ORIGINAL ARTICLE

Potential role of cyanidin 3-glucoside (C3G) in diabetic cardiomyopathy in diabetic rats: An in vivo approach

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Abstract The present study aimed to evaluate the importance of cyanidin 3-glucoside (C3G) of diabetic cardiomyopathy in diabetic rats. The rats were induced with diabetic using streptozotocin and total triglyceride (TG) and total cholesterol (TC) were determined. The range of myocardial enzymes such as aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LD) were also estimated, further, the Immuno histochemical analysis and western blot investigation were determined for the actual activity of C3G. Results indicated that the marker enzymes such as CK, LD and AST were significantly ($P < 0.05$) increased in STZ administered rats (DM group), while the levels of these elevated marker enzymes of cardiac injury significantly ($P < 0.05$) declined in the DM + C3G group, as compared to the diabetic group of rats. Additionally, a decrease in the level of TNF-alpha and interleukin-6, was noticed in the C3G treated group as compared to diabetic group. Finally, blotting analysis clearly confirmed that the C3G treatment resulted to higher level response of Bcl-2 and lower level response of caspase-3 and BAX. In conclusion, C3G a natural antioxidant may prevent cardiovascular complications by ameliorating oxidative damage, inflammation, metabolic dysfunctions and apoptosis pathways in type 2 diabetes.

1. Introduction

Globally, diabetes mellitus (DM) is a fast growing serious disease specifically in adult with prevalence of 135 million in the year 1995, with the expected prevalence of 300 million in 2025 (Kaul et al., 2012). DM causes above 80% of deaths in middle income countries. In 2030, DM will be the seventh foremost reason for death as per WHO projection (Mathers and
2. Materials and methods

2.1. Experimental animals

The current study was carried out with twenty-four male rats of wistar strain, weighing of 150–200 g. The rats were kept in a polypropylene laboratory cages, under a maintained environment of temperature (25 ± 2°C), humidity (50 ± 5%) and 12:12 h light–dark (LD) cycles. They were provided with usual laboratory food and tap water ad libitum before the experiments. All experimental procedures were carried out as per standard protocol evaluated and approved by I.A.E.C. (Institutional animal ethical committee).

2.2. Stimulation of DM type-2 and C3G treatment

The DM in rats was induced by fasting for 12 h. About 65 mg/kg streptozotocin (STZ) was solubilized in citrate buffer (0.1 M, pH 4.5) and administered intraperitoneally (i.p.). The rats were kept again on fasting for 12 h. Streptozotocin was injected on the 6th day and blood glucose level was determined by glucometer (Accu-Chek Go model GS; Roche Diagnostics GmbH, Mannheim, Germany) in entirely collected blood from the tail vein. Afterward, the rats containing more than 350 mg/dl glucose level were examined for subsequent studies. The animals were assigned into three groups as following; Group-1: Control (n = 8), Group-2: STZ-stimulated DM (n = 8) and Group-3: DM + C3G (n = 8). For the Group 3 (DM + C3G), 10 mg/kg C3G was solubilized in soybean oil and then administered orally at same time and each day for 7 days subsequent to the stimulation of DM.

2.3. Hematological evaluation

After the administration of STZ, levels of blood glucose were measured in entirely collected blood received from tail vein by a glucometer (Changsha Sinocare Inc., Changsha, China) at 72 h. The concentrations of total triglyceride (TG) and total cholesterol (TC) were measured by automatic biochemical analyzer (Olympus AU2700, Tokyo, Japan). The experimental rats were euthanized with CO2 inhalation after the 12 days of C3G treatment. The body weight was noted per day for 7 days.

2.4. Estimation of myocardial enzymes in serum

The samples of blood were obtained by artery of abdomen and serum was extracted by the centrifugation technique (1600×g, 10 min.) at 4°C. The aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LD) were estimated using the automatic biochemical analyzer (Olympus AU2700).

2.5. Analysis of SOD enzyme and MDA content

The hearts were isolated from the sacrificed rats, rinsed in isotonic saline and weighed. The myocardial tissues were homogenized with 0.1 M phosphate buffer (pH 7.4). The best suited diagnosis kits A003-1 for MDA content and A004-4 for SOD activity were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.6. Immunohistochemical analysis

The paraffin-embedded tissue sections (0.5 μm) were applied under immunohistochemistry using antigen-retrieval (microwave based) method. The tissue section was incubated using Anti-IL-6 (#ab6672; 1:500) with primary rabbit polyclonal Anti-TNF-α (#ab9635; dilution: 1 μg/ml) antibodies (Abcam, Cambridge, MA, USA) for the overnight, and further incubated with secondary antibody anti-mouse IgG (#7076) and with biotinylated anti-rabbit (#7074) for half an hour at 37°C. The lesser scanning microscope confocal FV10000 SPD (Olympus) was used to view the results and negative controls were applied as omission of the primary antibody.
2.7. Western blot investigation

