Original Article

Cytoprotective Effect of Silymarin against Diabetes-Induced Cardiomyocyte Apoptosis in Diabetic Rats

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Abstract

Objective The beneficial effects of silymarin have been extensively studied in the context of inflammation and cancer treatment, yet much less is known about its therapeutic effect on diabetes. The present study was aimed to investigate the cytoprotective activity of silymarin against diabetes-induced cardiomyocyte apoptosis.

Methods Rats were randomly divided into: control group, untreated diabetes group and diabetes group treated with silymarin (120 mg/kg·d) for 10 d. Rats were sacrificed, and the cardiac muscle specimens and blood samples were collected. The immunoreactivity of caspase-3 and Bcl-2 in the cardiomyocytes was measured. Total proteins, glucose, insulin, creatinine, AST, ALT, cholesterol, and triglycerides levels were estimated.

Results Unlike the treated diabetes group, cardiomyocyte apoptosis increased in the untreated rats, as evidenced by enhanced caspase-3 and declined Bcl-2 activities. The levels of glucose, creatinine, AST, ALT, cholesterol, and triglycerides declined in the treated rats. The declined levels of insulin were enhanced again after treatment of diabetic rats with silymarin, reflecting a restoration of the pancreatic β-cells activity.

Conclusion The findings of this study are of great importance, which confirmed for the first time that treatment of diabetic subjects with silymarin may protect cardiomyocytes against apoptosis and promote survival-restoration of the pancreatic β-cells.

Key words: Cardiomyocytes; Aspartate transaminase and Alanine transaminase ratio (AST/ALT); Creatinine; Caspase-3; Bcl-2; Cholesterol; Triglycerides

INTRODUCTION

Diabetes type 2 is a metabolic disorder that is primarily characterized by insulin resistance, altered insulin secretion and hyperglycemia[1]. It is also accompanied with disturbance of carbohydrate, fat and protein metabolism, which in turn leads to damage and failure of various organs and blood vessels[2-3]. Accordingly, diabetes is associated with cardiac abnormalities and pathological changes, since the increased levels of plasma glucose caused by diabetes may induce death of cardiomyocytes due to apoptosis[4]. Apoptosis in myocardial and skeletal muscle dysfunction have been observed in patients with chronic heart failure[5-8]. Apoptosis is a form of...

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programmed cell death occurring under certain physiological and pathological conditions as a common mechanism of cell replacement, tissue remodeling and elimination of damaged cells. As a cause of the homeostasis of mammalian tissues linked to enormous disorders, it plays a central role in normal cell cycle and tumor biology. Unlike necrosis, apoptosis is a form of death characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and genomic DNA fragmentation, which is essential for tissue development and homeostasis\(^9\). Apoptosis has been implicated in a number of diseases such as congestive heart failure and ischemic injury\(^10\). Cardiomyocyte death may also occur through necrosis other than apoptosis during cardiac ischemia and reperfusion\(^11-12\).

Silymarin is a flavonoid mixture extracted from *Silybum marium*\(^13\). The potential therapeutic properties of silymarin are attributed to its anti-inflammatory, antioxidant, and anti-cancer activities\(^14\). Recently, silymarin has been suggested to have an anti-diabetic activity in streptozotocin treated DM (type 1) in male albino rats\(^15\). In addition, various clinical studies have revealed that silymarin has potential anti-diabetic activities\(^16\). Recently, it has been confirmed that silymarin can prevent nephropathy-induced premature death in diabetic rats\(^17-18\). The present study was aimed to investigate the potential cardio-protective effect of silymarin against apoptotic death of cardiomyocytes associated with diabetes.

**METHODS**

**Animals**

Thirty adult male albino rats, each weighing 100±5 g, were used in the study. The animals were kept under the same natural environmental condition of temperature and photoperiod and with free access of food and water. All the procedures were in accordance with the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals. The animals were randomly divided into three groups (control group, diabetes group and diabetes group treated with silymarin). Each group had 10 rats.

