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# Fisetin ameliorates oxidative stress, inflammation and apoptosis in diabetic cardiomyopathy

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ABSTRACT

*Aims:* Hyperglycemia-mediated oxidative damage has been described as a major mechanism leading to pathologic changes associated with diabetic cardiomyopathy (DCM). Fisetin is a bioactive flavonol molecule found in many plants and possesses various biological activities. The present study investigated the protective effect of fisetin on diabetes-induced cardiac injury.

*Methods:* Diabetes was induced by streptozotocin (STZ) and both diabetic and control rats were treated with 2.5 mg/kg fisetin for six weeks.

*Key findings:* Diabetic rats exhibited hyperglycemia, and increased glycosylated hemoglobin and serum lipids accompanied with significant hypoinsulinism. In addition, diabetic rats showed several histological alterations in the myocardium, and significantly increased serum troponin I, creatine kinase-MB and lactate dehydrogenase. Oxidative stress, inflammation and apoptosis markers were increased, whereas antioxidant defenses were significantly reduced in the diabetic heart. Treatment with fisetin alleviated hyperglycemia, hyperlipidemia and heart function markers, and minimized histological alterations in the myocardium. Fisetin suppressed oxidative stress, prevented inflammation and apoptosis, and boosted antioxidant defenses in the heart of diabetic rats.

*Significance:* Fisetin attenuated the development of DCM *via* amelioration of hyperglycemia/hyperlipidemia-mediated oxidative stress, inflammation and apoptosis. Therefore, it might be worth considering the therapeutic potential of fisetin for human DCM.

## 1. Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and can cause long-term damage and dysfunction of different organs [1,2]. Diabetic cardiomyopathy (DCM) describes pathological changes in the myocardium in diabetes and is not directly attributable to coronary artery disease or hypertension [3,4]. The pathophysiological mechanisms underlying myocardial damage in diabetes are complex and multifactorial, including hyperglycemia-mediated oxidative stress, inflammation and activation of cell death pathways. These processes can eventually lead to heart failure [4–6].

Hyperglycemia-mediated oxidative stress may activate different inflammatory and cell death pathways which are involved in the development of DCM [5]. In addition to membrane lipid peroxidation and protein carbonylation, excess reactive oxygen species (ROS) activate the apoptotic signaling pathways in the diabetic heart, including mitochondrial apoptotic pathway [2,4]. Moreover, several reports showed that DCM was characterized by a significant increase in pro-inflammatory cytokines in the myocardium [7–9]. The levels of pro-inflammatory cytokines were positively correlated with ROS and diminished left ventricular (LV) function [10,11]. Since extensive evidence indicates the role of oxidative stress in the development of DCM, much attention has been paid to the usage of various antioxidants as a promising approach to attenuate DCM and other diabetes complications.

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a bioactive flavonol molecule found in many fruits and vegetables, such as, strawberry, apple, persimmon, grape, onion and cucumber [12]. Fisetin has gained great attention in the scientific community due to its antioxidant and ROS

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scavenging properties [12,13]. It has shown strong anti-inflammatory, antioxidant, antidiabetic, neuroprotective and cardioprotective effects in several preclinical studies [14–16]. Fisetin protected against cisplatin-induced renal injury by modulating nuclear factor-kappaB (NF-κB) activation, inhibition of apoptosis and restoration of antioxidant defenses [17]. In addition, fisetin attenuated ischemia/reperfusion (I/R)-induced cardiac injury through decreasing oxidative stress and apoptosis and restoration of the structure and function of mitochondria [18]. Besides, fisetin was shown to decrease ROS levels and up-regulate the expression of antioxidant genes in cardiomyocyte hypertrophy induced by phenylephrine (PE) *in vitro* [19]. In a recent study, fisetin protected against doxorubicin (DOX)-induced cardiac injury through inhibition of multiple processes, including oxidative stress and inflammation [20]. Despite the multiple pharmacological effects, the ameliorative effect of fisetin on DCM hasn't been reported yet. Therefore, the present study was designed to investigate the protective effect of fisetin on hyperglycemia-induced cardiac injury and to understand the mechanism underlying the beneficial effects of this bioactive flavonol using a well-established model of type I diabetes in rats.

## 2. Materials and methods

### 2.1. Experimental animals

Adult male Wistar rats, weighing 220–250 g, obtained from VACSERA (Cairo, Egypt), were used in this study. The animals were housed at normal temperature (23 ± 2 °C) on a 12 h light/dark cycle, received a standard diet and water *ad libitum*, and acclimatized to the laboratory conditions for one week prior to experiments. The experimental protocol was approved by the local animal care review committee, and all procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH publication no. 85-23, revised 2011).

### 2.2. Induction of type I diabetes

An experimental model of type I diabetes was induced in rats as previously described [1]. In brief, overnight fasted rats received a single intraperitoneal (i.p.) injection of 50 mg/kg streptozotocin (STZ; Sigma, St Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5). Diabetes was confirmed 7 days after STZ injection by measuring blood glucose levels and rats with > 200 mg/dl glucose were included in the study.

### 2.3. Experimental design and treatments

The normal and diabetic rats were randomly divided into four groups (N = 6) as following:

Group I (Control): normal rats received 10% dimethyl sulfoxide (DMSO).

Group II (Fisetin): normal rats received 2.5 mg/kg body weight fisetin dissolved in 10% DMSO [17].

Group III (Diabetic): diabetic rats received 10% DMSO.

Group IV (Diabetic + Fisetin): diabetic rats received 2.5 mg/kg body weight fisetin [17].

The dose of fisetin used in this study was based on a previous study reporting the antioxidant activity of fisetin *in vivo* [17], and was regularly adjusted based on the body weight changes. Both fisetin and the vehicle 10% DMSO were administered by oral gavage daily for six weeks.

### 2.4. Biochemical assays

#### 2.4.1. Determination of glucose, HbA1c, insulin and lipids

Glucose was estimated in serum following the method of Trinder [21] and glycosylated hemoglobin (HbA1c) was determined in blood collected on EDTA using assay kits purchased from Bio-Diagnostic Co. (Giza, Egypt) and Biosystems (Costa Brava, Barcelona, Spain), respectively. A specific ELISA kit (RayBiotech, Norcross, GA, USA) was used to assay serum insulin levels. Serum total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) were determined according to the standard methods using colorimetric assay kits (Spinreact, Girona, Spain). Low-density lipoprotein cholesterol (LDL-C) level in serum was estimated using Friedewald's formula: LDL-C (mg/dl) = TC − (HDL-C + (TG / 5)). Atherogenic index of plasma (AIP) and cardiovascular risk indices were calculated as following [22]: AIP = $Log_{10}$(TG / HDL-C), cardiovascular risk index 1 = TC / HDL-C and cardiovascular risk index 2 = LDL-C / HDL-C.