The refrigerated samples of tissue from left ventricle were homogenized with extremely cold lysis buffer solution (1 mM Na3VO4, 1% Triton X-100, 1 mM β-glycerophosphate, 20 mM Tris of pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mg/ml pepstatin aprotinin and leupeptin) followed by centrifugation (1600 × g, 15 min.) at 4 °C. The concentration of protein was examined in supernatant fluid by utilizing bicinechonic acid assay (Beyotime Institute of Biotechnology, Haimen, China). Equivalent quantities of protein had been utilized for conducting the western blot analysis in which following antibodies were used; β-actin (#sc-47778; 1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), BAX (#2772; 1:1000), Caspase-3 (#9661; 1:1000) with Bcl-2 (#2870; 1:1,000; all from Cell Signaling Technology, Inc.) The HRP associated secondary antibody had been incubated along with following membrane for one hour at 37 °C. The upgraded chemiluminescence kit was utilized for producing blots.

2.8. Data analysis

The data obtained from the studies are shown as mean ± SEM. The statistical analysis one-way ANOVA was performed using SPSS15.0 (SPSS, Inc., Chicago, IL, USA), where statistically significant difference is considered if $P < 0.05$.

3. Results

3.1. Effects of C3G on metabolic abnormalities

The current hematological studies exhibited the metabolic features of the investigational animals. In this experiment, STZ administered diabetic rats were observed with a significant ($P < 0.05$) lower body weight. Additionally, it was found that STZ injection significantly ($P < 0.05$) increased the level of blood glucose and heart-weight to body-weight ratio (HW/BW) as well as total cholesterol (TC) and total triglycerides (TG) in diabetic rats, when compared with Group-1(control group). However, C3G treatments exhibited a notably increased body weight along with decreased levels of TC, TG and HW/BW, compared to Group-2(DM group). Also, it was seen that levels of blood glucose were found significantly ($P < 0.05$) lowered (nearly control group) in the DM + C3G group as compared with Group-2 (DM group) (Table 1). These metabolic observations pointed out a good defensive action of cyanidin 3-glucoside in the DM.

3.2. Effects of C3G on markers of cardiac injury and oxidative stress

The biochemical marker enzymes of cardiac injury viz. CK, LD and AST were investigated in the current experiment. We found that these marker enzymes viz. CK, LD and AST were significantly ($P < 0.05$) raised in STZ administered rats (DM group). While the levels of these elevated marker enzymes of cardiac injury significantly ($P < 0.05$) declined in the DM + C3G group, as compared to diabetic group of rats (DM group) (Fig. 1A). These results also indicate a cardioprotective activity of cyanidin 3-glucoside in the diabetic rats. Furthermore, during the cardiac tissue examination, the diabetic group of rats (DM group) showed a significant ($P < 0.05$) decline in the SOD activity and a significant increase of MDA contents as shown in Fig. 1B and C. Though, C3G administered to diabetic rats resulted a significant ($P < 0.05$) up-regulation of SOD activity and MDA contents, and thus indicating a potential antioxidant attributes of cyanidin 3-glucoside.

3.3. C3G inhibits the production of TNF-α and interleukin-6

The expressions of inflammations were measured using Immunohistochemical technique. The results showed a dense brown colors staining as a result of increased levels of TNF-α and interleukin-6 in the STZ administered diabetic rats when compared to Group-1(control group). While the light colors staining expressing decrease level of TNF-alpha and interleukin-6, were noticed in the C3G treated group as compared to diabetic group (Fig. 2). These data indicate a strong defensive activity of C3G against the mediators of inflammation produced in DCM.

3.4. C3G prevents DM stimulated apoptosis of myocardial cells

An immunoblotting technique was used for the determination of expression levels of proteins viz. caspase-3, BAX, and Bcl-2. The blots demonstrated a lower response of Bcl-2 and higher response of caspase-3 and BAX in the diabetic group of rats compared to Group-1(control group). However, C3G treat-

| Experimental design   | Weight of body (g) | Heart-weight to body-weight ratio (HW/BW) (mg/g) | Blood glucose (mmol/l) | Total triglycerides (TG) (mmol/l) | Total cholesterol (TC) (mmol/l) |
|-----------------------|--------------------|-----------------------------------------------|------------------------|----------------------------------|-------------------------------|
| Group-1 (control)     | 412 ± 14           | 3.72 ± 0.12                                   | 6.4 ± 0.4              | 0.82 ± 0.04                      | 1.37 ± 0.04                   |
| Group-2 (DM)          | 245 ± 11           | 6.23 ± 0.11                                   | 20.6 ± 1.3             | 1.34 ± 0.15                      | 1.721 ± 0.05                  |
| Group-3 (DM + C3G)    | 294 ± 23           | 3.30 ± 0.14                                   | 7.2 ± 1.6             | 0.87 ± 0.06                      | 1.42 ± 0.30                   |

HW/BW ratios were determined on the sacrificed day of rats. The levels of TC, TG and blood glucose were determined in fasting basal state on the sacrificed day. Data are represented as the mean ± SEM.

