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Original article

Fisetin protects against streptozotocin-induced diabetic cardiomyopathy in rats by suppressing fatty acid oxidation and inhibiting protein kinase R

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ABSTRACT

This study examined if the Fisetin against streptozotocin-induced diabetic cardiomyopathy (DC) in rats involves regulating cardiac metabolism and suppressing protein kinase R (PKR). Male rats were divided (12/groups) as control (non-diabetic), control + Fisetin, T1DM, and T1DM + Fisetin. Fisetin was administered orally at a final dose of 2.5 mg/kg for 12 weeks. In T1DM1-induced rats, Fisetin prevented heart and final body weights loss, lowered circulatory levels troponin I and creatinine kinase-MB (CK-MB), increased fasting insulin levels, and improved ventricular systolic and diastolic functions. It also preserved the structure of the cardiomyocytes and reduced oxidative stress, fibrosis, protein levels of transforming growth factor-β1 (TGF-β1), collagenase 1A, caspase-3, and the activation of JNK, p53, and p38 MAPK. In the control and diabetic rats, Fisetin attenuated fasting hyperglycaemia, the increases in glucose levels after the oral and insulin tolerance tests, and HOMA-IR. It also increased cardiac glucose oxidation by increasing the activity of private dehydrogenase (PDH), phosphofructokinase (PFK), protein levels of PPAR-α and suppressed cardiac inflammation by inhibiting NF-κB. These effects were associated with a reduction in the activity of PKR and subsequent increase in the activity of eeukaryotic initiation factor 2 (eIF2) with a parallel increase in protein levels of p67, a cellular inhibitor of PKR. In cultured cardiomyocytes, Fisetin, prevented high glucose (HG)-induced activation of PKR and reduction in p67, in a dose-dependent manner. However, the effect of Fisetin on PKR was diminished in LG and HG-treated cardiomyocytes with p67-siRNA. In conclusion, Fisetin protects against DC in rats by improving cardiac glucose metabolism and suppressing PKR.

1. Introduction

Diabetic cardiomyopathy (DCM) is a common complication seen in diabetic patients and is a major cause of increased mortality (Leon, 2015). Clinically, DCM is known to cause alterations in the structure and function of the heart, which compromises the ventricular diastolic and systolic function and ends up in heart failure (HF) (Pappachan et al., 2013; Jia et al., 2018; Ritchie and Dale Abel, 2020). The pathophysiology of DCM among patients with the first type of diabetes mellitus (T1DM) is mostly understood in humans and animals (Jia et al., 2018; Ritchie and Dale Abel, 2020). Accordingly, insulin deficiency, hyperglycemia, and peripheral and cardiac insulin resistance (IR) are major culprits responsible for the pathogenesis of DCM among humans and animal models with T1DM (Jia et al., 2018).

In general, the major cardiac underlying mechanisms initiate DCM involves oxidative stress, inflammation, remodeling, and apoptosis (Pappachan et al., 2013; Ritchie and Dale Abel, 2020). However, reactive oxygen species (ROS) generated in the mitochondria due to the increasing influx of NADH, Flavin adenine

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dinucleotide (FAAD), and free fatty acids (FFAs) are well-believed to be the major stimulus that induces all the other damaging effects including inflammation, fibrosis, apoptosis (Anderson and Rodriguez, 2011; Jia et al., 2018). Besides, the mitochondria ROS depletes endogenous antioxidants (i.e GSH) and activates numerous ROS generation enzymes such as xanthine oxidase and NADPH oxidase by activating numerous metabolic pathways such as polyol pathway, advanced glycation end productions (AGEs), protein kinase C (PKC) signaling, the hexosamine pathway, and RAAS (Giacco and Brownlee, 2010; Jia et al., 2018). However, suppressing the mitochondria ROS generation by antioxidants and inhibitors, or overexpressing the antioxidant transcription factor still the most promising approaches to prevent DCM (Thandavarayan et al., 2011; Ji et al., 2017; Joseph et al., 2017; Ge et al., 2019; Ritchie and Dale Abel, 2020).

