ H_2O_2 vs C3G; §§ $P < 0.01$ $H_2O_2 + C3G$ vs C3G; ¶ $P < 0.01$ $H_2O_2 + C3G$ vs C3G.

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Table 2 Effect of H₂O₂ on the viability of NPI with or without C3G.

Conditions	Mean percentage of live NPI cells ($\pm$ S.E.M.)	Mean percentage of necrotic NPI cells ($\pm$ S.E.M.)	Mean percentage of apoptotic NPI cells ($\pm$ S.E.M.)
Untreated	91.8 $\pm$ 0.4	5.5 $\pm$ 0.6	2.7 $\pm$ 0.2
C3G	92.9 $\pm$ 0.7	4.4 $\pm$ 0.5	2.7 $\pm$ 0.2
H ₂ O ₂	35.2 $\pm$ 2.5*,§	54.5 $\pm$ 2.9*,§	10.3 $\pm$ 2.1*,§
H ₂ O ₂ +C3G	44.5 $\pm$ 2.9**,§§,¶	45.4 $\pm$ 4.1**,§§,¶	10.1 $\pm$ 2.4**,§§

The viability of NPI cells was determined using Annexin V-Cy3 Apoptosis detection kit and images of islets were analyzed using ImageJ software. Data shown in the table are averages of results obtained from four NPI samples in which five islets per NPI sample were analyzed per condition. The number of live, necrotic or apoptotic cells per NPI sample under each condition was counted and the numbers of these cells in four NPI samples were added and then divided by 4 to obtain the mean number of live, necrotic or apoptotic cells under each condition. The mean percentage of live, necrotic or apoptotic cells was calculated by dividing the mean number of live, necrotic or apoptotic cells by the mean total number of cells (live plus necrotic plus apoptotic) counted under each condition and multiplying the result by 100. There were no significant differences found between untreated and C3G-treated NPI under all conditions.

* $P < 0.05$ H₂O₂ vs untreated; ** $P < 0.05$ H₂O₂+C3G vs untreated; § $P < 0.05$ H₂O₂ vs C3G; §§ $P < 0.05$ H₂O₂+C3G vs C3G; ¶ $P < 0.05$ H₂O₂ vs H₂O₂+C3G.

the differences in these two groups were not found to be statistically significant (Table 1).

The cell viability assay showed that there were no significant differences in the proportions of live (91.8% $\pm$ 0.4% vs 92.9% $\pm$ 0.7%), necrotic (5.5% $\pm$ 0.6% vs 4.4% $\pm$ 0.5%) and apoptotic (2.7% $\pm$ 0.2% vs 2.7% $\pm$ 0.2%) cells between untreated and C3G-treated islets, respectively, confirming our finding that C3G was not toxic to NPI ($n=4$, Table 2). However, we found that the percentage of live cells in islets treated with H₂O₂ (35.2% $\pm$ 2.5%) was significantly ($P < 0.05$) lower than the percentages of live cells in untreated islets (91.8% $\pm$ 0.4%) and those islets that were treated with C3G (92.9% $\pm$ 0.7%). Similarly, the proportion of live cells in H₂O₂-treated islets with C3G (44.5% $\pm$ 2.9%) was also significantly ($P < 0.05$) lower than what was observed in untreated and C3G-treated islets (Table 2). We also found that the percentages of necrotic (54.5% $\pm$ 2.9%) and apoptotic (10.3% $\pm$ 2.1%) cells in H₂O₂-treated islets were significantly ($P < 0.05$) higher than the percentages of necrotic and apoptotic cells observed in untreated (5.5% $\pm$ 0.6% and 2.7% $\pm$ 0.2%, respectively) and C3G-treated islets (4.4% $\pm$ 0.5% and 2.7% $\pm$ 0.2%, respectively). The amounts of necrotic (45.4% $\pm$ 4.1%) and apoptotic (10.1% $\pm$ 2.4%) cells in H₂O₂-treated islets with C3G were also significantly ($P < 0.05$) higher than those found in untreated and C3G-treated islets (Table 2). We also found that the proportion of live cells in islets treated with H₂O₂ and C3G was significantly higher ($P < 0.05$) compared to the proportion of live cells in islets treated with H₂O₂ without C3G (44.5% $\pm$ 2.9% vs 35.2% $\pm$ 2.5%, respectively). The percentage of necrotic cells in islets treated with H₂O₂ and C3G (45.4% $\pm$ 4.1%) was significantly ($P < 0.05$) lower than the percentage of necrotic cells observed in islets treated with H₂O₂ without C3G (54.5% $\pm$ 2.9%). There was no significant difference

observed in the amount of apoptotic cells in islets treated with H₂O₂ with (10.1% $\pm$ 2.4%) and without C3G (10.3% $\pm$ 2.1%, Table 2).