*a* $P < 0.05$, against Group-1 control group.

*b* $P < 0.05$, against diabetic rats (DM group).
Figure 1  Cyanidin 3-glucoside attenuates cardiac damage and oxidative stress in DM. C3G was revealed to (A) reduce cardiac enzyme release in serum, (B) diminish the MDA content in cardiac tissue and (C) enhance SOD activity.
Diet is one of the significant factors related with development of type 2 (Franz et al., 2010). Several experimental findings indicate that a larger dietary intake of phytochemicals and polyphenolic compounds containing strong antioxidant capacity could be linked to lowering risk of diabetes as well as predisposing factors (Firdous, 2014). Our hematological finding revealed that C3G markedly ameliorated the STZ provoked metabolic abnormalities by increasing the body weight and decreasing the levels of blood glucose, TC, TG and HW/BW to closer the normal control rats.

4. Discussion

Considerable findings from numerous research of type 1 (Aziz et al., 2013) and type 2 (Singh et al., 2010; Serasanambati and Chilakapati, 2016) diabetes associate hyperglycemia which is known as a major clinical marker in DCM. In this study, higher levels of blood glucose were observed in the diabetic rats. It is considered that hyperglycemia may be possible as a result of reduction of pancreatic secretion of insulin. It is also well documented that STZ produced ROS, hinder the antioxidant defense system and causes oxidative destruction of β-cells in the pancreas (Tonne et al., 2013). However, increased levels of blood glucose were seen to be substantially lowered in the C3G treated group, compared to DM group. Though, these levels were not lowered to the same magnitude compared to control group. Numerous studies have suggested that dietary supplement plant extracts rich with C3G are involved with ameliorated insulin sensitivity in genetic and diet induced animal model of insulin resistance (Sasaki et al., 2007). Therefore, these metabolic observations indicate that protective effects of cyanidin 3-glucoside may be possible due to its ROS scavenging activity in the DM.

The biochemical investigation of marker enzymes of cardiac injury is usually an important parameter in the diagnosis of diabetic cardiomyopathy. An augmented level of serum marker enzymes viz. lactate dehydrogenase (LD), creatine kinase (CK) and aspartate aminotransferase (AST) leads to myocardial infarction (Khan et al., 2013). The increased levels of LD and CK in serum have been reported in the DCM (Yousaf and Powell, 2012). In this study, C3G significantly declined the levels of cardiac enzymes such as LD, CK and AST compared with diabetic rats signifying the cardioprotective activity of cyanidin 3-glucoside. Oxidative stress provoked by ROS is a major factor in the DCM etiology (Giacco and Brownlee, 2010). That the STZ decreases SOD activity and increases MDA content may be associated with increased for-
mations of ROS. Superoxide dismutase (SOD) is a well known significant defensive antioxidant enzyme which directly elimi-
ates the ROS (Fukai and Ushio-Fukai, 2011). Malondialde-
hyde (MDA), a marker of oxidative stress, is an end product of lipid peroxidation process. SOD level is declined and MDA content is raised in the serum of diabetic rats (Desai et al., 2015). Our finding demonstrated that cyanidin 3-glucoside administered to diabetic rats showed a significant up-regulation of SOD activity and MDA contents indicating an efficient ROS scavenging activity.

An increase in pro-inflammatory manifestation is majorly involved in the development of diabetic cardiomyopathy (Wang and Cai, 2006). The cytokines such as TNF-α, interleukin-1β and interleukin-6 are documented to produce myocardial injury. TNF-α is well accepted as an important mediator in heart failure (Tian et al., 2015). It stimulates inflammatory cytokines and causes myocardial fibrosis and hypertrophy, subsequently leads to LV dysfunction and remodeling (Hori and Nishida, 2009).

During the Immunohistochemical analysis, it was found that C3G remarkably attenuated inflammatory response of TNF-α and interleukin-6 in the diabetic animals. Thus C3G is indicating a strong protective activity of C3G against the inflammation produced in DCM. Furthermore, our immunoblotting technique showed that C3G treatment resulted in higher level responses of anti-apoptotic Bcl-2 along with lower level responses of pro-apoptotic caspase-3 and BAX (Fig. 3) in the diabetic group. Therefore, C3G amelio-
rated DM stimulated apoptosis of cardiomyocytes and demonstrat-
ing cardioprotective attributes in the diabetes.

5. Conclusion

In summary, cyanidin 3-glucoside demonstrated a beneficial potential therapeutic agent for the treatment of DCM. Our finding suggests that cyanidin 3-glucoside, a natural antioxid-
ant may prevents cardiovascular complications by ameliorating oxidative damage, inflammation, metabolic dysfunctions and apoptosis pathways in type 2 diabetes.

Conflict of interest

The authors declare that they have no conflicts of interest.

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