Currently, experimental and clinical evidence has identified protein kinase R (PKR) as an important pathological link between metabolic stress, obesity, and DM due to its important and potent pro-oxidant, pro-inflammatory, and apoptotic roles in most cells (Gal-Ben-Ari et al., 2019). Initially, PKR is activated during the viral infection to contain it. However, other stimuli such as ROS, inflammatory cytokines, and ER stress can activate PKR by activating the PKR activating protein (PACT) (Gal-Ben-Ari et al., 2019). On the other hand, under resting conditions, the activity of PKR is kept silenced. The most common inhibitors of PKR in the cells include p58IPK and p67/methionine aminopeptidase 2 (MetAP2 or p67) (Gil and Esteban, 2000). Once activated PKR can inhibit pancreatic cell proliferation, impair glucose hemostasis, and induce insulin IR (Gal-Ben-Ari et al., 2019). PKR also suppress protein synthesis by inhibiting the transcription factor eIF-2 and induces cell inflammation, ROS, and apoptosis by activating inflammasome/nuclear factor-kappa Beta (NF-κB) axis and upregulation of other pathways such as p53 and other mitogen-activated protein kinases including NH2-terminal Jun kinase (JNK), and p38 (p38-MAPK) (Nakamura et al., 2010, Carvalho-Filho et al., 2012; Mangali et al., 2019; Gal-Ben-Ari et al., 2019).

Since all these pathways are activated in the diabetic heart (Evans et al., 2002; Bugger and Abel, 2014; Huynh et al., 2014; Wilson et al., 2018; Zhang et al., 2018; Sharma et al., 2018), it has been suggested that PKR is a key player in DCM. Indeed, increased activity and expression of PKR were shown in vivo in the hearts of diabetic rats, as well as *in vitro* after exposure to high glucose (HG) or palmitic acid (Udumula et al., 2016, 2018; Mangali et al., 2019). Likewise, the levels and the activities of PKR were significantly increased in the failing humans' and animals' hearts (Kalra and Dhar, 2017; Udumula et al., 2018). However, pharmacological inhibition of PKR was shown to be an effective therapeutic strategy to preserve contractility, prevent oxidative stress, and suppress inflammation and apoptosis in the hearts of diabetic animals and HG-exposed cultured cells (Udumula et al., 2016, 2018; Mangali et al., 2019). Therefore, targeting PKR seems to be a reasonable promising strategy to prevent and treat DCM.

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a well-known non-toxic tolerable flavonoid that is available in large amounts in a variety of fruits and vegetables (Rodius et al., 2020). Numerous experimental studies confirmed many cardioprotective properties of Fisetin in various animal models of DCM, ischemia–reperfusion injury (I/R), atherosclerosis, myocardial infarction (MI), and HF (Shanmugam et al., 2018; Dong et al., 2018; Ma et al., Althunibat et al., 2019; Rodius et al., 2020). In the majority of these studies, the cardioprotective effect of Fisetin was attributed to its potent effect to suppress mitochondria ROS generation, inhibit NF-κB, and blocks apoptosis. In the streptozotocin (STZ)-induced DCM in rats, Fisetin reduced fasting hyperglycemia, increased insulin levels, ameliorated dyslipidemia, and suppressed cardiomyocytes oxidative stress and inflammation with no effect on these markers in control rats (Althunibat et al., 2019).

However, if the cardioprotective potential of Fisetin against DCM is mediated by insulin/glucose-independent mechanisms (i.e modulating the activity of PKR) was never tested before. Therefore, in this study, we have tested the hypothesis that the Fisetin protects against DCM in streptozotocin-T1DM by inhibiting cardiac PKR activation, an effect that could be mediated by modulating the expression of MetAP2 (p67), p58IPK, and PACT.

2. Material and methods

2.1. Animals

Male Wistar albino rats weighing 170 ± 10 g and aged 8 weeks were provided from/maintained in the animal department unit at King Khalid University, Kingdom of Saudi Arabia (KSA). The animals were always being housed at 21 ± 2℃ and 61–64% humidity with 12/12 h dark/light cycle. All rats normal diet AIN-93G (Cat. No. D110700, Dytes, USA). Drinking water was available all through the day for all rats, *ad libetum*. Ethical approval for the use, treatment, and sampling was confirmed issued by the official Review Board at Princess Nourah University, Riyadh, KSA (IRB Number 20–0096), which follows the regulations issued by the National Institutes of Health (publication no. 85–23, revised 2011).

2.2. Induction of T1DM

T1DM was induced using STZ as previously published by others (Lo et al., 2017; Wang-Fischer et al., 2018). Briefly, STZ (Cat. No. 572201, Sigma Aldrich, MO, USA) was prepared in 0.05 M (pH 4.5) citrate buffer (Cat. No. S0130, Sigma Aldrich, MO, USA) to a final concentration of 300 mg/mL. Rats were administered, intraperitoneally, with this STZ solution at a final dose of 65 mg/kg. A 5% glucose solution was administered to rats for the first 12 h to prevent sudden hypoglycemia. Rats have blood glucose > 300 mg/kg measured after 48 h were considered to have T1DM and were used further for the experimental procedure.