**Induction of Diabetes**

Diabetes was induced with two intraperitoneal (i.p.) injections of alloxan (Sigma) previously dissolved in ice cold phosphate-buffered saline, pH 6.8 (Merck). The first dose was 150 mg/kg as recommended by Bromme et al.\(^19\), while the second dose was 100 mg/kg given two days after the first dose to ensure the induction of diabetes throughout the experimental duration. Diabetes threshold was serum glucose level >250 mg/dL (Diagnostics, Indianapolis, IN, USA). The rats in control group were injected with normal saline. Silymarin was from Sigma Chemical Co. (St. Louis, MO, USA). Silymarin was dissolved in sodium hydroxide with pH <7.8. Seven days after diabetes induction, the rats in silymarin treated group were given i.p. injection of silymarin for ten days at dosage of 120 mg/kg·d. At the end of experiment, the rats were sacrificed by decapitation after 12 h fasting, and the blood and cardiac tissues samples were collected for further analysis.

**Plasma Measurements**

Total protein contents were determined according to the method of Lowry et al. using bovine serum albumin as standard\(^20\). Aspartate transaminase (AST) and Alanine transaminase (ALT) activites were tested as previously described\(^21\). Creatinine level was measured by kinetic method as described by Henry\(^22\). Cholesterol level was estimated using available commercial kit (Sigma-Aldrich-MAK043). Triglycerides level was determined according to the method of Buccolo and David\(^23\). Serum glucose level was determined according to the instructions of kit’s manufacturer (ab65333). Finally, insulin level was determined according to the instructions of kit’s manufacturer (Mercodia-10-1251-01).

**Histological and Immunohistochemical Studies**

For haematoxylin and eosin dye (H&E) staining, the cardiac muscle specimens from the left ventricles of the animals in all groups were fixed in 10% neutral formalin, dehydrated through alcohols, cleared in xylene, and then embedded in paraffin in order to obtain 5 μm sections. The immunohistochemical staining for detection of the immunoreactivity of caspase-3 and Bcl-2 proteins was done as previously described\(^24\). Immunohistochemical reaction was carried out by using avidin biotin peroxidase method by Nova Castra Laboratories Ltd, UK. Endogenous peroxidase activity was inhibited by incubation with 0.3% \(\text{H}_2\text{O}_2\) in methanol for 30 min. The sections were blocked with normal goat serum for 1 h to prevent non-specific binding followed by incubation with the primary antibody caspase-3 (apoptotic marker) or Bcl-2 protein (antiapoptotic marker) for 1 h at room
temperature. The sections were incubated with the secondary antibody (biotinylated anti-mouse IgM) for 30 min. The sections were then incubated with ExtrAvidin (Sigma) for 45 min at 37 °C. Staining was visualized using diaminobenzidine (DAB, Sigma), then slides were washed and counterstained with haematoxylin, cleared, mounted and examined by light microscopy. Finally, the caspase-3 or Bcl-2 cytoplasmic site of reaction was stained brown and nuclei stained blue. The quantification of caspase-3 and Bcl-2 intensity was carried out with NIH ImageJ software. In order to investigate whether the over expression of caspase-3 in the diabetes group was equivalent to apoptosis, the total and fragmented DNA in the cardiomyocyte was measured colorimetrically as previously described\textsuperscript{[25]}. 

**Statistical Analysis**

The data were analyzed with Sigma Plot 10 software (Systat Software Inc.), and Prism 3.0 package (GraphPad Software, Inc, San Diego, CA, USA). One-way ANOVA Newman Keuls multiple test was used as a post-hoc comparison test. P values were calculated using student t-test and a significant difference was determined as P<0.05.