C3G has no deleterious effect on the natural resistance of NPI to hypoxia-induced apoptosis

The effect of C3G on NPI in hypoxic environments was examined and NPI showed natural resistance to hypoxia as was previously reported (Emamaullee *et al.* 2006). Cell viability assay showed that the proportions of live cells in untreated and C3G-treated islets exposed to normoxia, and 3% and 1% hypoxia were not significantly different (Table 3). There was a slight decrease in the percentage of live cells when NPI were exposed to 1% hypoxic condition compared to islets cultured in normoxia, but the difference was not statistically significant. There were also no significant differences observed in the proportions of necrotic and apoptotic cells in untreated and C3G-treated islets incubated under normal, and 3% and 1% oxygen conditions (Table 3).

C3G upregulated the expression of *HO1* but not *BCL2* and *SURVIVIN* genes

To investigate the possible mechanism of protection of C3G on NPI, we evaluated the gene expression of antioxidant enzyme *HO1* and anti-apoptotic molecules *BCL2* and *SURVIVIN* by RT-qPCR. Treatment of NPI with C3G for 24 h at physiological conditions significantly enhanced the *HO1* gene expression at low concentrations and this effect appears to reach maximum at 1.0 μ M concentration (Fig. 3A). There were no significant differences found in the gene expression of *BCL2* and *SURVIVIN* between untreated and C3G-treated islets (Fig. 3B and C).

Research	C3G and others	C3G protects neonatal porcine islets	235:3	244
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Table 3 Effect of hypoxia on NPI with or without C3G.

Conditions	Mean percentage of live NPI cells ($\pm$ S.E.M.)	Mean percentage of necrotic NPI cells ($\pm$ S.E.M.)	Mean percentage of apoptotic NPI cells ($\pm$ S.E.M.)
Normoxia			
Untreated	92.6 $\pm$ 0.6	4.8 $\pm$ 2.6	2.6 $\pm$ 0.3
C3G	93.4 $\pm$ 1.1	4.3 $\pm$ 2.3	2.3 $\pm$ 0.2
3% Hypoxia			
Untreated	93.2 $\pm$ 1.2	4.1 $\pm$ 0.7	2.7 $\pm$ 0.6
C3G	92.6 $\pm$ 0.2	4.8 $\pm$ 0.1	2.6 $\pm$ 0.3
1% Hypoxia			
Untreated	90.7 $\pm$ 0.2	5.9 $\pm$ 3.4	3.4 $\pm$ 0.1
C3G	92.0 $\pm$ 0.2	4.9 $\pm$ 3.1	3.1 $\pm$ 0.1

Data shown in the table are averages of four NPI samples obtained from four isolations. The total number of NPI counted for each NPI sample under normoxic and hypoxic conditions with or without C3G was 20 (i.e., five islets per NPI sample). The viability of NPI cells was determined using Annexin V-Cy3™ Apoptosis detection kit and images of islets were analyzed using ImageJ software. The mean percentage of live, necrotic or apoptotic cells was calculated by dividing the mean number of live, necrotic or apoptotic cells by the mean total number of cells counted under each condition and multiplying the result by 100. No significant differences were observed between untreated and C3G-treated islets under all conditions compared using two-way analysis of variance.

C3G enhanced ERK1/2 and PI3K/Akt-induced NRF2-mediated HO1 induction

To further elucidate the underlying protective mechanisms of C3G on NPI, the protein expressions of molecules involved in major mechanisms of cellular defense against oxidative stress were determined. We initially examined the effect of C3G on ERK1/2 and PI3K/AKT kinases. As shown in Fig. 4A and D, C3G induced the phosphorylation of ERK1/2 and PI3K/AKT at a concentration of greater than or equal to 0.5 μ M, and the phosphorylation of ERK1/2 and PI3K/AKT reached maximum at 6–12 h (Fig. 4B and E). In addition, we investigated the effect of C3G on NRF2 nuclear translocation and the subsequent HO1 induction. After 24 h of treatment with various concentrations of C3G, the cytosolic and nuclear proteins were isolated for the Western blot analysis. As shown in Fig. 4C and F, C3G significantly elevated the level of nuclear NRF2 and the subsequent HO1 induction at concentrations of 0.5 and 1.0 μ M.