#### 2.4.2. Assay of cardiac function markers

The activities of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) were estimated using kits supplied by Spinreact (Girona, Spain) and BioVision (Milpitas, CA, USA), respectively. Troponin-I (cTnI) in serum was determined using ELISA kit purchased from Kamiya Biomedical Company (Tukwila, WA, USA). All assays were performed in accordance with the manufacturer's instructions.

#### 2.4.3. Determination of malondialdehyde (MDA), protein carbonyl and antioxidants

MDA [23] and protein carbonyl [24] levels were measured in the heart homogenate (10% w/v in 10 mM ice-cold Tris–HCl buffer (pH 7.4)). Reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) were determined according to the methods of Beutler et al. [25], Nishikimi et al. [26] and Aebi [27], respectively.

#### 2.4.4. Assay of pro-inflammatory cytokines and apoptosis markers

The levels of interleukin (IL)-1β and IL-6 in the heart homogenate were assessed using ELISA kits provided by RayBiotech (Norcross, GA, USA). Tumor necrosis factor (TNF)-α in the heart homogenate was estimated by using ELISA kit purchased from ALPCO (Salem, NH, USA). Bax, Bcl-2, caspase-9 and caspase-3 were estimated using ELISA kits (MyBioSource, San Diego, CA, USA). The protein content in the heart homogenate was assayed according to the previously described method of Lowry et al. [28].

### 2.5. Histopathological and immunohistochemical examination

Specimens from the heart of control and diabetic rats were collected and fixed in 10% neutral buffered formalin solution for 48 h and processed for paraffin embedding. Five-micron thick sections were sliced using a microtome (Leica RM 2155, England). The sections were routinely stained with hematoxylin and eosin (H&E) for examination. Other sections were stained with rabbit anti-NF-κB antibody (Santa Cruz Biotechnology, USA). In brief, heart sections were blocked in 3% hydrogen peroxide ($H_2O_2$), washed, blocked with protein block (Novocastra) and probed with rabbit anti-NF-κB antibody. After washing, the sections were incubated with the secondary antibody and then counterstained with hematoxylin.

### 2.6. Statistical analysis

All the values are represented as mean ± standard error of the mean (SEM) and all statistical comparisons were performed using the one-way ANOVA test followed by Tukey's *post-hoc* analysis. A $P$ value < 0.05 was considered significant. GraphPad Prism 5 software (San Diego, CA, USA) was used for the statistical analysis.

## 3. Results

### 3.1. Fisetin prevents body weight loss and ameliorates hyperglycemia in diabetic rats

At the end of 6 weeks, diabetic rats showed a significant

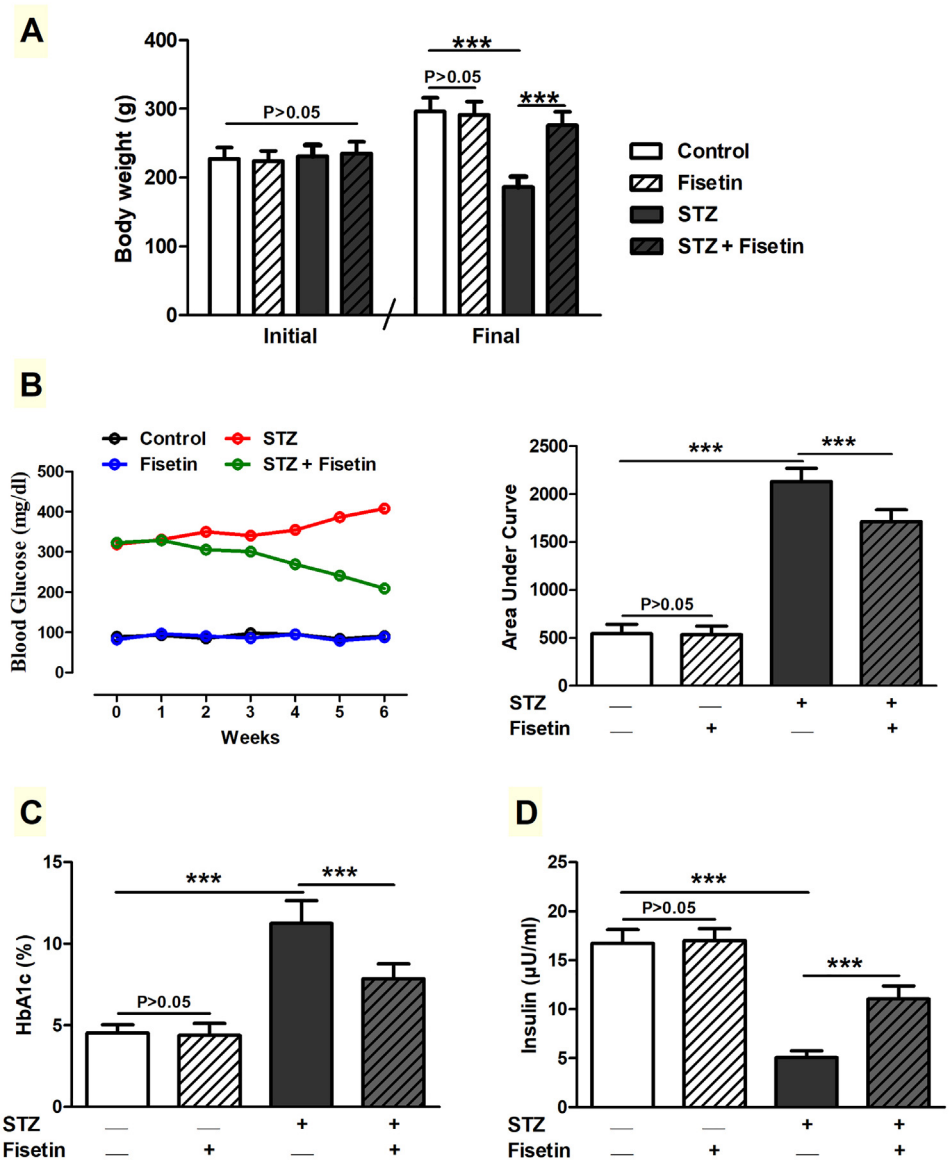


**Fig. 1.** Fisetin improves body weight and ameliorates hyperglycemia in diabetic rats. (A) Fisetin prevented body weight loss in diabetic rats. (B) Blood glucose was significantly increased in diabetic rats and treatment with fisetin for 6 weeks remarkably ameliorated blood glucose levels. Fisetin decreased HbA1c% (C) and increased serum insulin (D) levels in diabetic rats. Treatment with fisetin for 6 weeks didn't affect glucose, HbA1c% or insulin levels in normal rats. Data are expressed as Mean ± SEM, n = 6. ***$P < 0.001$.

