Silymarin ameliorates Metabolic Risk Factors and Protects against Cardiac Apoptosis in Streptozotocin-induced Diabetic Rats

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Abstract The effect of silymarin (SMN) in reducing metabolic risk factors and amelioration of cardiac apoptosis in an experimental model of diabetes was investigated. Streptozotocin (STZ) was injected into male rats (50 mg/kg, ip), and after diabetic induction SMN (80mg/kg) was orally administered for 21 days. Diabetic rats showed a significant decrease in insulin level, hyperglycemia, elevated HOMA-IR, glycosylated hemoglobin (HBA1c), and lipid profile. A significant increase in the lipid peroxidation product malondialdehyde (MDA) and a decrease in the glutathione (GSH) concentration were demonstrated in the heart of the diabetic rats. A significant increase in the activities of creatin kinase-MB (CK-MB) and lactate dehydrogenase (LDH), aspartate amino transferase (GOT), alanine amino transferase (GPT) in the serum of the diabetic rats were demonstrated. Increased numbers of apoptotic cells with down-regulation of Bcl-2 and activation of Bax as well as the caspases-9 and 3 were demonstrated in the diabetic rats. SMN treatment of the diabetic rats ameliorated hyperglycemia, HBA1c, increased insulin secretion, improved HOMA-IR and lipid profile. SMN significantly increased serum HDL. SMN also blunted the increase in MDA and stimulated the GSH production in the heart of STZ-treated rats and improvedredox state. SMN enhanced Bcl-2 expression, blocked the increase of Baxand the caspases 9 and 3. This study demonstrates the effectiveness of SMN in ameliorating myocardial injury in experimental diabetes. This may be related to its antioxidative and anti-apoptotic properties and can be used as antidiabetic complement in case of diabetes mellitus to avoid cardiac complications.

Keywords: *antioxidants, apoptosis, oxidative stress, glutathione*

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1. Introduction

Association of hyperglycemia with various cardiac complications is the main cause of morbidity in the diabetic population and produce significant costs stress for the healthcare system [1]. Diabetes and its cardiac complications are associated with increased oxidative stress by increasing production of mitochondrial ROS, by glucose autoxidation and by non-enzymatic glycation of proteins [2]. Cell death is a major result of myocardial abnormalities and an important cause of various cardiomyopathies. Mitochondria regulate apoptosis that have been shown to play an important role in the development of cardiomyopathy and atherosclerosis [3,4]. The anti-apoptotic function of Bcl-2 is to prevent the pro-apoptotic family members and blocking the oligomerization required for the initiation of apoptosis [5]. Bcl-2 family members are membrane bound, with their major sites of actions on the mitochondrial outer membrane and the membrane of the endoplasmic reticulum [6].

Several evidences documented that reducing oxidative stress through antioxidant treatment might be an effective strategy for decreasing diabetic cardiomyopathy [7]. The use of complementary and alternative medicine is widespread. Medicinal plants is an important source of active natural products which differ widely due to their structure and biological properties and play an important role in the management of various human diseases, including diabetes and cardiovascular diseases [8]. Many plant extracts and their purified compounds have been reported to control blood glucose and reverse physiological abnormalities [9]. Silymarin (SMN) is a flavonoid mixture extracted from Silybummarianum L. (milk thistle) that grows in different parts of the world. SMN contains functionally important flavolignans such as silybin, silycristin, silydianin and isosilybin as the major constituents [10]. SMN functions as a free radical scavenger, increasing glutathione content via the glutathione system. The protective effect of SMN on tissue damage may be attributed to an increase in the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, and inhibition of lipid peroxidation that, in addition to the glutathione system, constitute the efficient defense mechanisms against damage by free radicals [11,12]. SMN owns a variety of pharmacological activities, such as anti-inflammatory,

immunomodulatory, and antidiabeticimpact [13,14,15]. The mechanism of action for these beneficial effects of SMN is unknown. However, antioxidant activity is a leading theory. Hence, the aim of the present study is to investigate the cardioprotective effect of SMN on metabolic risk factors, oxidative stress, cardiac apoptosis and its regulating proteins in the heart of the experimentally-induced diabetic rats.

2. Material and Methods

All chemicals were of the highest purity available. Streptozotocin (STZ) and silymarin (SMN) were purchased from Sigma Chemical (St Louis, MO).

Male Wister rats, weighing 220–240 g, were housed in cages at 22–24 °C with a 12-h light/dark cycle with free access to food and drinking water. All experiments were carried out in accordance with protocols approved by the local experimental animal ethics committee, College of Science, Princess Nora University, Riyadh, Saudi Arabia. The animals were divided into four groups of 6 rats each. The first group served as a control, the second group received every other day a dose of SMN (80 mg kg^{-1}, i.g.) for 21 days, the third group received a single injection of 50 mg kg^{-1} STZ i.p., the fourth group received a single injection of STZ (50 mg kg^{-1}, i.p.) followed by SMN (80 mg kg^{-1}, i.g.) daily for 21 days.