C3G enhanced the function of NPI transplanted under the kidney capsule of diabetic mice

To determine whether C3G could enhance the function of NPI xenografts, NPI were transplanted under the kidney capsule of diabetic mice. As shown in Fig. 5A, recipients of 2000 IEQs of C3G-treated NPI with or without C3G-supplemented drinking water showed significantly ($P<0.05$) lower blood glucose levels compared to recipients of untreated islets. Mice that received C3G-treated NPI with or without C3G-supplemented drinking water achieved normoglycemia at 8 and 10 weeks post-transplantation, respectively. Mice that received the same amount of untreated NPI achieved normoglycemia at 12 weeks post-transplantation. The blood glucose levels of mice that received C3G-treated islets were significantly ($P<0.05$) lower at 5, 9 and 10 weeks post-transplantation compared to the blood glucose levels of mice that received untreated islets. Similarly, the blood glucose levels of mice that received C3G-treated NPI and C3G-supplemented drinking water were significantly ($P<0.05$)

Figure 3

Gene expression of *HO1*, *BCL2* and *SURVIVIN* on islets treated with or without various concentrations of C3G before transplantation. Islets were incubated in Ham's F10 medium containing various concentrations of C3G for 24 h, and the mRNA levels of *HO1* (A), *BCL2* (B) and *SURVIVIN* (C) were assessed by RT-qPCR assays. Gene expressions were shown as means $\pm$ S.E.M. and relative to the untreated islets ($n=5$). * $P<0.05$ vs the untreated islets.

Figure 4

Effects of C3G on the protein levels of ERK1/2, PI3K/Akt and Nrf2 in NPI. Phosphorylation of ERK1/2 and PI3K/Akt in islets treated with various concentrations of C3G for 24 h (A). Phosphorylation of ERK1/2 and PI3K/Akt at indicated times in islets treated with 1.0 μM C3G for 24 h (B). Protein levels of Nrf2 and HO-1 in islets treated with various concentrations of C3G for 24 h (C). Bar graphs showing the band intensities relative to the levels detected in untreated islets by densitometric quantification (D, E and F). Data are expressed as means ± S.E.M. (n = 3). *P < 0.05 and **P < 0.001 vs the untreated islets.

lower at 5–10 weeks post-transplantation compared to the blood glucose levels of mice that received untreated islets. In addition, mice that received C3G-treated islets and C3G-supplemented drinking water displayed significantly ($P < 0.05$) lower blood glucose levels at 7 and 8 weeks post-transplantation compared to the mice that received C3G-treated islets. All recipients of NPI responded well to the glucose challenge (i.e., lower blood glucose levels at 30 min post-glucose challenge) and there were no significant differences found among the three recipient groups (Fig. 5B). As shown in Fig. 5A, the blood glucose

levels of all mice increased dramatically after the removal of the kidney bearing the islet graft, indicating that the islet transplant was responsible for maintaining the normal blood glucose levels.

Immunohistochemistry analysis of the NPI xenografts

At the end of the study (>15 weeks post-transplantation), the islet grafts and pancreas were harvested from all recipient mice. All mice that received NPI under the kidney capsule showed abundant insulin (Fig. 6A, B and C)

Figure 5

Average blood glucose levels of STZ-induced diabetic B6 *rag1*^{-/-} mice before and after islet transplantation. Untreated islets or islets treated with 1.0 μM C3G for 24 h were transplanted under the kidney capsule of diabetic mice (n = 4 per group), and their blood glucose levels were monitored once every week (A). Two weeks after all recipients achieved stable normoglycemia, IPGTT was conducted in the mice (n = 4) in each group (B). *P < 0.05: C3G oral vs untreated; §P < 0.05: C3G oral vs C3G culture; #P < 0.05: C3G culture vs untreated.

Figure 6

Immunohistochemical analysis of islet grafts under the kidney capsule of recipient mice. Brown structures and dots show insulin (A, B and C) or glucagon (D, E and F) positive stained cells (indicated with black arrows). Representative images of NPI xenografts under the kidney capsule are shown. (A) and (D) represent untreated islet grafts; (B) and (E) represent C3G-treated islet grafts and (C) and (F) represent C3G-treated islet grafts from mice provided with C3G-supplemented drinking water. Scale bar represents 200 μ m. Expressions of *HO-1*, *BCL-2* and *SURVIVIN* genes on NPI xenografts are shown in (G). Gene expressions are shown as means $\pm$ s.e.m. and relative to the untreated islets ($n=4$). * $P<0.05$ vs the untreated islet grafts.

and glucagon (Fig. 6D, E and F) positive stained cells. In addition, insulin positive cells were not found in the recipients' pancreas, further confirming that maintenance of normoglycemia in these recipients was due to the transplanted islets (data not shown).