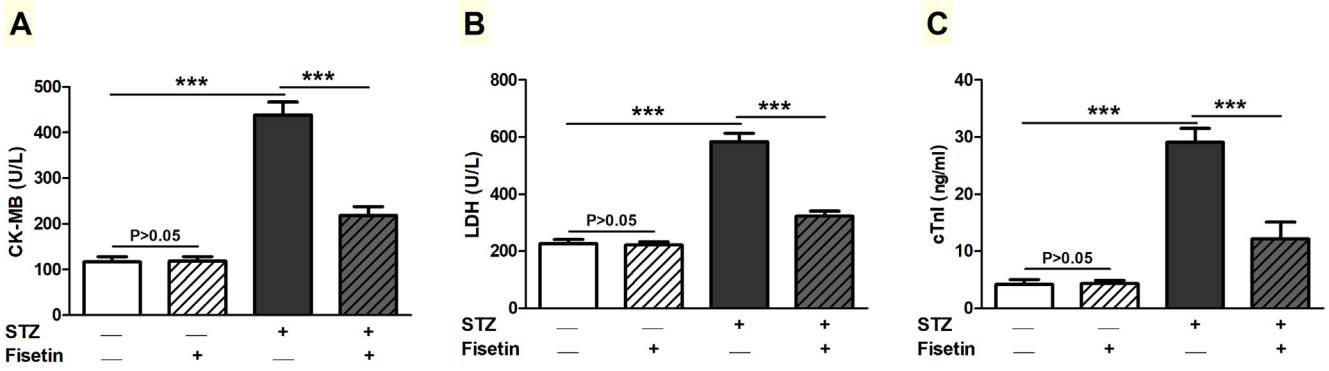


**Fig. 2.** Fisetin attenuates myocardial damage in diabetic rats. Treatment with fisetin for 6 weeks ameliorated serum levels of (A) CK-MB, (B) LDH and (C) cTnI in diabetic rats. Data are expressed as Mean ± SEM, n = 6. ***$P < 0.001$.

($P < 0.001$) reduction in body weight as compared to that of the control rats. Fisetin prevented the body weight loss in diabetic rats as represented in Fig. 1A.

Blood glucose measurements revealed a significant ($P < 0.001$) increase in glucose levels of the diabetic rats during the experimental period (Fig. 1B). In addition, diabetic rats showed a significant increase in HbA1c ($P < 0.001$; Fig. 1C), whereas serum insulin was significantly reduced ($P < 0.001$; Fig. 1D). In contrast, treatment of diabetic rats with fisetin significantly ameliorated ($P < 0.001$) glucose, HbA1c% and insulin levels. Oral administration of fisetin for 6 weeks didn't affect glucose, HbA1c or insulin levels in normal rats.

### 3.2. Fisetin attenuates myocardial damage in diabetic rats

To evaluate the ameliorative effect of fisetin on diabetes-induced myocardial injury, we assayed the circulating levels of the cardiac function markers, CK-MB, LDH and cTnI, and performed a histological study. Diabetic rats exhibited a significant ($P < 0.001$) increase in CK-MB (Fig. 2A), LDH (Fig. 2B) and cTnI (Fig. 2C) when compared with the control rats. Treatment of the diabetic rats with fisetin for 6 weeks resulted in a significant ($P < 0.001$) amelioration in the circulating levels of CK-MB, LDH and cTnI. Rats treated with fisetin for 6 weeks showed non-significant changes in serum levels of the assayed cardiac function markers ($P > 0.05$).

The cardioprotective effect of fisetin was further confirmed by the histological findings. H&E-stained sections in the heart of both control (Fig. 3A) and fisetin-supplemented rats (Fig. 3B) showed normal structure of the myocardium and no histological alterations. In contrast, sections in the heart of diabetic rats (Fig. 3C–E) revealed several histological alterations, including Zenker's necrosis, myocarditis, hyaline degeneration, pericarditis, vacuolations, edema, inflammatory cells infiltration, congested blood capillaries, extravasated erythrocytes and other manifestations (Table 1). Treatment with fisetin attenuated the diabetes-induced myocardial injury as depicted in Fig. 3F and Table 1.

**Table 1**
Lesion scores of different histological alterations among all experimental groups.

| | Control | Fisetin | STZ | STZ + Fisetin |
|---|---|---|---|---|
| Vacuolation of sarcoplasm | – | – | + + + | – |
| Zenker's necrosis | – | – | + + + | – |
| Pericarditis | – | – | + + + | – |
| Myocarditis | – | – | + + + | – |
| Hyaline degeneration | – | – | + + + | + |
| Intramuscular edema | – | – | + + | – |
| Congested blood capillary | – | – | – | – |
| Extravasated erythrocytes | – | – | + + + | – |

### 3.3. Fisetin ameliorates serum lipids and cardiovascular risk indices in diabetic rats

STZ diabetic rats exhibited a notable increase in serum TG (Fig. 4A; $P < 0.001$), TC (Fig. 4B; $P < 0.001$), LDL-C (Fig. 4C; $P < 0.001$) and vLDL-C (Fig. 4D; $P < 0.001$) when compared with the control group. Serum HDL-C was remarkably reduced in STZ diabetic rats as represented in Fig. 4E ($P < 0.001$). Diabetic rats treated with fisetin for 6 weeks showed a significant decrease in TG ($P < 0.001$), TC ($P < 0.001$), LDL-C ($P < 0.001$) and vLDL-C ($P < 0.001$), and increased HDL-C ($P < 0.05$). Normal rats received fisetin showed non-significant ($P > 0.05$) changes in serum lipids.

Given the role of altered serum lipids in the development of cardiac abnormalities, we calculated the cardiovascular risk indices in diabetic and fisetin supplemented rats. Diabetic rats exhibited significantly ($P < 0.001$) increased TC/HDL-C (Fig. 4F; $P < 0.001$), LDL-C/HDL-C (Fig. 4G; $P < 0.001$) and AIP (Fig. 4H; $P < 0.001$). Treatment with fisetin for 6 weeks improved the cardiovascular risk indices in diabetic rats ($P < 0.001$), but not in the normal rats.