To induce diabetes, the rats in diabetic groups were injected intraperitoneally (i.p.) with freshly prepared streptozotocin in 0.01 M citrate buffer, pH 4.5, at a single dose of 50 mg/kg body wt.The animals were considered diabetic if the blood glucose level was greater than 250 mg/dl.Blood glucose level was determined every 3 days for two weeks after STZ injection using a glucose monitor set (Elegance, CT-X10, Convergent Technologies GmbH & Co. KG, Marburg, Germany). The initial and final Body weights were determined.

At the end of the experimental period of 21 days and after an overnight fast, rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.)and blood was collected by cardiac puncture. Sera were separated by centrifugation (500×g) (HERMLE LABORTECHNIK Z326K, Germany) for 5 min for biochemical determinations. Each heart fragment was homogenized in 10 volumes of 50 mM sodium phosphate buffer (pH 7.4) at 4 °C for 30 s using a Polytron homogenizer. The homogenate was centrifuged (1088×g) for 5 min in a refrigerated centrifuge. The resulting supernatant was used for the measurements of glutathione and lipid peroxidation.

2.1. Biochemical Analysis

The levels of serum glucose and lipid fractions were assayed using colorimetric assay kit according to the manufacturer's instructions (Spinreact, Esteved'en Bas Girona, Spain). The serum insulin level was measured by ELISA method using DRG Elisa insulin kit (Rat ELISA Kit, Cambridge, MA, USA). The homeostasis model assessment for insulin resistance (HOMA-IR) index was calculated as descried previously [16]. Activities of lactate dehydrogenase (LDH), creatin kinase (CK-MB), asparate amino transferase (GOT), alanine amino transferase (GPT) in the serum were measured using a kit from Elitech Group, Puteaux, France. Lipid peroxidation product,

malondialdehyde (MDA), was measured by thiobarbituric acid assay, which is based on MDA reaction with thiobarbituric acid to give thiobarbituric acid reactive substances (TBARS) [17]. Glutathione (GSH) level was determined by the method of Sedlak& Lindsay et al.[18]. Protein concentrations were determined as described byLowry et al. [19].

2.2. Molecular Analysis

Hearts samples were prepared for flow cytometer described by Gong et al. [20]. The cells obtained were suspended in PBS with BSA, divided into aliquots collected in round-bottom tubes (Becton Dickinson) and stored at 4 °C for flow cytometric analyses. The flow cytometer analyses were performed on a FACSCalibur^{TM} cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software (Becton Dickinson) for data acquisition and analysis [21].

2.2.1. Flow Cytometric Analysis of Apoptosis with Annexin V–FITC Staining

For the annexin V assay, heart samples were stained with fluorescein isothiocyanate-conjugated annexin V using the ApoAlert kit from Clontech (Palo Alto, CA) according to the manufacturer's instructions.

2.2.2.. Flow Cytometric Analysis of Bcl-2 and Bax

Heart samples were prepared with PBS/BSA buffer then were incubated with Anti-Bcl-2 [100/D5] antibody (ab692) or Anti-Bax [6A7] antibody (ab5714) for 15 minutes at room temperature. Cells finally was re-suspended in 0.5% paraformaldehyde in PBS/BSA and analyzed by flow cytometer.

2.2.3. Flow Cytometric Analysis of Caspases-3 and 9.

The heart samples were prepared with a PBS/BSA buffer were incubated with antibody (FITC Rabbit Anti-Active Caspase-3 (CPP32; Yama; Apopain, BD Bioscience) or Rabbit monoclonalAnti-Caspase-9 (E23) antibody (ab32539) mixed well and incubated for 30 min at room temperature. The cells were washed with BD Perm/Wash (BD Bioscience), centrifuged at 400 xg for 5 min and the supernatant was discarded. Cells finally were re-suspended in BD Perm/Wash and analyzed using flow cytometer.

2.3. Statistical Analysis

Data were presented as the mean**±** S.D. In all cases, n refers to the number of animals in each treatment group. The statistical analyses were performed by one-way ANOVA followed by Students Newman–Keul's post hoc test. Statistical significance was considered as P < 0.05.

3. Results

After three weeks of treatment, the body weight of the rats in the diabetic group significantly decreased compared to the control group. The diabetic rats treated with SMN (80mg/kg) showed significant increase in the body weight compared with the diabetic group (Figure 1), however it still significantly lower than the control group.

The body weight of the rats significantly increased in the SMN group compared with the control group at the end of the experimental period.