**RESULTS**

**Total Plasma Protein Content and Creatinine, AST and ALT levels**

As shown in Figure 1, Data shown a slightly significance decrease (P<0.05) in total plasma protein content in untreated diabetic group compared with the others groups. However, the level of plasma creatinine significantly increased (P<0.001) in untreated diabetes group (1.8±0.09 mg/dl) compared with control group (0.6±0.07). Although the level of plasma creatinine declined (P<0.001) in treated diabetes group (0.9±0.06) in comparison with untreated diabetic group, it was still much higher than that in control group (P<0.05). This obvious increase of the plasma creatinine level in untreated diabetic rats by 3 and 2 folds in comparison with other groups reflected a failure of the creatinine clearance system in the untreated diabetic rats. The levels of AST and ALT and their relative ratio were calculated since diabetes is associated with elevated AST/ALT ratio. The data revealed an obvious increase in the levels of AST and ALT in all groups (18.2±1.99 and 15.3±2.3 IU/dl in untreated diabetes group, 7.5±0.74 and 10.2±1.5 IU/dl in control group, 13.3±0.98 and 12.8±1.65 IU/dl in treated diabetes groups). The treatment with silymarin for diabetic rats resulted in a significant decrease in the levels of AST and ALT, however still there was a significant increase in AST level compared with control group. The AST/ALT ratio was calculated, since the mild to moderate increase in AST/ALT ratio was considered a specific indicator closely related to diabetes type 2 and metabolic syndromes. It was noteworthy that in both untreated and treated diabetes groups, the AST/ALT ratio was >1, while it was <1 in control group (Figure 2).

**Blood Glucose and Insulin Alteration**

The level of plasma glucose could provide direct and full information about diabetes status. As shown by

![Figure 1](image_url)
the data, the level of blood glucose significantly decreased (P<0.001) in silymarin treated diabetes group (224±43.84 mg/dL) compared with untreated diabetes group (437±67.5 mg/dL) and control group (154±38.2 mg/dL). It was noteworthy that the level of glycemia increased in the diabetic rats by factor 2.83 and reduced to 1.45 after treatment with silymarin (Figure 3), which indicated that the level of glycemia significantly declined by a factor 1.95 in silymarin treated diabetes group compared with untreated diabetes group. In order to confirm these findings, the insulin level was measured in each group. The level of insulin in control group was recorded to be 15.4±3.22 mg/dL, and in untreated diabetes group it significantly declined to 7.5±1.27 mg/dL (P<0.001). The level of insulin significantly increased to 11.2±2.31 mg/dL in treated diabetes group after the treatment with silymarin (P<0.001), which might reflect survival and restoration of the pancreatic β-cells in the diabetic rats due to the restoration of insulin secretion after treatment with silymarin.

**Cholesterol and Triglycerides**

The recorded levels of cholesterol and triglycerides were 58.2±7 and 80.6±12.34 mg/dL in control group, respectively. A significant elevation of cholesterol and triglycerides levels in the untreated and treated diabetes groups was recorded (Figure 4). In the untreated diabetes group the levels of cholesterol and triglycerides were 140.5±13.81 and 210.1±21.57 mg/dL, respectively, while the levels of cholesterol and triglycerides in treated diabetes group declined to 95±15.96 and 120±9.22 mg/dL, respectively.

**Histological Observations**

In rats in control group, by using H&E, the myocardium was striated and arranged in a linear array that branched and anatomized in a specific pattern giving the appearance of a sheet. The cardiac muscle fibres were joined together by intercalated discs. They contained acidophilic cytoplasm with oval centrally located nuclei. The cardiac muscle fibres were separated by delicate layer of connective tissue with well evidenced myocardial blood capillaries (Figure 5A). In diabetic rats, many cardiac myocytes showed remarkable disorganization and fragmentation. Also, sarcoplasmic vacuolation, loose of cross striation of cardiac muscles, appearance of necrotic areas and inflammatory cells, disappearance of intercalated discs in many cardiac myocytes, pyknotic nuclei and marked congestion with dilatation of the myocardial blood vessels were observed. The treatment with silymarin at dosage of 120 mg/kg·d for 10 d in diabetic

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**Figure 2.** AST/ALT ratio in plasma of control group, untreated diabetes group, and silymarin treated diabetes group.

**Figure 3.** Total plasma insulin (A) and glucose (B) in the control group, untreated diabetes group and diabetes group treated with silymarin. Insulin level significantly declined in diabetes group compared with control group (**P, 0.01**) and increased after silymarin treatment (**P, 0.01**). A significant decrease in the level of insulin was observed in the treated diabetic group compared with control group (**P, 0.05**). In the contrary, the total plasma glucose increased in diabetes group compared with normal control and treated diabetic rats (**P, 0.001**). Although plasma glucose declined after silymarin treatment compared with diabetic rats (**P, 0.001**), but there was still a significant difference compared with normal control rats (**P, 0.01**).
rats resulted in an obvious recovery and restoration of the normal architecture of the muscle fibres.