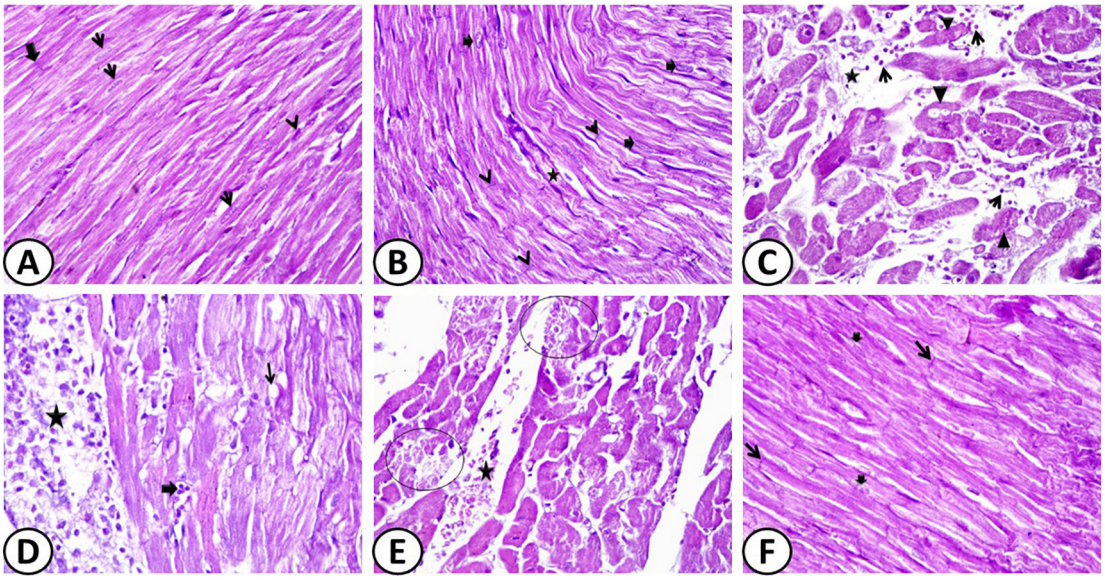


**Fig. 3.** Fisetin prevents histological alterations in the heart of diabetic rats. (A) Representative photomicrograph of the heart of the control rats showing normal cardiomyocytes which appeared as striated muscle fibers with normal nuclei (thin arrows), normal intercalated discs (thick arrow) and normal capillary endothelium (arrow head). (B) Photomicrograph of sections in the heart of normal rats treated with fisetin for 6 week showing normal cardiomyocytes with normal nuclei (thick arrows), normal intercalated discs (arrow heads) and normal intramuscular capillary (star). (C–E) Sections in the heart of diabetic rats showing [C] Zenker's necrosis (arrow heads), edema (star), inflammatory cells infiltration (thin arrows), [D] pericarditis (star), myocarditis characterized by infiltration of inflammatory cells mainly lymphocytes (thick arrow), degenerated muscle fibers and vacuolations (thin arrow), [E] multifocal myomalacia with vacuolated sarcoplasm (circles), and extravasated erythrocytes and few lymphocytes (star). (F) Representative photomicrograph of the heart of diabetic rats treated with fisetin for 6 weeks showing restoration of the normal appearance of cardiomyocytes, nuclei (thick arrows), striation, sarcolemma and intercalated discs (thin arrows). (H&E, ×400).

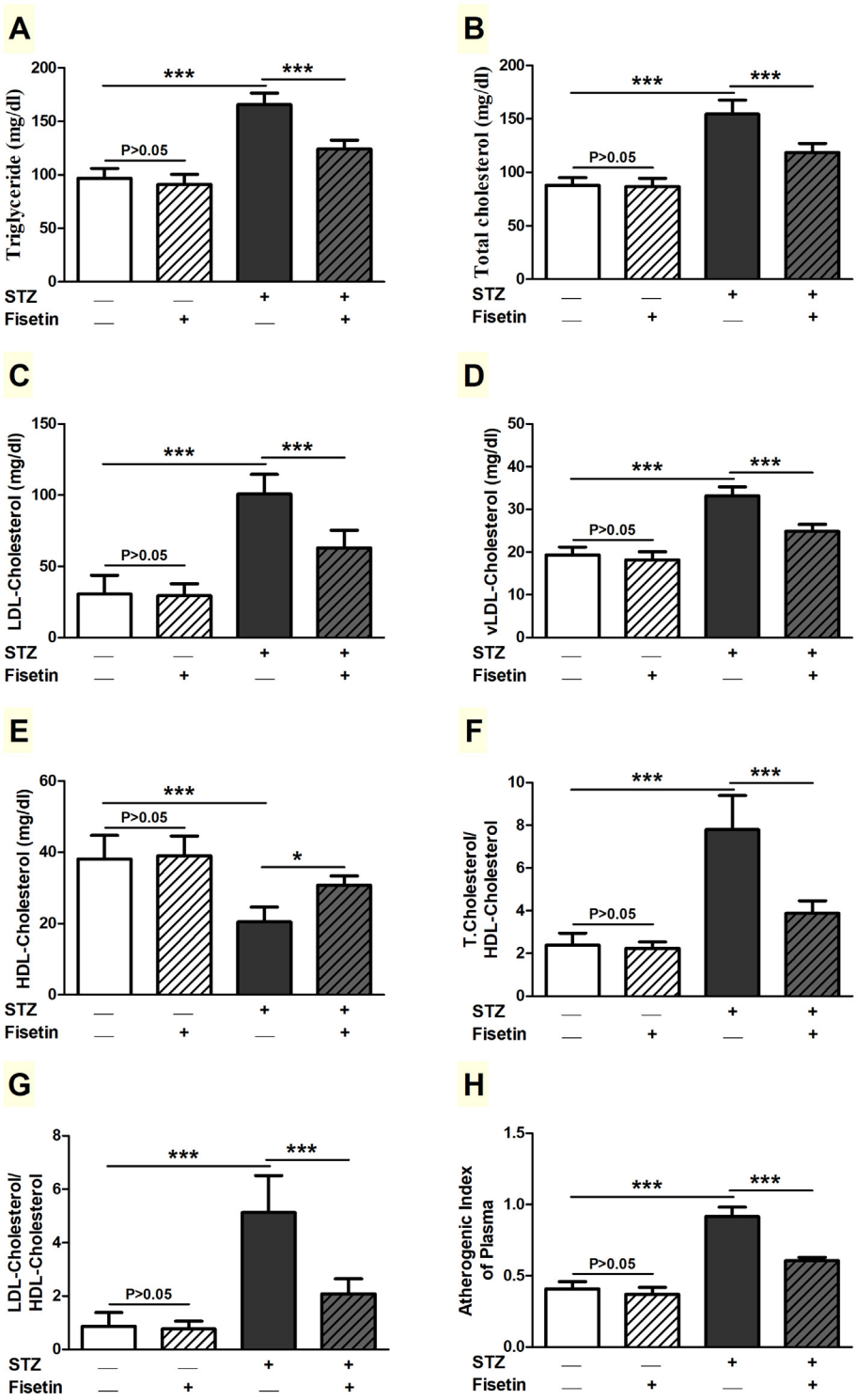


**Fig. 4.** Fisetin ameliorates serum lipids and cardiovascular risk indices in diabetic rats. Treatment of the diabetic rats with fisetin for 6 weeks significantly decreased serum (A) TG, (B) TC, (C) LDL-C, and (D) vLDL-C, and increased (E) HDL-C levels. Diabetic rats treated with fisetin showed a significant decrease in the cardiovascular risk indices (F) TC/HDL-C and (G) LDL-C/HDL-C, and (H) atherogenic index of plasma. Data are expressed as Mean ± SEM, n = 6. $^{*}P < 0.05$ and $^{***}P < 0.001$.