The STZ treatment produced a significant elevation in the serum glucose and HBA1c 21 days after injection (Figure 2a & Figure 2b). Rats treated with STZ had significantly higher HOMA-IR and secreted significantly less insulin than the control group (Figure 2c& Figure 2d). The oral treatment of the diabetic rats with SMN displayed a significant control of serum glucose and HBA1c levels and showed significantly higher insulin and HOMA-IR values compared with that of the diabetic rats.

In the diabetic rats, total lipids, triglycerides, total cholesterol, LDL and VLDL were significantly higher while HDL was significantly lower than the control group (Table 1). The treatment of the diabetic rats with SMN exerted significant amelioration of lipid fractions toward

the control levels compared with that of the diabetic group (Table 1).
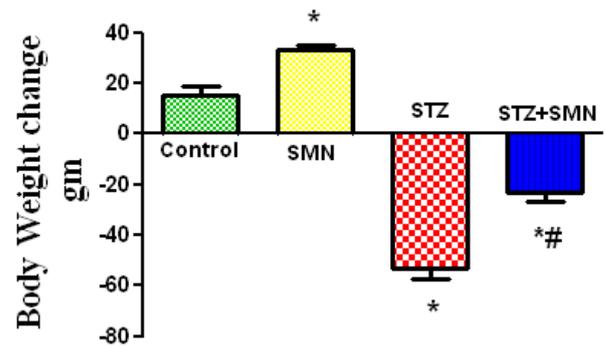

Figure 1. Effect of silymarin on body weight in different animal groups at the end of the experimental period. *P < 0.05 with respect to control group. #P < 0.05 with respect to diabetic group
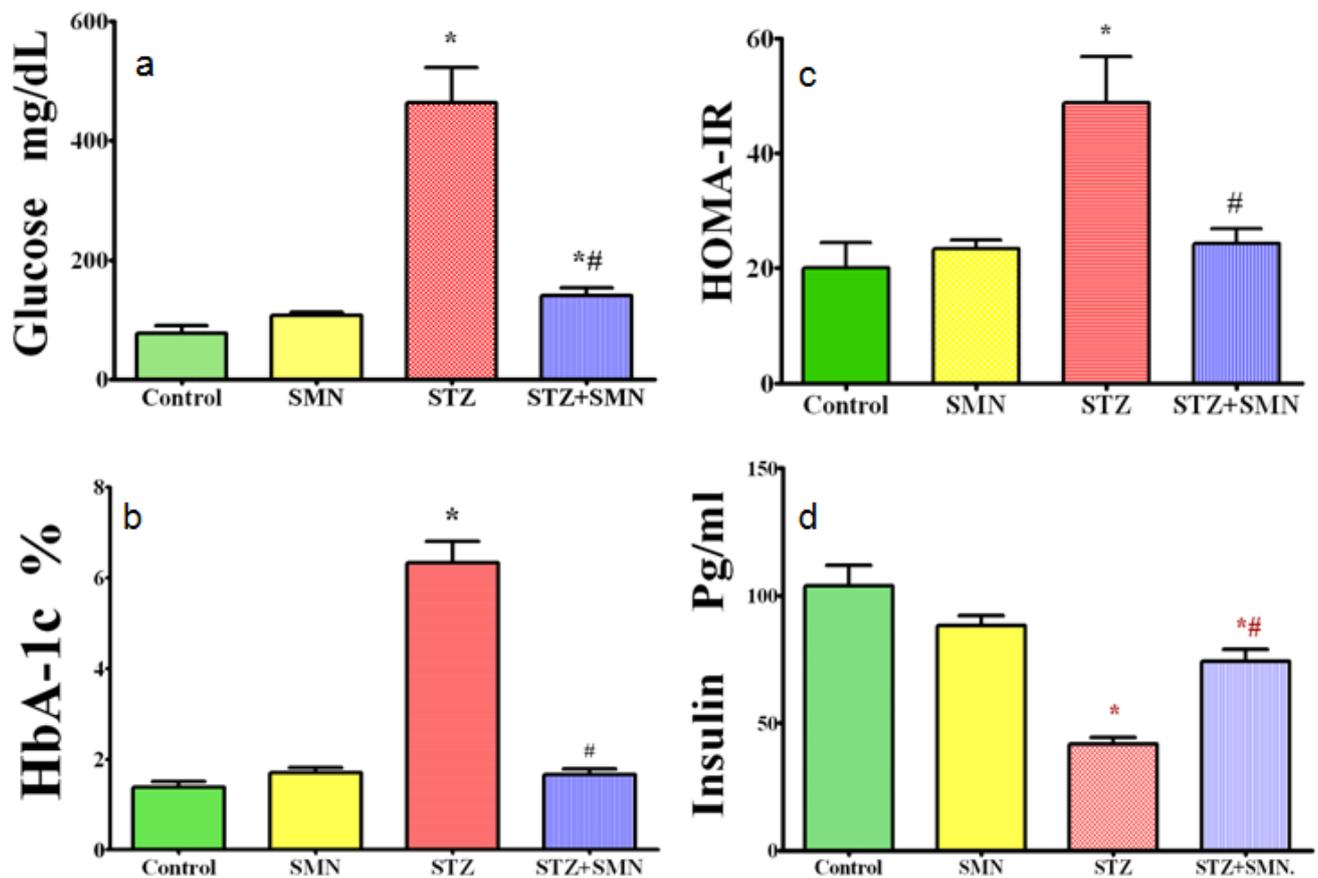