C3G elevated the expression of *HO1* gene of NPI xenografts

The gene expressions of *HO1*, *BCL2* and *SURVIVIN* in the islet xenografts were also quantified by RT-qPCR assay. The *HO1* gene expression in grafts of mice that received C3G-treated NPI with or without C3G-supplemented drinking water was significantly higher compared to those detected in untreated islet grafts (Fig. 6G). There were no significant differences found in the gene expressions of *BCL2* and *SURVIVIN* among the three groups, similar to what we observed in the islets prior to transplantation.

Discussion

In the present study, we showed that C3G is able to protect NPI. We found that treatment of NPI with low C3G concentrations (0.1–1.0 μ M) for 24 h was non-toxic to NPI, which was consistent with our previous studies on mouse islets and pancreatic β cells (Zhang *et al.* 2011, Cai *et al.* 2015). However, 5.0 μ M C3G treatment did not significantly improve NPI cell viability, which was different from what we found in mouse islets (Cai *et al.* 2015). One cause for the difference, we suppose, is the

species diversity; another one may be due to the immature nature of NPI being more resistant to damage compared to mature mouse islet cells. Although NPI was previously demonstrated to have natural resistance to hypoxia-induced apoptosis (Emamaullee *et al.* 2006), which was confirmed in our present study, we found that NPI are susceptible to damage induced by ROS (H_2O_2) as another group (Yeom *et al.* 2012) has also demonstrated. We found that NPI treated with H_2O_2 displayed light staining with dithizone suggesting that H_2O_2 may trigger degranulation of the β cells. In addition, numerous single cells and significantly higher amounts of disaggregated and lower amounts of non-disaggregated islets were observed in H_2O_2 -treated islets compared to islets that were not treated with H_2O_2 . However, we also found that islets treated with H_2O_2 and C3G displayed slightly intense staining with dithizone compared to H_2O_2 -treated islets without C3G. Moreover, we found that significantly, more live cells and less necrotic cells were detected in NPI treated with H_2O_2 and C3G compared to H_2O_2 -treated islets without C3G suggesting that C3G may have a protective effect on H_2O_2 -induced NPI cell death.

We also found that mouse recipients of C3G-treated islets that were provided with additional C3G in their drinking water achieved normoglycemia earlier than those mice that received only C3G-treated islets, followed by mice that received untreated islets. In addition, mice that received C3G-treated NPI and C3G-supplemented drinking water had significantly lower blood glucose levels at seven and eight weeks post-transplantation compared to mice that received C3G-treated islets,

indicating that additional administration of C3G could enhance the *in vivo* function of NPI. Two weeks after all the recipients achieved stable normoglycemia, an IPGTT was performed to evaluate the ability of transplanted islets to respond to glucose challenge. We found no significant difference among the three groups compared in terms of the recipients' response to glucose challenge possibly due to the sufficient number of NPI that have engrafted and matured over the follow-up period. As shown in Fig. 6 mature insulin-producing cells were present in abundance in the islet xenografts of these mice. As such, these mature NPI are now able to reverse diabetes and maintain stable normoglycemia in transplant recipients (Korbutt *et al.* 1996). Since the number of animals in each group was small, it remains to be determined in future studies whether this trend will continue when larger numbers of animals are included.

HO1 is among the most critical components of cellular defense system against oxidative stress-induced injury, and it is thought to play a key role in maintaining antioxidant/oxidant imbalance (Maines 1997). Indeed, some studies showed that HO1 induction could promote graft survival when islets are transplanted under the kidney capsule (Bottino *et al.* 2004, McCall & Shapiro 2012). In addition, it has been shown that NPI transduced with adenovirus construct containing HO1 gene displayed significantly reduced apoptosis after treatment with H₂O₂ in a dose-dependent manner (Yeom *et al.* 2012). Fibroblasts obtained from transgenic pigs that expressed human HO1 protein also demonstrated significantly higher viability after treatment with H₂O₂ compared with fibroblasts obtained from wild type control pigs (Yeom *et al.* 2012). Our results show that NPI treated with C3G had a significantly higher expression of HO1 genes before and after transplantation. It is interesting to note that short-term treatment of NPI with C3G *in vitro* could result in higher HO1 gene expression in C3G-treated islet grafts compared to untreated islet grafts several months post-transplantation. It is known that during post-natal development, β cells adapt to higher insulin demands in response to increased body weight of a healthy individual and growing islets require oxygen and nutrients (Eberhard *et al.* 2010). As such, islets developed mechanisms to expand its microvasculature to provide oxygen and nutrients to the cells within the growing islets (Eberhard *et al.* 2010). It is possible that when NPI are isolated from the pancreas, the harmful effects of the isolation procedure could trigger

