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Preventive effect of phycocyanin from *Spirulina platensis* on alloxan-injured mice

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ABSTRACT

The preventive effect of phycocyanin (obtained from *Spirulina platensis*) on alloxan-injured mice is investigated. Oral administration of phycocyanin was started two weeks before an alloxan injury and continued until four weeks later. Tests resulted in the following positive results of oral phycocyanin administration on alloxan-injured mice: decrease fasting blood glucose and glycosylated serum protein (GSP); maintain total antioxidative capability (T-AOC); avert malondialdehyde (MDA) formation in the liver, kidney, and pancreas; decrease total cholesterol (TC) level and triglycerides (TG) level in serum and liver; increase the levels of hepatic glycogen level; maintain glucokinase (GK) expression in the liver and decrease p53 expression in the pancreas at mRNA level. The histological observations also supported the above results. Acute toxicity study further shows that phycocyanin is relatively safe. These results led to the conclusion that phycocyanin has significant preventive effect on alloxan-injured mice. The inhibition of p53 pathway could be one of the mechanisms that led to the protection of pancreatic islets from alloxan injury. We also proposed that GK expression that functions to promote liver glycogen synthesis could be the reason for reduced blood glucose level. The encouraging results are the first step in studying the potential of phycocyanin as a clinical measure in preventing diabetes.

1. Introduction

Diabetes mellitus is one of the most common chronic diseases worldwide, and continues to increase in numbers and significance, as economic development and urbanization lead to changing lifestyles characterized by reduced physical activity. Latest study from the International Diabetes Federation (IDF) shows that the global diabetes epidemic continues to grow (Whiting et al., 2011). Therefore, the search for more effective, safer and better oral hypoglycemic agents has been, and continues to be an important area of active research.

Phycocyanin (PC), a blue photosynthetic pigment, has been used as a food colorant for chewing gum, ice sherbets, soft drinks, candies, and cosmetics including lipstick and eyeliners. Small quantities of phycocyanin are also used as biochemical tracers in immunoassays due to its fluorescent properties (Chaiklahan et al., 2011). Furthermore, phycocyanin has been proven to have therapeutic properties including antioxidant, anti-inflammatory, neuroprotective, hepatoprotective, and anti-cancer activities (Eriksen, 2008; Ou et al., 2010; Pentón-Rol et al., 2011). However, there is little information regarding the anti-diabetic activity of phycocyanin.

Early detection and prompt treatment of diabetes can delay the onset or prevent the progression of complications associated with diabetes. Several studies have shown that tissue (particularly in the liver and the kidney) antioxidant status may be an important factor in the etiology of diabetes

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and that antioxidant treatment reduces diabetic complications (Kakkar et al., 1998). Much interest has placed on examining the role and usage of natural antioxidants as a means to prevent oxidative damage in diabetes patients with high oxidative stress (Qi et al., 2008; Yu et al., 2009). The antioxidant activity of phycocyanin leads us to hypothesize that phycocyanin may be beneficial for diabetic prevention and cure. In this study, we evaluated the preventive effect of phycocyanin for alloxan-injured mice. The mechanism of the preventive effect of phycocyanin was also discussed.

2. Materials and methods

2.1. Preparation of phycocyanin from Spirulina platensis

Phycocyanin was extracted and purified from *S. platensis*. The process of extraction of phycocyanin included homogenization, centrifugation, and precipitation with ammonium sulphate. DEAE-Sepharose Fast Flow chromatography and hydroxylatite chromatography were applied during the purification process (Ou et al., 2004).