Figure 2. Effect of silymarin on the serum level of glucose (mg/dl), insulin (pg.ml), blood HBA1c % and HOMA-IR % of experimental groups. All values are expressed as mean ±SD. Each group consists of six albino rats. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group

Table 1. Effect of silymarin on serum lipid profile (total lipids (TL), cholesterol (Ch), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), and very low lipoprotein (VLDL) expressed as mg/dl in the different animal groups. All values are expressed as mean ±S.D. Each group consists of six albino rats. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group

	Total lipids	Triglycerides	Cholesterol	LDL	VLDL	HDL
Control	437± 15	58.1± 6.8	81.5± 6.0	28.5± 5.1	11.6± 1.4	41.4± 2.5
SMN	441± 27	71.8± 8.1	96.0 ±8.7	16.7± 2.5*	14.4± 1.6	64.9± 9.6*
STZ	1055± 26*	146.8± 18.8*	145.6± 6.7*	94.7± 8.9*	27.7± 3.4*	23.7± 1.6*
STZ+SMN	631± 38*#	97.3± 8.5*#	103.2± 3.3*#	19.4± 6.1*#	19.5± 1.7*#	59.8± 5.4*#

Animals that injected with STZ had significantly higher activities of CK-MB, LDH, GOT, and GPT, when compared with the control values. The oral treatment with SMN after diabetic induction ameliorated the increase in

the activities of these enzymes in the serum and displayed insignificant changes when compared with those of the control (Table 2).

A significant increase in lipid peroxidation was demonstrated in the heart of the diabetic rats indicated by a rise of the MDA concentration after STZ treatment (Table 3). The present results also revealed a marked amelioration of lipid peroxidation in the heart against diabetic-induced rise in MDA level by SMN administration after STZ treatment. The concentration of GSH in the heart significantly decreased after the diabetic induction by STZ. This effect was significantly prevented in the heart by SMN administration into diabetic rats.

Table 2. Effect of silymarin on the activities of serum CK-MB, LDH, GOT and GPT expressed as (U/ml) in the different animal groups. All values are expressed as mean ±S.D. Each group consists of six albino rats. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group

	CK-MB	LDH	GOT	GPT
Control	135 ± 8.8	201 ± 31.9	131 ±16.9	85 ±9.4
SMN	136 ± 9.6	157 ± 30.9	149 ±4.6	84 ±13.7
STZ	479 ± 34.9*	494 ± 25.9*	473 ± 8.3*	188 ±15.2*
STZ+SMN	175 ± 6.9*#	172 ± 14.1#	176 ±17.5#	94 ± 6.3#

Table 3. Effect of silymarin on lipid peroxidation product (MDA) (nmol/mg protein) and glutathione (GSH) (mg/g protein) levels in the heart of experimental group. All values are expressed as mean ±S.D. Each group consists of six albino rats. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group

	MDA	GSH
Control	6.46 ± 0.65	9.38 ± 0.42
SMN	5.33 ± 0.41	9.20 ± 0.11
STZ	14.87 ± 0.79*	3.48 ± 0.79*
STZ+SMN	4.51 ± 0.38*#	9.44 ± 0.10*#