2.3. Experimental groups

Rats were segregated randomly into 4 groups (12 rats/group) as **1) Control:** were non-diabetic rats and received 0.1% dimethyl sulfoxide (DMSO) (Cat. No 276855, MO, USA)) diluted with phosphate buffer saline (PBS), a vehicle, **2) Control + Fisetin:** were non-diabetic rats and administered Fisetin (2.5 mg/kg) (Cat. NO. ab142429, Cambridge, UK), **3) T1DM:** administered the diluted 0.1% DMSO, and 4) **T1DM + Fisetin:** were diabetic rats that were administered Fisetin (2.5 mg/kg). All treatments were administered to treated rats, orally and on a daily basis, for a total period of 8 weeks, a period that has been previously shown to induced DCM in rats (Wang-Fischer and Garyantes, 2018). The dose of Fisetin was adopted from similar studies which have confirmed its cardioprotective effect against DC and short-term cisplatin-induced nephrotoxicity in rats (Sahu et al., 2014).

2.4. Glucose and insulin tolerance

By the end of week 8, All rats were fasted on two separate days (12 h) and then exposed to either OGTT (oral glucose tolerance test; 2 g/kg) or IPITT (intraperitoneal insulin tolerant test; 0.75 units/kg) as described by others (Wong et al., 2015). In both cases, a blood sample (250 μl) were collected by cutting the tail, at 0.0, 15, 30, 60, and 120 min, in EDTA (10 μl of 10% of K3-EDTA/ml blood) Eppendorf tubes, centrifuged at 1000 × g for 5 min to collect

plasma. All plasma samples were kept at −20℃ and used later to measure plasma glucose (Cat. No. 81,693 Crystal Chem, IL, USA, respectively).

2.5. Evaluation of left ventricular function

One day after the glucose tolerance test and over 3 days period, all rats were anesthetized with ketamine hydrochloride/xylazine hydrochloride solution (1.9 mg/kg) (Cat. No. K-113, Sigma Aldrich, St Louis, MO, USA). Once anesthesia was confirmed in rats, both eyes of each rat were kept moist by applying a small amount of an eye ointment. After placing the rat on a heating table, the right carotid artery was located and separated from the surrounding tissue and nerves. A pre-calibrated Millar catheter, connected to a Power Lab data acquisition system (AD instrument, Australia), was directly forwarded to the LV. LV pressure was recorded for 30 min on the Power lab system and the recorded signal was analyzed using the associated LabChart-8 software. LV parameters derived from this signal were LV systolic and diastolic pressures and the maximum increase or decrease in pressure change over time (LVSP, LVEDP, dP/dt_{max}, and dP/dt_{min}, respectively).

2.6. Serum and tissue collection

After LV hemodynamics measurement, cardiac puncture protocol was used to collect the blood (1 ml) which were centrifuged (1500 × g/10 min) and used to collect serum. All serum samples were stored at −20℃ and used later for some biochemical analysis. Then, the rats were ethically killed and their hearts were rapidly extracted on ice and their LVs were cut into smaller pieces. Pieces of these LVs were directly placed in 10% buffered formalin or cacodylate buffer (containing 1% osmium tetroxide) for the morphological and ultrastructure studies. Other parts were stored at −80℃ for any further biochemical analysis.

2.7. Preparation of tissue homogenates and the cytoplasmic/nuclear fractions.

At the time of the use, frozen-LVs (25 mg) were homogenized in an appropriate volume of ice-cold phosphate-buffered saline (PBS/pH = 7.4) (Cat. No. 20012043) to which an appropriate volume of protease inhibitor (Cat. No A32965, ThermoFisher scientific) was added. Samples were centrifuged (1300 xg/4℃/ 10 min) and supernatants were stored at −70℃ until measurements. Nuclear and cytoplasmic fractions were prepared using a special tissue kit (Cat. No. 78835, ThermoFisher) per manufacturer's instructions.