2.2. Animals and treatments

Male ICR mice (18–22 g) were obtained from the Comparative Medical Center of Yangzhou University (Yangzhou, China), and were allowed one week to be quarantined and acclimated prior to experimentation. Then the mice were randomly divided into four groups with each consisting of 12 mice. The 4 groups are the control group, the alloxan group, the alloxan + 100 mg phycocyanin/kg body weight (referred to as the PC100 group) and the alloxan + 200 mg phycocyanin/kg body weight (referred to as the PC200 group). Mice in the control group and the alloxan group took physiological saline for 6 weeks. Mice in the PC100 group and the PC200 group took phycocyanin at the specified dose once a day for the 6 week period. The alloxan injury was introduced at the end of 2 weeks by injecting freshly prepared alloxan at a dose of 150 mg/kg body weight after a 12 h fasting to all groups except the control group. Levels of fasting plasma glucose were measured on the last day of the 2nd week before the time that alloxan was introduced to establish the baseline levels for all four groups. At the end of the 6-week period, the mice were fasted overnight (12 h), anaesthetized with 3% sodium pentobarbital (ip) and sacrificed by decapitation. Blood was placed into a centrifuge tube and allowed to clot to obtain the serum. The serum was separated by centrifugation at 1400 × *g* for 10 min and stored at −20 °C until assayed as described below. The liver, the kidney, and half of the pancreas were excised from the mice and stored at liquid nitrogen until use. The other pancreas was excised and fixed in 10% formalin solution for histopathologic analysis.

In the following analysis, the day that the alloxan injury was introduced is marked as day 0.

2.3. Biochemical analysis

Blood glucose levels were measured at day 0, day 7, day 14, day 21, and day 28 after injection of alloxan. Blood samples were obtained from the mice' eyes and their glucose levels were tested by blood glucose test kit (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China).

Separated sera were used for the estimation of glycosylated serum protein (GSP), triglyceride (TG) and total cholesterol (TC). Samples of 200 mg liver or muscle were homogenized in 5 mL of a cold 0.1 M phosphate buffer with a pH value of 7.4. Tissue homogenates were prepared in a glass tissue homogenizer. The homogenate was centrifuged at 10,000 × *g* for 15 min and the supernatant was used for the measurement of total protein, total antioxidative capability (T-AOC), TG, TC, and glycogen. All biochemical parameters were determined using commercial kits (Nanjing Jiangcheng Bioengineering Institute, Nanjing, China) according to the enclosed guidelines.

2.4. Histological analyses

Classical procedure was used for histology. After fixation in Bouin solution, pieces of fixed tissue were embedded into paraffin, cut into slices and colored with hematoxyline–eosine.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA in the liver or the pancreas was isolated using a total RNA isolation kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China). The total RNA (1.0 μg) was reverse-transcribed using Oligo(dT)$_{18}$ as a primer and M-MLV reverse transcriptase (Promega, USA) to produce the cDNAs. PCR was performed using the selective primers for p53 protein (sense: 5′-CCACCATCCACTACAACTACAT-3′, antisense: 5′-GCAAGCAAGGGTTCAAAGAC-3′), glucokinase (GK) (sense: 5′-AAAGATGTTGCCCACCTAC-3′, antisense: 5′-GAAGTCCCACGATGTTGTT-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (sense: 5′-CATCACCATCTTCCAGGAGC-3′, antisense: 5′-TAAGCAGTTGTTGTTGCAGG-3′). PCR was performed for 30 cycles using the following conditions: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1 min. The band intensities of the amplified DNAs were compared after visualization.

2.6. Acute toxicity study

Female and male ICR mice (18–22 g) were obtained from the Comparative Medical Center of Yangzhou University (Yangzhou, China), and were allowed one week to be quarantined and acclimated prior to experimentation. Before treatment, the mice were fasted overnight. Then the mice were randomly divided into two groups with each consisting of 20 mice (10 females and 10 males). Phycocyanin was suspended into distilled water and was administrated to the mice at a dose of 5000 mg/kg body weight *via* oral gavages. The

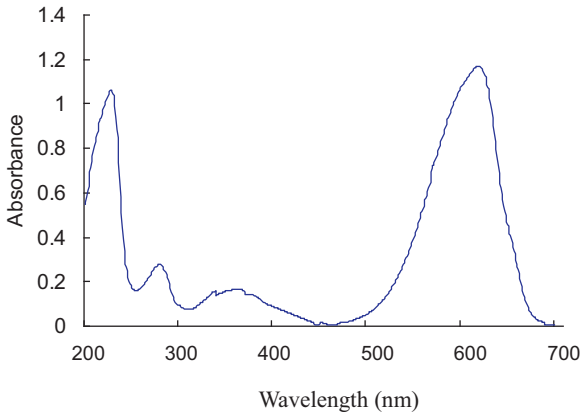

Fig. 1 – UV–vis absorption overlay spectrum of phycocyanin from *Spirulina platensis*. The single peak at 620 nm suggested the absorbance maxima (λ_{max}) of purified phycocyanin.