### *3.4. Fisetin attenuates diabetes-induced myocardial oxidative stress in rats*

Hyperglycemia and hyperlipidemia are associated with increased production of ROS; therefore, we examined the effect of fisetin treatment on oxidative stress markers and antioxidants in the heart of rats. STZ-induced rats showed a significant increase in the cardiac MDA (Fig. 5A; $P < 0.001$) and protein carbonyl levels (Fig. 5B; $P < 0.001$). The heart of diabetic rats received fisetin for 6 weeks exhibited a significant amelioration of both MDA and protein carbonyl levels. In the diabetic heart, GSH content (Fig. 5C), and activity of both SOD (Fig. 5D) and CAT (Fig. 5E) were significantly diminished ($P < 0.001$). Conversely, diabetic rats treated with fisetin for 6 weeks showed an improvement in cardiac GSH ($P < 0.01$), SOD ($P < 0.01$) and CAT ($P < 0.001$).

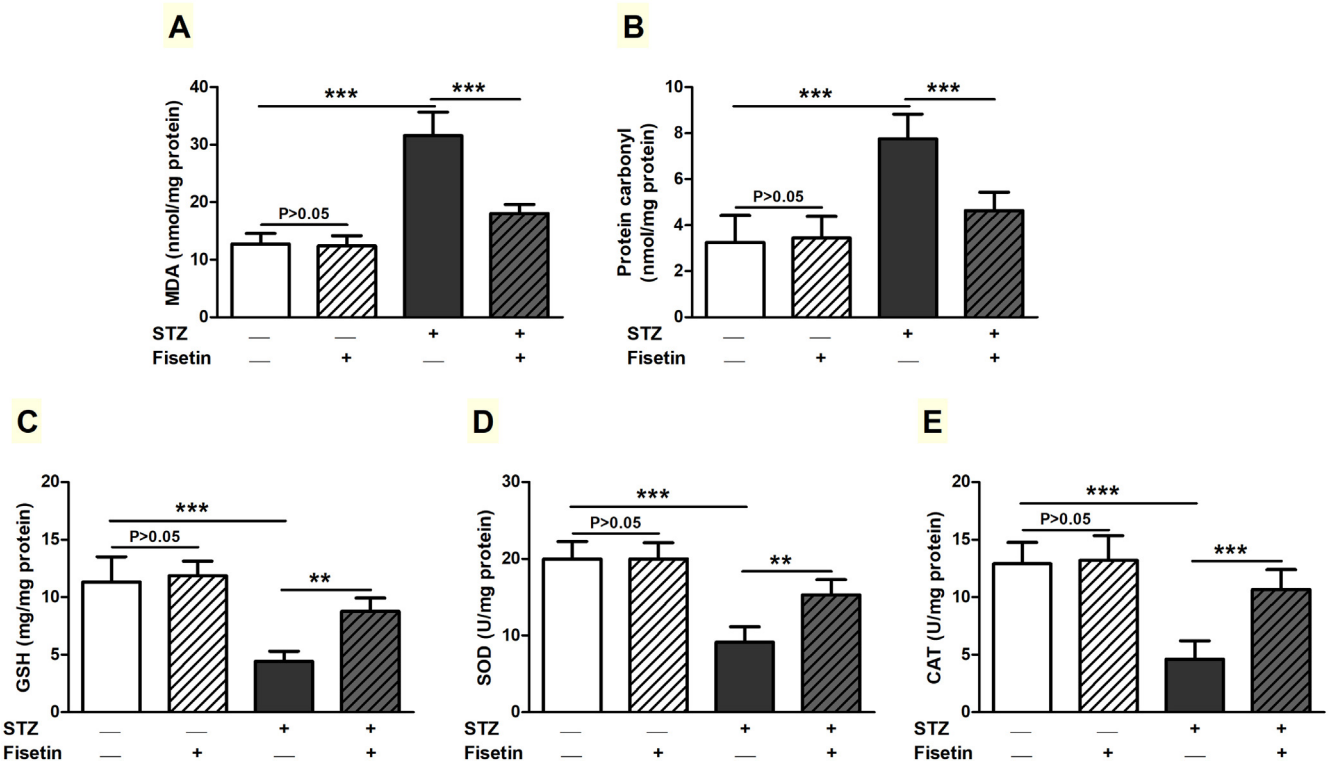


**Fig. 5.** Fisetin attenuates diabetes-induced myocardial oxidative stress in rats. The heart of diabetic rats treated with fisetin showed a significant decrease in the levels of (A) MDA and (B) protein carbonyl, and increased (C) GSH content, (D) SOD activity and (E) CAT activity. Data are expressed as Mean ± SEM, n = 6. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Normal rats treated with fisetin for 6 weeks showed non-significant changes in the cardiac levels of lipid peroxidation, protein carbonyl or antioxidants.

### 3.5. Fisetin suppresses inflammation in the diabetic heart

Diabetic rats showed a significant increase in myocardial NF-κB expression ($P < 0.001$; Fig. 6A–B), whereas fisetin treatment significantly decreased the expression of myocardial NF-κB ($P < 0.001$; Fig. 6A–B).

The pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were increased significantly ($P < 0.001$) in the heart of diabetic rats. In contrast, diabetic rats received oral administration of fisetin for 6 weeks showed a significant amelioration of the cardiac levels of IL-1β (Fig. 6C; $P < 0.001$), IL-6 (Fig. 6D; $P < 0.001$) and TNF-α (Fig. 6E; $P < 0.001$). Both cardiac NF-κB and pro-inflammatory cytokines were non-significantly changed in normal rats treated with fisetin for 6 weeks.

### 3.6. Fisetin prevents myocardial apoptosis in diabetic rats

To evaluate the beneficial effect of fisetin in attenuating apoptosis in the heart of diabetic rats, we assayed the expression levels of Bcl-2, Bax, caspase-9 and caspase-3. The anti-apoptotic protein Bcl-2 was significantly ($P < 0.001$) decreased in the heart of diabetic rats, whereas, treatment with fisetin increased it significantly ($P < 0.01$) as represented in Fig. 7A. The pro-apoptotic protein Bax (Fig. 7B) as well as the Bax/Bcl-2 ratio (Fig. 7C) were significantly ($P < 0.001$; $P < 0.001$) increased in the heart of diabetic rats, an effect that was reversed in rats treated with fisetin ($P < 0.001$). Caspase-9 (Fig. 7D) and caspase-3 (Fig. 7E) showed a significant ($P < 0.001$; $P < 0.001$) increase in the diabetic heart. In contrast, fisetin supplementation decreased both caspase-9 ($P < 0.001$) and caspase-3 ($P < 0.001$). All apoptotic markers showed non-significant changes in the heart of rats treated with fisetin for 6 weeks (Fig. 7).