To determine whether hyperglycemia led to apoptosis, the heart was subjected to flow cytometric analysis using Annexin V-FITC staining (Figure 3). The data show that the percentage of apoptotic cells significantly increased in the heart of diabetic rats compared with the controls. The treatment with SMN significantly minimized the number of apoptotic cells compared to the diabetic rats. The effects of diabetes on specific proteins that are related to apoptotic processes and the ameliorative ability of SMN against diabetes cardiomyopathy were assessed. The analysis demonstrated that the anti-apoptotic protein Bcl-2 significantly decreased while the apoptotic protein Bax was increased in the heart of diabetic rats and the treatment with SMN significantly normalized the changes in the expression of these proteins (Figure 4).
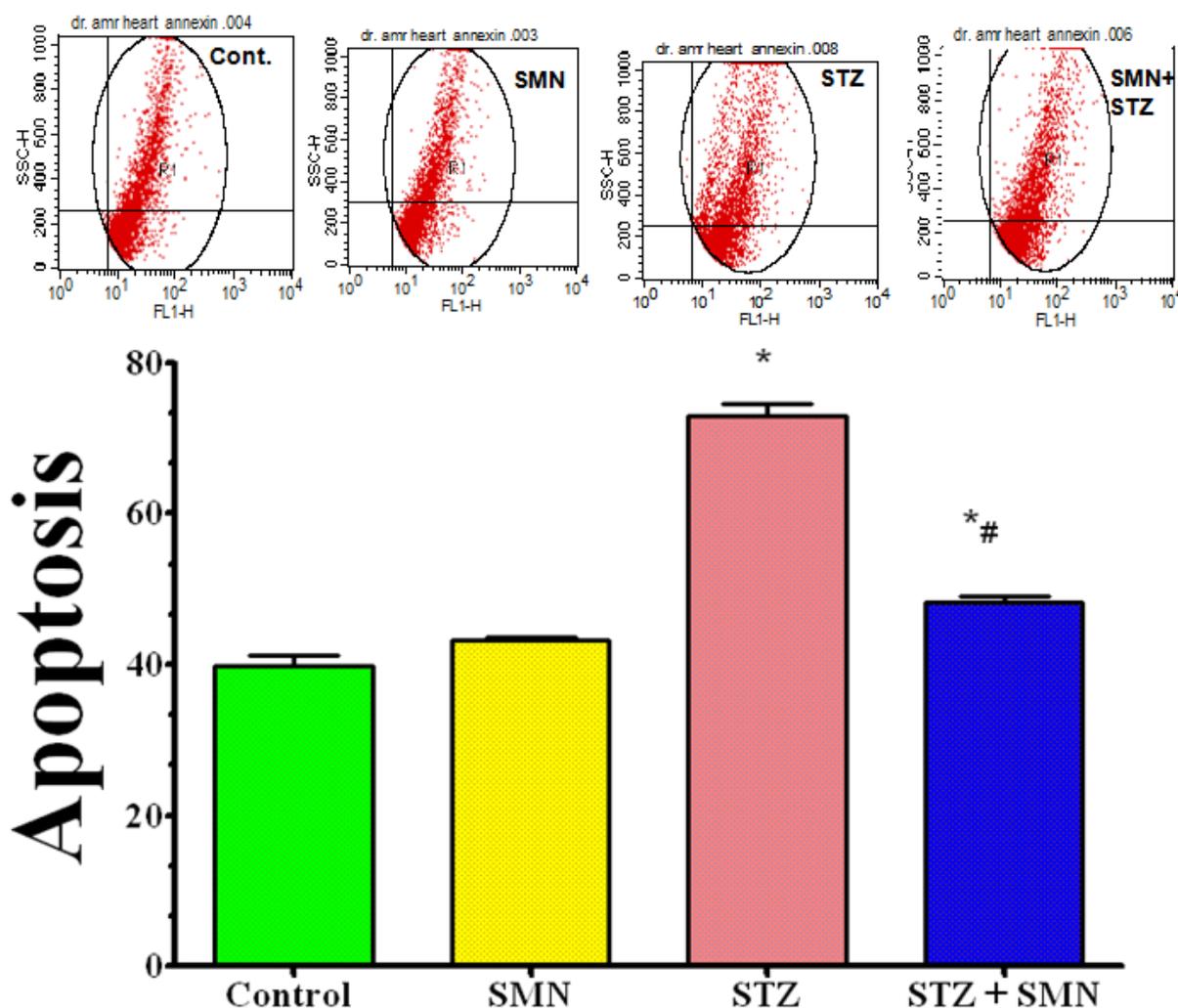