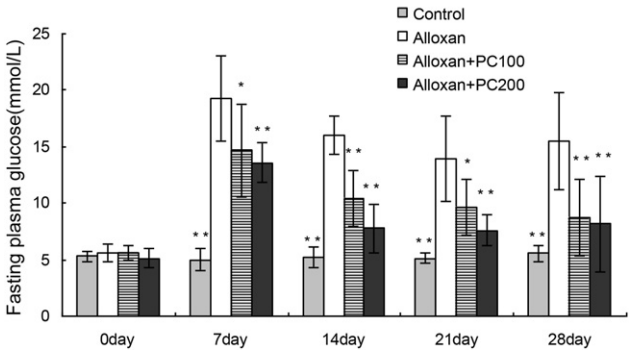

Fig. 2 – Effect of phycocyanin-treated on blood glucose level (mmol/L) in experimental mice. Details were described in the text. Data were shown as means ± S.D. (n = 10–12). *P < 0.05, **P < 0.01 *vs.* alloxan group.

mice were observed for 1 h continuously, then hourly after 4 h, and finally every 24 h for 2 weeks. All external morphological, behavioral, neurologic and autonomic changes as well as toxic effects were recorded.

2.7. Statistical analysis

Animal experimental data was analyzed by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons, which was used to evaluate the difference between two chosen groups. The data was expressed in the format of "mean value ± Standard Deviation (S.D.)", and differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Purity of phycocyanin

The purity of phycocyanin is generally evaluated based on the absorbance ratio of A_{620}/A_{280}. Phycocyanin of purity 0.7 is considered as food grade, 3.9 as reactive grade, and greater than 4.0 as analytical grade (Rito-Palomares et al., 2001). The absorption spectrum of phycocyanin (Fig. 1) showed that the absorbance ratio of A_{620}/A_{280} was 4.2. This indicated that we obtained analytical grade phycocyanin.

3.2. Serum glucose level of experimental mice

Referring to Fig. 2, the serum glucose levels of all groups were about the same at day 0, indicating that administration of phycocyanin does not affect basal serum glucose level. After alloxan was introduced (day 0), the alloxan group showed a significant increase in fasting blood glucose while the two PC groups have managed to maintain the blood glucose levels.

3.3. Glycosylated serum protein (GSP) levels of experimental mice

Comparing the alloxan group and the control group, alloxan caused significant increase ($P < 0.01$) in the GSP level (2.72 ± 0.22 *vs.* 2.06 ± 0.18, mmol/L). Compared the PC groups with the alloxan group, phycocyanin counteracted the effect of alloxan and brought the GSP level back down (2.43 ± 0.15 with PC100, $P < 0.05$; 2.28 ± 0.2 with PC200, $P < 0.01$).

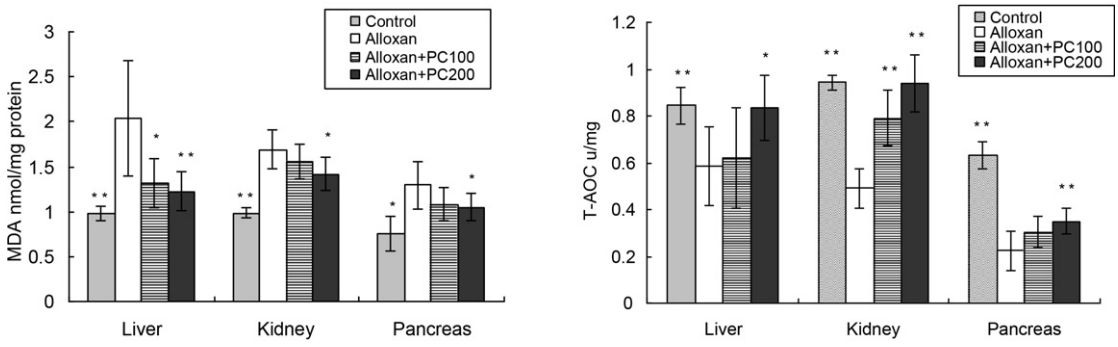

Fig. 3 – Effects of phycocyanin on the level of MDA and T-AOC of the liver, kidney and pancreas in the mice. Data were shown as means ± S.D. (n = 10–12). *P < 0.05, **P < 0.01 *vs.* alloxan group.