## 4. Discussion

Hyperglycemia-mediated oxidative stress in the diabetic heart may contribute to increased cardiomyocyte death and this could explain the increased cardiac related morbidity and mortality in diabetes. Despite the significant advances in understanding the pathophysiology of DCM, the therapeutic options are still very limited. In the current study, we demonstrated that the flavonoid fisetin attenuated hyperglycemia-induced cardiac injury by mitigating oxidative stress, inflammation and apoptosis.

A STZ type 1 diabetic rat model was used to investigate the protective effect of fisetin against cardiomyopathy. Recently, we have reported diabetes-associated myocardial injury in STZ-induced rats [7,8]. STZ has been extensively used to induce experimental diabetes to investigate the anti-diabetic potential of therapeutic agents *in vivo*. STZ causes pancreatic β-cells necrosis by increasing the production of hydrogen peroxide and hydroxyl radicals, leading to oxidative damage of lipids, proteins and DNA [29]. This explains the observed hyperglycemia which was associated with decreased insulin levels in STZ injected groups in the present study. Furthermore, chronic hyperglycemia is associated with overproduction of oxidative agents causing progressive damage to pancreas and other tissues. Due to their low levels of antioxidants, β-cells are highly susceptible to further oxidative damage which in turn can worsen hyperglycemia [30]. Herein, STZ-induced rats exhibited marked hyperglycemia evidenced by increased HbA1c% along with hypoinsulinism. Treatment with fisetin for 6 weeks ameliorated glucose, HbA1c% and insulin levels. This anti-hyperglycemic effect of fisetin could be partially explained in terms of its antioxidant effect. Fisetin can protect the pancreatic β-cells against hyperglycemia-induced oxidative stress and hence preserve their functionality and insulin release. In this context, Prasath et al. [31] have demonstrated that fisetin suppressed oxidative stress and inflammation in the

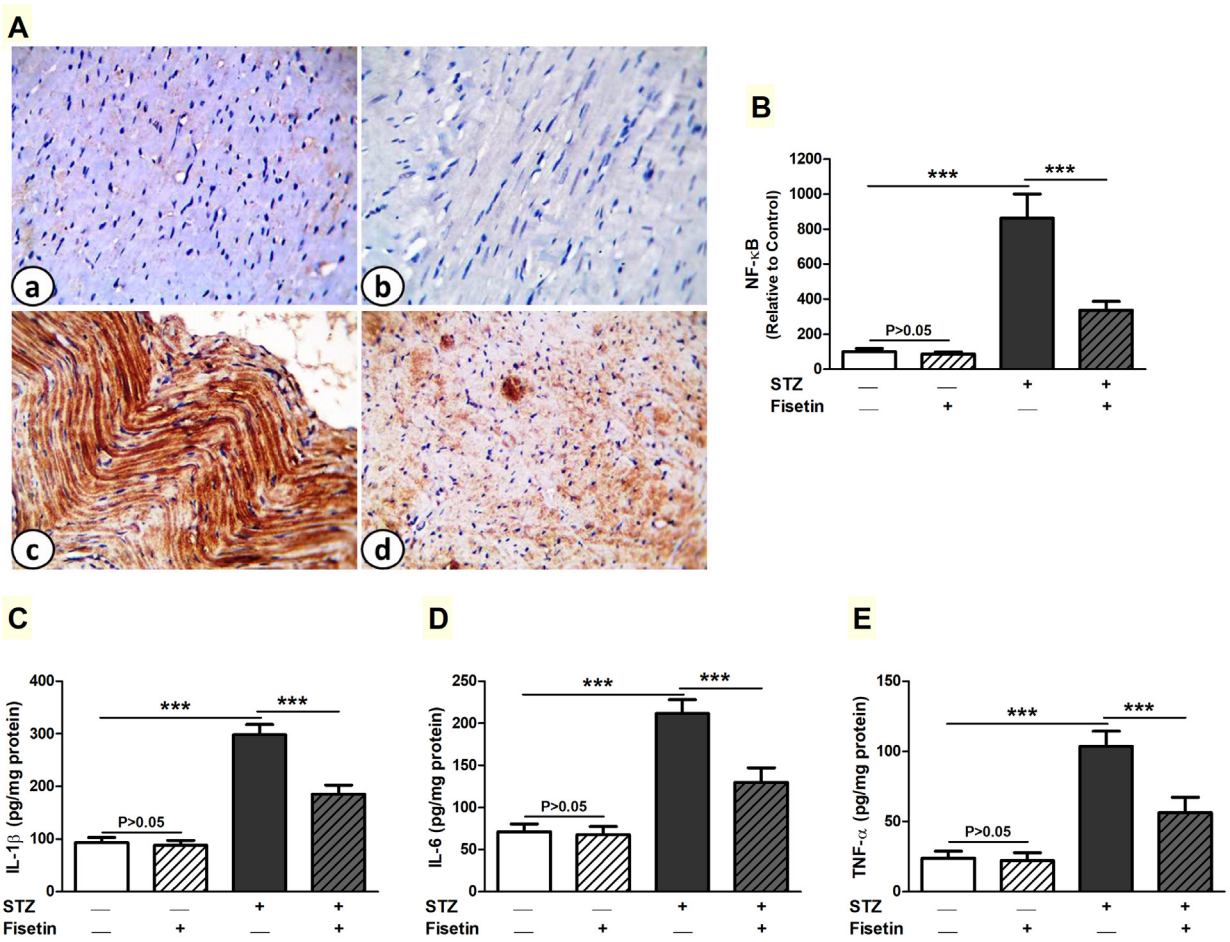


**Fig. 6.** Fisetin suppresses inflammation in the diabetic heart. (A) Immunohistochemical staining of NF-κB in the heart of (a) control and (b) normal rats treated with fisetin showing very weak reactivity, (c) diabetic rats showing intense immune-positive reaction of cardiomyocytes, and (d) diabetic rats treated with fisetin showing mild immune-positive reactivity of NF-κB (× 400). (B) Quantification of NF-κB immunostaining with ImageJ showing a significant increase in NF-κB expression in the heart of diabetic rats and the ameliorative effect of fisetin. (C–D) Pro-inflammatory cytokines in the heart of control and treated rats. Fisetin decreased the levels of (C) IL-1β, (D) IL-6 and (E) TNF-α in the myocardium of diabetic rats. Data are expressed as Mean ± SEM, n = 6. ***$P < 0.001$.

pancreas and subsequently increased plasma insulin and ameliorated glucose levels in STZ diabetic rats. Fisetin has also been shown to suppress hepatic glucose production *via* down-regulation of the mRNA expression of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) [14]. In addition, diabetic rats received 10 mg/kg fisetin for 30 days exhibited a significant improvement in the activity of hepatic and renal hexokinase, glucose-6-phosphate dehydrogenase, glycogen synthase, glycogen phosphorylase, pyruvate kinase and fructose-1,6-bisphosphatase, and subsequently ameliorated blood glucose levels [32]. Therefore, fisetin suppresses hyperglycemia *via* its dual action to protect pancreatic β-cells against oxidative damage and modulation of the carbohydrate metabolizing enzymes.