the NPI cells to adapt and develop mechanisms to help them survive under this harmful condition. Since NPI are still in the post-natal development stage, it is conceivable that their abilities to adapt to changing environment and to develop mechanisms important for their survival under harmful conditions are greater compared to adult islets. These abilities of NPI could be further enhanced in the presence of C3G during the 24-h period. As NPI remained outside the pancreas (i.e., post-transplantation under the kidney capsule), it is possible that NPI maintained the abilities they developed during the 24h period exposure to C3G for their survival outside the pancreas. As such, treatment of NPI with C3G for 24h could result in NPI maintaining high HO1 gene expression even several months after transplantation under the kidney capsule. However, it remains to be determined whether the high HO1 gene expression would equate to high HO1 protein expression in the NPI grafts.

BCL2 and SURVIVIN are well-known molecules that inhibit cell apoptosis. Studies have been reported that these two molecules can protect pancreatic β cells and islets from apoptosis-induced cell death (Tran *et al.* 2003, Dohi *et al.* 2006). Therefore, we also assessed the gene expression of BCL2 and SURVIVIN in NPI. We found no significant changes between the C3G-treated and untreated islets, which were consistent with what we found in mouse islets (Cai *et al.* 2015). It was also previously reported that NPI do not express detectable BCL2 protein and that an anti-apoptotic molecule that may be of significance in porcine islets, in particular NPI would be the X-linked inhibitor of apoptosis (Emamaullee *et al.* 2006).

NRF2, a basic region leucine-zipper transcription factor, has been demonstrated to be involved in the transcriptional regulation of HO1 gene (Alam & Cook 2003, Li & Kong 2009, Pi *et al.* 2010). NRF2 contains a transcriptional domain that can positively regulate the transcription of HO1 gene. When NRF2 is activated, it dissociates from its docking protein named KEAP1 in the cytoplasm and migrates into the nucleus, where it binds to the antioxidant responsive element in the promoter and subsequently stimulates the expression of various antioxidant genes including HO1 (Dhakshinamoorthy & Jaiswal 2001, Satoh *et al.* 2006, Kaspar *et al.* 2009). It is also known that the phosphorylation of several signal transduction pathways including ERK1/2 and PI3K/AKT could activate NRF2, leading to the translocation of NRF2 (Numazawa *et al.* 2003, Zhao *et al.* 2015). The present results showed that when

NPI were treated with C3G, the ERK1/2 and PI3K/Akt signal pathways were activated, the nuclear NRF2 was upregulated and the HO1 expression was increased, which were consistent with our previous studies of pancreatic β cells (Zhang *et al.* 2011, 2013). Thus, based on the previous and current findings, we postulate that the mechanism of protection by C3G is through the ERK1/2 and PI3K/AKT signaling pathway-induced NRF2-mediated HO1 expression. Further study, which would involve inhibition or deletion of this pathway, will confirm our hypothesis that the ERK1/2 and PI3K/AKT signaling pathway-induced NRF2-mediated HO1 expression is indeed critical in the protection induced by C3G on NPI.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the University of Alberta Office of the Provost and VP Academic, Alberta Diabetes Institute, Alberta Diabetes Foundation, National Natural Science Foundation of China (Nos. 81301889, 81570698 and 81700682) and Zhejiang Natural Science Foundation (2014C33187 and LY16H070003). The Special Foundation for Innovative Talents Training Project of Zhejiang University '985 program' provided funding support for C L and B Y. Funding support for Z X was provided by the University of Alberta Office of the Provost and VP Academic and the Faculty of Medicine and Dentistry. The Alberta Innovation Health Solutions provided funding support for M B and the Li Ka Shing Foundation provided support for W H.

Author contribution statement

C L participated in designing and performing the research, analyzing the data and writing the manuscript. B Y, Z X, E B, M B, and W H participated in designing, performing and analyzing the data. B X and P W participated in performing the research. B Z, X L and K C participated in preparing the Cyanidin-3-O-Glucoside. Y W participated in designing the experiments. G R R participated in designing the research, analyzing the data and writing the manuscript.

Acknowledgements

The authors are grateful to Dr Wen Fu from the Department of Neurology, University of Alberta, for his excellent technical assistance in Western blot analysis, Jose Rodriguez-Silva for his technical assistance on immunohistochemistry images and Alexander Szojka and Dr Adesida Adetola for their technical assistance in performing the hypoxia experiments. The authors also thank Ken and Denise Cantor, John and Ewa Burton and Martine Farand for their generous donations to support this work.

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Received in final form 7 September 2017

Accepted 20 September 2017

Accepted Preprint published online 20 September 2017