Figure 3. Flow cytometry analysis of rat cardiomyocytes showing the percentage of apoptosis in different animal groups. Flow cytograms showing examples of data generated by FAC on top of each histogram. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group
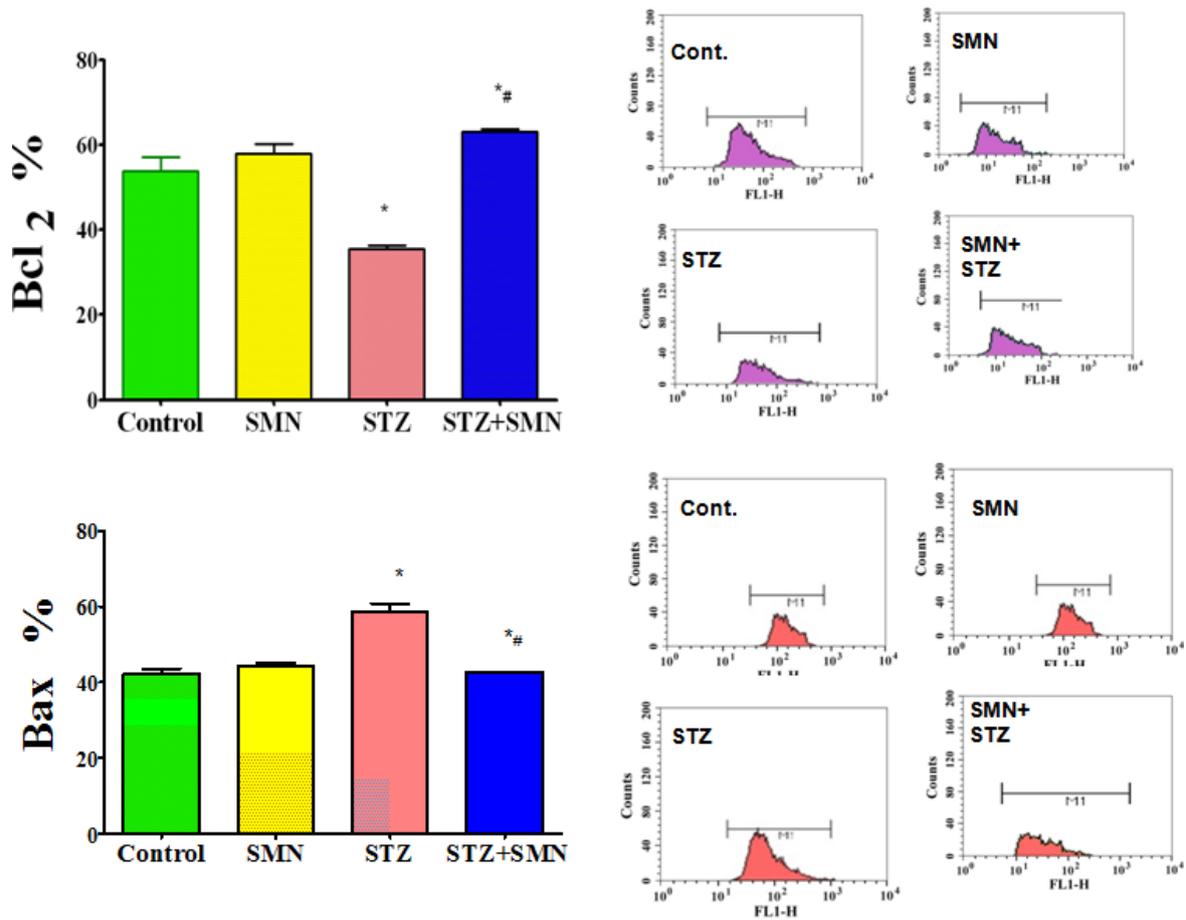
Figure 4. Flow cytometry analysis of rat cardiomyocytes showing the percentage of Bcl-2 and Bax in different animal groups. Flow cytograms showing examples of data generated by FAC at the side of each histogram. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group