**Immunohistochemical Observations**

Immunohistochemical observations of apoptosis-related proteins by measuring caspase-3 showed a strong positive reactivity of its expression in the sarcoplasm of cardiomyocytes and the walls of blood vessels of diabetic rats. The data showed that Bcl-2 antiapoptosis-related proteins were expressed intensively in the sarcoplasm of cardiomyocytes of normal control rats. The expression of caspase-3 immunoreactive protein in the sarcoplasm of cardiomyocytes exhibited a marked positive expression in diabetic rats, much higher than that in normal control rats, indicating a weak immunoreactivity (Figure 5A). After the treatment with silymarin for diabetic rats, a marked reduction of immunoreaction for caspase-3 expression was observed, which was similar to that in normal control rats. However, the immunohistochemical expression of Bcl-2 in cardiomyocytes showed significant difference and strong positive reaction in

![Figure 4](image_url). Plasma cholesterol and triglycerides levels in the control group, untreated diabetes group and silymarin treated diabetes group. The plasma cholesterol level increased in either untreated diabetes group or treated diabetes group compared with control group (**P, 0.001). However, compared with untreated silymarin treatment decreased plasma cholesterol level in treated diabetes group (***P, 0.001). The level of triglycerides significantly increased in untreated diabetic rats compared with normal controls (**P, 0.001), but significantly declined in silymarin treated diabetic rats (***P, 0.001) compared with untreated.

![Figure 5](image_url). (A), H&E and immunohistochemical stained cardiac tissue sections in control group, untreated diabetes group and treated diabetes group (Bar=6.25 µm). (B), Caspase-3 immunoreactivity increased in untreated diabetes group by quantifying staining intensity compared with control group (**P, 0.01), meanwhile, the difference was not significant between treated diabetic rats and normal control rats. In the contrary, the immunoreactivity of Bcl-2 in diabetic rats significantly declined compared with normal control rats and treated diabetic rats (***P, 0.001), but increased after sylmarin treatment (**P, 0.001). The level of caspase-3 increased in untreated diabetes group by 1.38 and 1.3 folds compared with control and treated diabetic groups, respectively. Of note, the level of Bcl-2 decreased by 1.6 fold in the untreated diabetic rats compared with either control rats or treated diabetic rats.
the sarcoplasm of cardiomyocytes of normal control rats, and weak immunoreactivity in the case of diabetic rats. Silymarin treatment for diabetic rats showed a marked restoration of positive reaction in the sarcoplasm approximately similar to normal appearance. The level of caspase-3 increased in untreated diabetes group by 1.38 and 1.3 folds compared with that in the control and treated diabetes groups, respectively. However, the level of Bcl-2 decreased by 1.6 folds in untreated diabetes group compared with that in both control group and treated diabetes group (Figure 5B).

Since all cells expressed caspase-3 and its expression might not be equivalent to apoptosis, the ratio of DNA fragmentation, a gold standard for the incidence of apoptosis, was measured, which reflected the DNA damage. The data revealed an obvious increase in the level of DNA damage in the cardiac tissues of diabetes group compared with other groups (P<0.001). There was also a significant increase in the DNA damage level in silymarin treated diabetes group compared with control group (P<0.01). However, the level of the fragmented DNA significantly declined in silymarin treated diabetes group compared with untreated diabetes group (P<0.001). In summary, silymarin might have DNA protective activities against diabetes induced damage, and thus could prevent its severe long-term effects.

**DISCUSSION**

A recent study has concluded that the increased level of plasma glucose could promote apoptosis in β-cells of human pancreas.[26] In the present study, the level of hyperglycemia in treated diabetes group declined twice, suggesting a hypoglycemic activity of silymarin. The hypoglycemic effect of silymarin could be attributed to the restoration of insulin secretion and its elevated levels in the treated diabetes group, reflecting an additional cytoprotective effect of silymarin on the pancreatic β-cells of diabetic subjects. On the other hand, the data showed high elevated level of plasma creatinine in untreated diabetes group compared with other groups. Hyperglycemia also causes osmotic diuresis and depletion of extracellular fluid volume[27] which could explain the elevated plasma creatinine in untreated diabetes group. It is noteworthy that creatinine is a metabolic product of creatine and phosphocreatine found mainly in the muscles. Thus, creatinine could directly provide a clear picture for any muscle mass alteration. Furthermore, creatinine does not bind to plasma proteins, and is freely filtered by the glomerulus of the kidney. That has an important clinical implication for overestimating creatinine clearance of kidney function[28-29]. The data showed high elevated levels of cholesterol and triglycerides in diabetic rats which declined after the treatment with silymarin. This finding might suggest a hypcholesterolemic activity of silymarin.