In addition to its anti-hyperglycemic effect, fisetin conferred protection against cardiomyocyte injury in diabetic rats. Diabetes associated cardiac injury in our study was evidenced by the elevated circulating levels of CK-MB, LDH and cTnI, and was further confirmed by the histological manifestations. CK-MB is a well-known sensitive marker of cardiomyocyte damage and a positive correlation between the myofibrillar disintegration and increased serum levels of CK-MB and cTnI has been reported [33]. In support of these findings, previous work from our lab has demonstrated elevated serum CK-MB and cTnI in experimental DCM in rats [7,8]. Interestingly, treatment with fisetin significantly ameliorated the circulating CK-MB, LDH and cTnI levels, and prevented hyperglycemia-induced cardiomyocyte injury, demonstrating its cardioprotective efficacy. The protective effect of fisetin has been recently reported in a rat model of DOX-induced cardiotoxicity [20]. Fisetin suppressed DOX-induced oxidative stress and inflammation in the heart of rats, and decreased serum levels of LDH and CK-MB [20]. Given the key role of dyslipidemia in provoking diabetic heart disease [34,35], the cardioprotective effect of fisetin in diabetic rats could be directly connected to its anti-hyperlipidemic effect. Here, diabetic rats showed an atherogenic lipid profile characterized by increased TG, TC, LDL-C and vLDL-C, and decreased HDL-C as we previously reported [7,8]. The negative impact of dyslipidemia on the heart has been demonstrated *via* assessment of the cardiovascular risk indices. Diabetic rats exhibited increased TC/HDL-C and LDL-C/HDL-C ratios as well as AIP, a frequent predictor of atherosclerosis. Hyperlipidemia in diabetes can induce ROS production and elicit ectopic lipid accumulation and subsequently lipoapoptosis, fibrosis and contractile dysfunction [36]. Furthermore, dyslipidemia and abnormal lipid metabolism were associated with both systolic and diastolic dysfunction in diabetic mice [37]. Therefore, management of hyperlipidemia is a key component to reduce the cardiovascular risk in diabetics [38]. Indeed, dietary polyphenols consumption can prevent the progression of heart disease *via* lowering serum lipids [2,39]. Treatment of the diabetic rats with fisetin markedly ameliorated serum lipids and decreased cardiovascular risk indices. In agreement with these findings, fisetin exerted anti-hyperlipidemic effect in diabetic rats [40] and displayed anti-hypercholesterolemic effects in high fat diet-induced rats

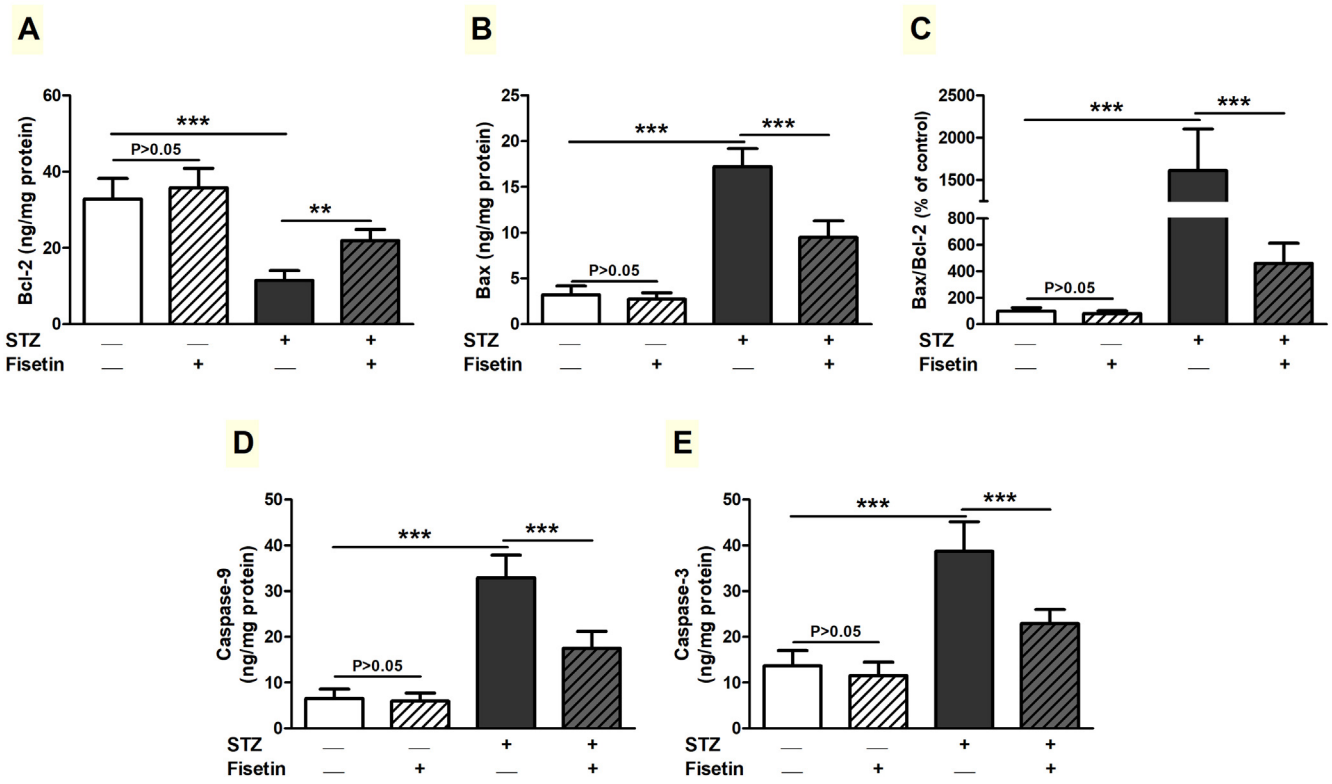


**Fig. 7.** Fisetin prevents myocardial apoptosis in diabetic rats. Treatment with fisetin increased Bcl-2 levels (A), and decreased Bax (B), Bax/Bcl-2 ratio (C), caspase-9 (D) and caspase-3 (E). Data are expressed as Mean ± SEM, n = 6. $^{**}P < 0.01$ and $^{***}P < 0.001$.