Figure 5. Flow cytometry analysis of rat cardiomyocytes showing the percentage of caspases 3 and 9 in different animal groups. Flow cytograms showing examples of data generated by FAC at the side of each histogram. *P < 0.05 with respect to control group. # P < 0.05 with respect to diabetic group
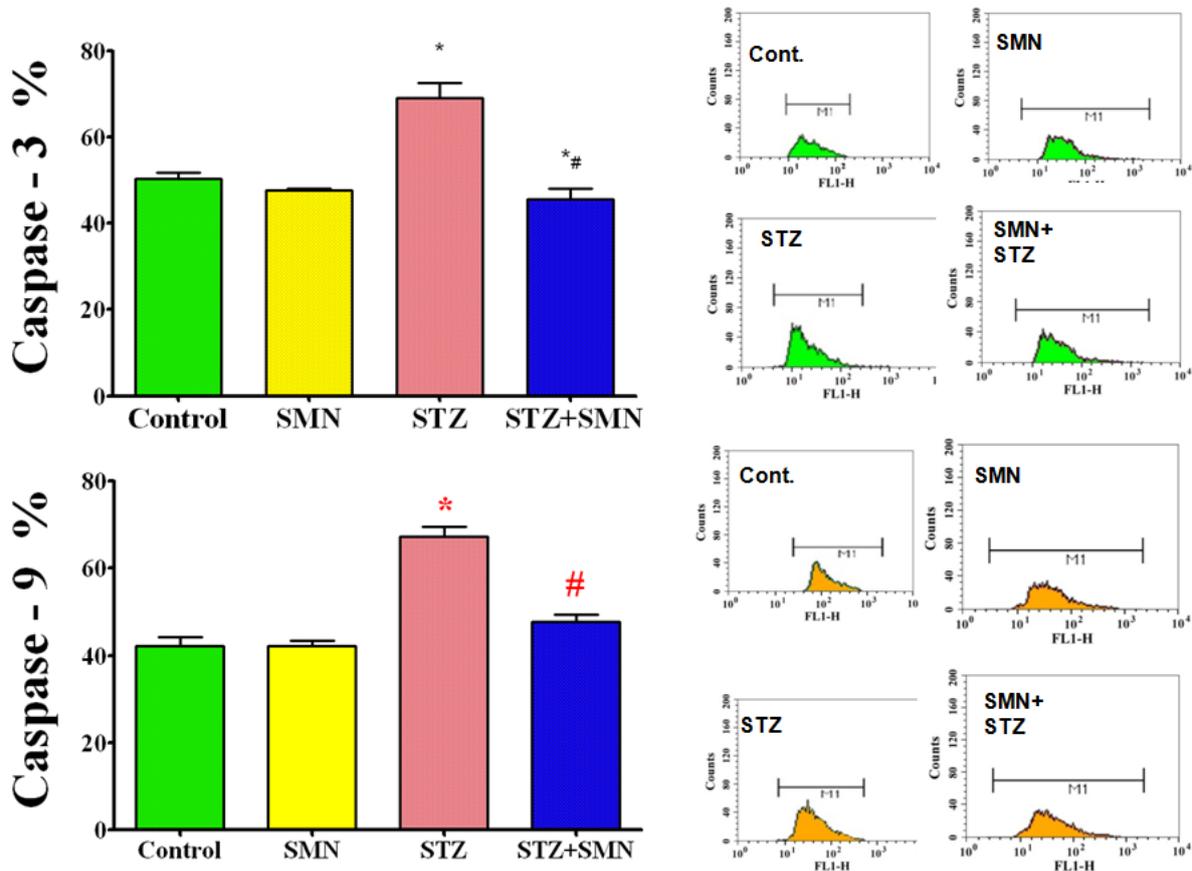
The expressions of caspases 9 and 3 were strongly detected in the heart from the diabetic rats, and the SMN treatment significantly inhibited the diabetes-induced activation of these apoptotic proteins (Figure 5).

4. Discussion

The escalating epidemic of cardiac injury is a pressing issue in clinical medicine worldwide. This is due to overwhelming increases in the incidence and prevalence of diabetes. Several studies demonstrate metabolic and cardiac health beneficial effects for many medicinal plants. However, precise molecular mechanisms of action are largely incomplete. The results of the present study showed that in the SMN-treated diabetic rats the body weights increased significantly compared with diabetic rats probably due to the protective effect of SMN in decreasing the gluconeogenesis and regulating muscle lose [22,23].

The blood glucose levels of diabetic rats significantly increased, but in SMN-treated group, it significantly decreased compared with blood glucose level in control rats. The present results support previous results that SMN showed a significant decrease in the serum glucose level [24,25] and a decrease in HBA1c concentration in the SMN-treated group compared to the diabetic group [22]. SMN also maintained serum insulin secretion and markedly improved HOMA-IR values. The present data support recent study in which treatment with silybin decreased HOMA-IR, serum ALT activity and markedly improved hepatic and myocardial damage [26].

Furthermore, significant increases in the serum total lipids, total cholesterol and triglycerides demonstrated in the diabetic rats compared to the control group. It is well known that lowering of serum lipid levels through dietary is major therapeutic trend to decrease the risk of cardiovascular disease and related complications. In the present study, when SMN was given to diabetic rats, the levels of serum lipid risk factors levels significantly reduced, while HDL level markedly increased in SMN-treated group. This finding is supportive of the SMN beneficial effect, since HDL levels are reported to correlate closely and inversely with the risk of heart disease [27]. HDL is strongly protective against atherosclerosis ensuring sufficient blood supply to the heart. Furthermore, SMN is a well-known hepatoprotective herbal medicine [28]. Thus, the improvement of liver function following SMN administration may positively control lipid and glucose metabolism.

Cytosolic enzymes leak into the intracellular space indicates cell membrane damage. Increased SGOT, SGPT, LDH, and CK-MB are well-known diagnostic markers of myocardial defect. In the present study, significant rises in the activities of these enzymes in the serum of the diabetic group suggest the incidence of significant membrane damage in cardimyocytes compared with the control group. These findings confirm the onset of myocardial defects and leaking out of the marker enzymes from heart to blood [29]. Treatment with SMN resulted in a significant reduction in the activities of these enzymes toward near normal levels compared with the diabetic group. This demonstrated that SMN could maintain membrane integrity thereby restricting the leakage of these enzymes.