On the other hand, diabetes is associated with elevated AST/ALT ratio. Since the mild to moderate increase in AST/ALT ratio have been considered a signal closely related to diabetes type 2, and also a metabolic syndrome[30-31]. In an open-label uncontrolled clinical trial using exenatide to assess the safety of the drug in patients with diabetes, aspartate aminotransferase (AST) and insulin sensitivity were improved over a 3.5-year follow-up period[32]. In this study, the AST/ALT ratio in control group was <1, while >1 in both untreated diabetes group and treated diabetes group. This finding is of great importance because AST/ALT ratio >1 is usually considered a determinant of progressing liver damage and cirrhosis[33]. Therefore, AST/ALT ratio could be part of the evaluation and prediction of diabetes type 2 development[34]. Currently, some studies have revealed that cardiovascular disease is closely related to the elevated AST levels[35].

In the present study we investigated the protective effect of silymarin against apoptotic death of cardiomyocytes associated with diabetes. The expression of caspase-3 immunoreactive protein in the sarcoplasm of cardiomyocytes was markedly elevated in diabetic rats compared with rats in other groups. As shown in Figure 5B, the level of caspase-3 increased in untreated diabetes group by 1.38 and 1.3 folds compared with control and treated diabetes groups, respectively. However, as a result of
treatment with silymarin, a marked reduction of immunoreaction for caspase-3 expression was observed in diabetic rats, similar to that in control group. The activity of the anti-apoptotic Bcl-2 protein could explain the previous findings. Since Bcl-2 is an antiapoptotic protein which is present in mitochondria, endoplasmic reticulum, and nuclear membrane, different protective mechanism may lie behind[36-38]. In untreated diabetic rats, the level of Bcl-2 decreased by 1.6 folds compared with either control group or treated diabetes group (Figure 5B). The data showed a strong positive expression of Bcl-2 in the sarcoplasm of cardiomyocytes of normal control rats, and weak immunoreactivity in diabetic rats. Our findings are in consistent with the results by Bhan and his colleagues that diabetes was associated with increased apoptosis and less expression of Bcl-2 protein on diabetic rat wounds[39]. Meanwhile, silymarin treatment for diabetic rats showed a marked restoration of positive reaction in the sarcoplasm similar to normal appearance. It is extremely important that Bcl-2 percentage of cardiomyocytes in diseased heart is higher than in the control heart which may maintain cell survival in myocarditis[40]. Moreover, Yan and his colleagues demonstrated that Bcl-2 increased after cardiopulmonary resuscitation in rats[41]. Bcl-2 regulates mitochondrial permeability processes by regulating mitochondrial protein release into the cytoplasm, and so it constitutes a key point for the mitochondrial pathway of apoptosis[42], thus preventing intracellular oxidation which triggers apoptotic program[42-43].

The data have also revealed that silymarin has a DNA protective activity against damage induced by diabetes, which provide more information for developing new therapeutic strategy to protect diabetes patients from severe long-term effects on cardio-vascular system.

**CONCLUSION**

The data have revealed that silymarin has histoprotective and cellular restoration activities not only for the pancreatic β-cells by promoting insulin secretion restoration in the treated diabetic rats, but also for the cardiomyocytes by preventing apoptosis associated with diabetes. Finally, the AST/ALT ratio is an extremely important indicator for health, since either in human or animal it must be less than 1 in normal subjects. The effects of silymarin as described above are obvious and might be of significant clinical importance given the large number of diabetes patients and the severe long-term effects on cardio-vascular system and the well-known safety characteristics of silymarin as a therapeutic and prophylactic compound. Preclinical and clinical trials using silymarin as an adjuvant supplement for diabetes therapy are urgently needed to facilitate the clinical use of this fascinating agent.

**CONFLICT OF INTERESTS**

The corresponding author declares no conflict of interest.

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