[41]. Modulation of genes involved in the metabolism of cholesterol and bile acids mediated the cholesterol-lowering effect of fisetin [41]. Thus, amelioration of hyperlipidemia plays a central role in the protective effect of fisetin against diabetic heart disease.

The coexistence of hyperglycemia and hyperlipidemia is associated with increased ROS production and oxidative damage. Hyperglycemia/hyperlipidemia-mediated oxidative stress is implicated in the pathogenesis and progression of DCM and other cardiovascular diseases [2,5,42]. Increased ROS generation accompanied with diminished antioxidant defenses promote oxidative stress in the diabetic heart [4,10]. Consistent with previous studies, the diabetic heart exhibited an increase in MDA and protein oxidation, coupled with reduced GSH and activity of antioxidant enzymes [2,7,8,12]. Lipid peroxidation can disrupt integrity of the phospholipid bilayer and inactivate membrane-bound receptors and enzymes, leading to increased cell permeability and death [43]. Moreover, ROS can diminish the cellular antioxidant capacity by promoting oxidation of the antioxidant enzymes [44]. Therefore, maintenance of the cellular redox balance represents an effective strategy to attenuate oxidative stress in various diseases. Herein, fisetin boosted antioxidant defenses and attenuated oxidative stress in the diabetic heart. In accordance, fisetin attenuated I/R- [18] and DOX-induced cardiac injury [20] by suppressing lipid peroxidation and restoration of endogenous antioxidants [18]. Additionally, activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) and other cytoprotective proteins might has a role in mediating the antioxidant potential of fisetin. In this context, fisetin protected against neuropathy in diabetic animals *via* the modulation of Nrf2 and NF-κB signaling [45]. In endothelial cells, fisetin up-regulated heme oxygenase (HO)-1 and protected against oxidative stress by up-regulating Nrf2 [46]. Nrf2 is a transcription factor controlling the expression of multiple antioxidant defenses to protect against ROS-induced oxidative damage [47–51]. A recent *in vitro* study has pointed to the role of sirtuins and forkhead box O3a modulation in mediating the antioxidant effect of fisetin [52]. Furthermore, the antioxidant potential of fisetin could be related to the *O*-dihydroxy structure in the B ring and the 3-hydroxy group and 2,3-double bond in the C ring [53]. These findings demonstrated that fisetin can attenuate myocardial injury in diabetes *via* attenuation of oxidative stress.

Several studies have demonstrated a strong correlation between oxidative stress, inflammation and apoptosis in diabetes. Hyperglycemia provokes the production of pro-inflammatory cytokines as a result of increased ROS levels [54]. Here, we demonstrated increased NF-κB expression in the diabetic heart in accordance with our previous findings [7]. NF-κB is a transcription factor which upon activation results in increased expression and release of pro-inflammatory cytokines. Elevated ROS, activation of extracellular signal-regulated kinase (Erk)1/2, up-regulation of mitogen-activated protein kinase (MAPK) signaling and degradation of IκB are the mechanisms underlying hyperglycemia-induced NF-κB activation and subsequent production of inflammatory mediators [55,56]. In addition, hyperlipidemia has been suggested as a contributing factor in NF-κB activation [57]. Diabetic rats in the present study exhibited an increase in cardiac levels of IL-1β, IL-6 and TNF-α. These inflammatory mediators play a role in diabetic heart disease and previous studies reported a positive correlation between their levels, oxidative stress and declined LV function in experimental DCM [58,59]. TNF-α has been implicated in cardiac hypertrophy and dysfunction. *In vivo* administration of TNF-α resulted in cardiac inflammation and dysfunction [60]. Similar effects have been reported in cardiomyocyte-specific TNF-α overexpression in mice [61]. In addition, treatment of diabetic rats with anti-TNF-α monoclonal antibody suppressed myocardial inflammation and fibrosis [62]. Hence, strategies to minimize pro-inflammatory cytokines production might have direct cardioprotective effects in diabetes [2,6]. Treatment of diabetic rats in the present study with fisetin markedly decreased the cardiac levels of pro-inflammatory cytokines. Accordingly, fisetin reduced TNF-α and IL-6 levels, myeloperoxidase activity and expression of inducible nitric oxide synthase (iNOS) in the kidney of cisplatin-treated rats [17] and suppressed inflammation in the heart

of DOX-induced rats [20]. Fisetin has suppressed the production of cytokines through multiple epigenetic changes including inhibition of NF-κB in high glucose-induced monocytes [63]. These findings corroborate our hypothesis that fisetin suppresses pro-inflammatory cytokines production in the diabetic heart and confers protection against DCM.

To further elucidate the cardioprotective efficacy of fisetin against diabetes-induced cardiac injury, the present study examined its effect on the expression of pro- and anti-apoptotic proteins. In the diabetic heart, several mechanisms are implicated in provoking apoptosis. These include hyperglycemia, dyslipidemia, excessive ROS generation, inflammation and mitochondrial dysfunction [64,65]. Herein, diabetic rats exhibited significantly increased cardiac levels of Bax, caspase-9 and caspase-3, coupled with decreased Bcl-2. Bax is a pro-apoptotic protein that is inserted into the mitochondrial membrane, leading to formation of channels and release of cytochrome *c* which binds to apoptotic protease-activating factor (APAF)-1 and caspase-9 in the cytosol to form the apoptosome complex [66]. Apoptosome activates caspase-3 which induces cell death by promoting the activation of caspase-3-activated DNase (CAD), resulting in DNA fragmentation [67]. Fisetin down-regulated Bax, caspase-9 and caspase-3, and increased Bcl-2 levels in the heart of diabetic rats. Bcl-2 protects against apoptosis by preventing cytochrome *c* release from the mitochondria [68]. This anti-apoptotic effect of fisetin against hyperglycemia-mediated activation of the mitochondrial pathways of apoptosis was supported by previous *in vivo* studies [17,20]. The anti-inflammatory potential of fisetin could be explained as a direct result of its anti-hyperglycemic, anti-hyperlipidemic and antioxidant potential.

In conclusion, this study indicates that fisetin attenuates cardiomyopathy *via* amelioration of hyperglycemia and attenuation of oxidative stress, inflammation and consequent apoptotic cell death. Thus, fisetin possesses a therapeutic potential for the treatment and/or prevention of DCM. However, further studies are needed to determine the exact molecular mechanisms underlying the therapeutic potential of fisetin.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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