Table 1 – Effects of phycocyanin on triglyceride (TG) and total cholesterol (TC) levels in serum and liver.

Group	Serum triglyceride (mmol/L)	Serum total cholesterol (mmol/L)	Liver triglyceride (mg/g tissue)	Liver total cholesterol (mg/g tissue)
Control	1.17 ± 0.17**	2.50 ± 0.26**	3.27 ± 0.94**	1.87 ± 0.29**
Alloxan	2.14 ± 0.31	3.21 ± 0.24	8.36 ± 1.62	2.35 ± 0.30
Alloxan + PC100	1.68 ± 0.27*	2.95 ± 0.22*	5.28 ± 1.51**	1.97 ± 0.15*
Alloxan + PC200	1.35 ± 0.26**	2.60 ± 0.11**	3.47 ± 0.73**	1.91 ± 0.19**

Data were shown as means ± S.D. (n = 10–12).
* $P < 0.05$.
** $P < 0.01$ *vs.* alloxan group.

3.4. *Levels of MDA, T-AOC, TG and TC*

As shown in Fig. 3, there were significant difference on the level of MDA and T-AOC in the liver, kidney, and pancreas between the control group and the alloxan group ($P < 0.05$ or $P < 0.01$). Comparing to the alloxan group, the PC 200 group had significant higher levels of T-AOC and lower MDA content. As shown in Table 1, the alloxan group had higher TG and TC levels in serum and liver comparing to the control group, while the PC groups reversed the effect of the alloxan, as seen by markedly decreased TG and TC levels.

3.5. *Levels of hepatic glycogen in experimental mice*

There were significant difference on the hepatic glycogen contents (mg/g tissue) between the control group and the alloxan group (19.15 ± 3.62 *vs.* 13.26 ± 1.78, $P < 0.01$). PC counteracts the effect of alloxan as seen by the markedly higher hepatic glycogen contented in the PC groups in comparison with that in the alloxan group (17.13 ± 2.40 with PC100, 17.97 ± 2.49 with PC200, $P < 0.01$).

3.6. *Morphological analysis*

The morphological analysis result is shown in Fig. 4. Pancreatic tissues in the control group showed typical septal and intralobular ducts distribution, as well as conjunctive tissue lobules. By contrast, pancreatic tissues in the alloxan group showed few traces of endocrine tissue, depauperate pancreatic islets, and an obscure demarcation between islets and the surrounding acini. Results in the PC groups showed that phycocyanin improved the morphology of pancreatic islets and restored the amount of cytoplasmic secretory granules of insulin-producing β-cells.

3.7. *GK mRNA expression in the liver and p53 mRNA expression in the pancreas*

We investigated the expression of GK at the mRNA levels in mice liver. As shown in Fig. 5, compared to the control group, the level of GK mRNAs were significantly lower in the alloxan group. In comparison, the level of GK mRNAs in the PC groups