Increased lipid peroxidation and decreased endogenous antioxidants are a well-known mechanism of cellular injury and has been used as an indicator of oxidative stress that leads to cardiomyopathy[7,30]. In support of these findings, a significant elevation of MDA and a decrease in GSH levels in the heart tissue, with significant increases in the CK-MB and LDH activities in the diabetic group were demonstrated compared with the control group. The results clearly indicate increased production of free radicals in STZ-induced diabetes mellitus leading oxidative injury because there is a loss of membrane integrity, with disruption of phospholipids in the membrane bilayer which potentially account for the increased serum enzymes including GOT, GPT, CK-MB and LDH. In group treated with SMN, the elevated oxidative stress and the activity of functional enzyme indicators were markedly ameliorated indicating the beneficial role of SMN on heart function. Similar study has established that SMN treatment prevented tissue damage and restored the activity and gene expression of antioxidant enzymes after alloxanadministration [31].

It has been shown that SMN prevents the damage induced by oxidative agents in hepatic membranes, microsomes and mitochondria [32,33]. These observations of the effect of SMN of hepatocyte protection may contribute for explaining why SMN has a protective effect on cardiac lipid peroxidation with the recovery of the CK-MB and LDH activities. This in turn may contribute to amelioration of heart function. Study performed by other investigators showed that SMN has a protective effect on pancreatic lipid peroxidation and cytokine mediated toxicity [11] with the recovery of the ß-cells function and restoration of serum glucose level [24]. The present result agrees with these reports and further demonstrated restoration of insulin level and HOMA-IR. Thus, the present results indicate a cytoameliorative effect of SMN in cardiac cells and that SMN may be therapeutically beneficial to decrease early diabetic cardiomyopathy.

Concomitant with the altered redox state in the heart, there was a significant increase in the expression of apoptotic protein Bax and a decrease in the anti-apoptotic protein Bcl2 with activation of caspase-9, and 3 supporting the increase in the number of apoptotic cells in the diabetic group. The mitochondrial pathway is the main pathway involved in the cardioprotection of SMN [34]. The disruption of these proteins indicates apoptosis developing through mitochondrial pathway of apoptosis. STZ-induced diabetes increased caspases 9 and 3 activities in the diabetic heart. The present data suggest that caspase-3 may be activated by the mitochondrial pathway following diabetes. Active caspase-3 ultimately leads to mobilization of caspase activated DNase into the nucleus, DNA fragmentation, and end-stage apoptosis [35].

Apoptosis of cardiomyoctes can be prevented by the inhibition caspase-dependent or independent pathways. Conversely, the survival of cardiac cells is generally regulated by antioxidant and anti-apoptotic proteins[36]. In this study, anti-apoptotic expression of Bcl2 and blocking Bax and caspases 9 & 3 were distinctive in the heart of the SMN-treated diabetic group, but that was not the case for those in the diabetic group indicating a control of apoptosis through mitochondrial pathway. Recently

SMN showed to modulate the apoptosis-regulating proteins through inhibition of diabetes-induced initiation of caspase-activated DNAse activity indicating the abolition of diabetes-dependent activation of genome-degrading pathways [37]. Additionally, SMN may prevent the activation of caspase-3 by controlling its upstream mitochondrial cytochrome c pathway [38]. In SMN-treated diabetic rats, the down-regulation of the caspases and up-regulation of Bcl-2 indicated that SMN directly blocks main steps in the mitochondrial apoptotic pathways.

Recent studies suggest that the lowered production of ROS in cells treated with SMN could prevent apoptotic signaling in the mitochondria through specific targeting of Bcl2/caspase-3 and improved mitochondrial membrane potential and membrane fluidity [38]. The stability and expression levels of Bcl-2 protein can be regulated by ROS through various mechanisms[39].SMN appears to maintain the amount of ROS generation and free radical detoxification systems within the levels required for optimal anti-apoptotic processes. This important balance has crucial effects on the control of redox-sensitive signaling proteins and apoptotic signals through the redox-sensitive pathways of apoptosis.

In conclusion, the present investigation confirmed that SMN sustains the integrity of membrane indicated by maintaining the activities of marker enzymes in the serum of diabetes-induced early cardiotoxicity in rats. This might be due to anti-lipoperoxidative and antioxidant effects of SMN. SMN normalized apoptotic regulating proteins and consequently protected heart against apoptosis. These data suggest that SMN control crucial steps in the mitochondrial pathway through its antioxidant properties. Thus, SMN may be a promising pharmacological agent for preventing the potential cardiotoxicity associated with diabetes.

Conflict of Interest

The author declares that there is no conflict of interest.

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