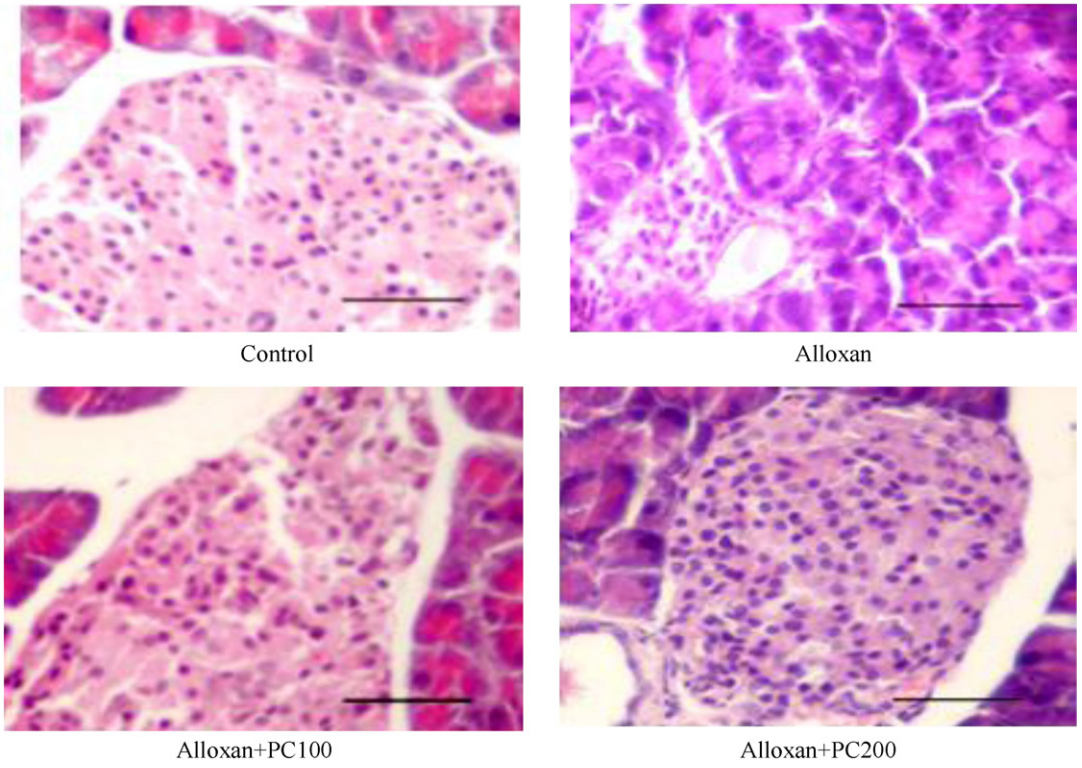

Fig. 4 – Effects of phycocyanin on the morphological features in the mice pancreas (Bar = 50 μm).

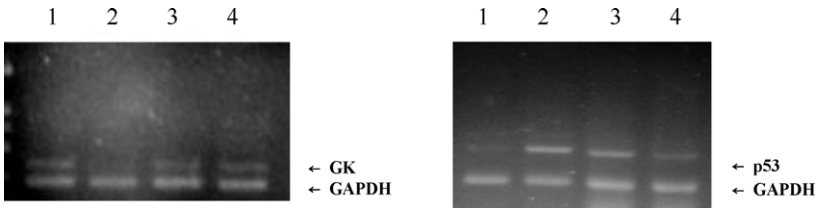

Fig. 5 – mRNA expression level analysis of GK in mice liver and p53 in mice pancreas by reverse transcription-PCR. Expression of GAPDH mRNA was used as the internal standard. Lane 1: control group, lane 2: alloxan group, lane 3: 100 mg/kg PC + alloxan group, lane 4: 200 mg/kg PC + alloxan group.

were recovered to close to that in the control group. We also investigated the expression of p53 at the mRNA levels in the pancreas. p53 mRNA expression was very low in the control group. It is much higher in the alloxan group, and the level comes back down in the PC groups. This illustrates the effectiveness of PC in counteracting the effect of alloxan (Fig. 5).

3.8. *Acute toxicity*

The mice treated with phycocyanin at the dosage of 5000 mg/kg body weight did not show any drug-induced toxic physical signs during the 2-week period and no deaths were registered.

4. Discussion

There are various diabetic animal models which can be used to investigate the pathogenesis and evolution of diabetes, and can possibly be used to screen new anti-diabetic drugs. Alloxan is a commonly used chemical in studies of experimental diabetes. It induces chemical diabetes by damaging the insulin-secreting β-cells of the pancreas, and it could cause time- and concentration-dependent degenerative lesions of the pancreatic β-cells (Lenzen and Panten, 1998). Alloxan also induces free radicals, especially superoxide radicals, which are generally accepted as the trigger that leads to progressive damage and ultimately leads to pancreatic β-cell death and hypoinsulinemia, which leads to a the decreased use of glucose by the tissues (Zhang et al., 2009). In the present study, administration of phycocyanin showed a significant decreased effect on fasting blood glucose levels in alloxan-induced diabetic mice. This result suggest s that phycocyanin protects β-cells of pancreatic islets from alloxan induced injury and/or promotes pancreatic β-cell regeneration (Jothivel et al., 2007). This was confirmed by morphological analysis of pancreatic islets (Fig. 5). Phycocyanin decreases GSP content in diabetic mice. This is encouraging for future study of clinical usage because the increase of GSP in patients with diabetes mellitus is directly proportional to the fasting plasma glucose level (Jackson et al., 1979).

Diabetes mellitus exhibit high oxidative stress leading to oxidative damage particularly in the liver, the kidney, and the pancreas. Weakened anti-oxidative defense system promotes free radical generation (Kakkar et al., 1998; Ihara et al., 1999; Kuyvenhoven and Meinders, 1999). Reduced antioxidant levels and increased lipid peroxidation level in experimental diabetes have been reported (El Naggar et al., 2005). Our present study confirmed that there was a strong correlation between oxidative stress and diabetes occurrence, and administration of phycocyanin exerted beneficial antioxidant defense actions against the condition of diabetes mellitus.

Insulin deficiency leads to a variety of derangements in metabolic and regulatory processes, which in turn leads to accumulation of lipids such as TC and TG in diabetic patients (Goldberg, 1981). Administration of pycocyanin to the diabetic mice resulted in a significant reduction in serum and liver TG and TC levels. Thus it is an effective hypocholesterolemic and hypertriglyceridaemic agent.

One of the factors for elevating blood glucose may be the reduction of glycogen synthesis and the acceleration of glycogen disassimilation in the liver. Our results showed that co-administration with phycocyanin could increase hepatic glycogen synthesis significantly. This suggests that phycocyanin is involved in promoting the synthesis of glycogen, and thus inhibits elevation of blood glucose. Glucokinase, also called hexokinase IV, is the rate-limiting enzyme which catalyses glucose phosphorylation in pancreatic β-cells and hepatocytes, and plays a fundamental role in glucose homeostasis (Tiedge et al., 2000). Alloxan intake evidently decreases glucokinase expression in the liver, suggesting that alloxan's toxicity is not specific to the pancreas. Given the important role of glucokinase as a glucose sensor, and the effects of alloxon on liver glucokinase activity, alloxan-induced diabetes can be used to examine the anti-diabetic effects of compounds prompting insulin secretion and increasing liver-specific glucokinase activity. As alloxan toxicity to the liver or as a consequence of the damage to pancreas, liver glucokinase plays a crucial role in alloxan-induced diabetes (Zhang et al., 2009). In our experiment, liver glucokinase expression in alloxan-injured mice decreases evidently as expected. Moreover, in contrast to unusually high level of glucose, the hepatic glycogen content in alloxan-injured mice is not increased compared to normal mice. The lack of increase in glycogen levels may be due to the decreased glucokinase activity in the liver (Bollen et al., 1998). The present study showed that co-administration with phycocyanin could increase glucokinase expression significantly in the liver of alloxan-injured mice. This suggested that phycocyanin might promote hepatic glycogen synthesis by enhancing glucokinase expression.

It is known that the p53 tumor suppressor directs cells into either death or cell-cycle arrest (Haupt et al., 2003). Our

results indicated that alloxan enhanced p53 expression in the pancreas, while co-administration of phycocyanin decreased p53 expression evidently. We would like to think that phycocyanin may protect pancreastic islets from alloxan injury by inhibiting the p53 pathway, although we found little discussion in the open literature about p53 expression in pancreas.

5. Conclusion

In this experimental study, the authors demonstrated for the first time that phycocyanin had significant preventive effect on alloxan-injured mice. All test results showed that phycocyanin counteracts alloxan's negative effects. We discussed the potential use of phycocyanin as a clinical agent for treating diabetes. We proposed hypothesis on the mechanism of phycocyanin's prevention and cure effect to be further studied. The hypothesized mechanisms are: phycocyanin enhances GK expression, which in turn promote liver glycogen synthesis; phycocyanin inhibits p53 pathway, which in turn protect the pancreastic islets from alloxan toxicity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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