2.8. Biochemical analysis in the serum and the LVs:

Serum Levels of troponin-I and creatinine kinase-MB (CKMB) were measured using special ELISA kits for rats (Cat. No. E4737 and Cat. NO. E4608, BioVision, CA, USA). Total levels of reduced glutathione (GSH) were measured using an assay kit (Cat. No. K454 (BioVision, CA, USA). Levels of interleukin-6 (IL-6) were measured using an ELISA kit (Cat. No. K4145, BioVision, CA, USA). Levels of superoxide dismutase (SOD) were measured using a rat's assay kit (Cat. No. E458, BioVision, CA, USA). Levels of tumor necrosis factor-alpha (TNF-α) were determined by an ELISA kit (Cat. No., K1052, BioVision, CA, USA). Levels of malondialdehyde (MDA) were determined using an assay kit (Cat. No. K454, BioVision, CA, USA). ROS levels were measured using a special fluorometric kit (Cat. No. E-BC-K138-F, Elabscience, USA). The activity of PKR was measured using a special rat's ELISA kit (Catalog No. LS-F16140, Lifespan BioSience Inc. (LSBio, Seattle, USA). The nuclear activity of NF-κB p65 was measured using an ELISA kit (Cat. No. Trans AM 40596, Active Motif, Tokyo, Japan). The absorbance and fluorescence signals in all analyses were measured by the M2 Spectramax plate reader (Molecular Devices, USA) or (FL600Bio fluorescent reader (Tek Instruments, Inc., USA). All analyses/kits were conducted for n = 6/group and following the manufacturer's instructions.

2.8.1. Isolation of cardiomyocytes

Isolated LV cardiomyocytes were prepared from the hearts of adult rats (Eid et al., 2019). After isolation, the cells (4×10^5) in 25-cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin under 95% air, 5% CO2, and 37 °C. The fibroblasts were removed by changing the culture media after 3 and 21 h. The cells were kept in the culture media for 8 days where the media was changed every 2 days.

2.8.2. siRNA

All reagents used for this study were supplied by Santa Cruze Biotechnology. Based on the results obtained from the in vivo part of this study, we have silenced the expression of MetAP2 using the siRNA (Cat. No. sc-61024, Santa Cruze Biotechnology, USA). The siRNA protocol was conducted as described by the manufacturer. In brief, the siRNA and control siRNA (Cat. No. sc-37007) were dissolved in RNAase free water and diluted to a final concentration of 10 μM in the siRNA Dilution Buffer (Cat. No. sc-29527). To prepare the transfection reagent, 6 μl of siRNA and 8 μl of the control siRNA of this prepared concentration, as well as 6 μl of the transfection reagent (Cat. No. sc-29528) were diluted, individually, with 100 μl of the transfection media and then all components were mixed at room temperature for 30 min. For the transfection, the isolated cardiomyocytes (2×10^5) were grown in humidified conditions 2 ml FBS supplemented antibiotic-free normal growth medium. Once reaching a confluence of 60–80%, the cells were washed twice with 2 ml transfection media (Cat. No. sc-36868) and then incubated with the working reagent (transfection reagent plus 0.8 ml transfection media) for 6 h in the Co2 incubator. To each transfection well, 1 ml of the 2X growth media was l and incubated under the same conditions for an extra 20 h. Then, the media was replaced with 1 × growth media and incubated for the next 48 h. The expression of MetAP2 was confirmed by western blotting.

2.8.3. Cell treatment and measurements.

Cells ($1X10^4$) were cultured in 60 mm culture dishes in either low glucose (LG) (5-μM) or high glucose (HG) (25-μM) with or without treatment with Fisetin (20 μM) for 24 h. In our preliminary data and using a dose–response experiment (5, 10, 20 μM), we have found that Fisetin is not toxic to LG-cultured cardiomyocytes and caused a dose–response increase in the levels of MetAP2 (p67) (supplementary Fig. 1). For this reason, we have selected the dose of 20 μM for this part. Cell survival was determined by the cell counting kit-8 (CCK-8) (Cat No. CK04-13, Dojindo, Japan). The levels of single-stranded DNA (ssDNA), a commonly used marker for cell apoptosis, were determined using an ELISA kit (Cat No. APT225, Millipore, USA). Besides, cells of all treatments were frozen at −80℃ and used later for western blotting experiments. All in vitro experiments were performed in three trials each performed in duplicate.

2.8.4. Preparation of cell homogenates for western blotting

Parts of the LVs or cultured cells were homogenized in RIPA buffer (Cat. No. 20012043, and Cat. No. 89900, respectively, ThermoFisher scientific) containing a protease inhibitor (Cat. No A32965, ThermoFisher Scientific) and centrifuged (1000 × g/10 min/4℃) to collect the supernatant containing the protein fractions. Protein concentration in the total cell homogenates and nuclear fractions were using an assay kit (Cat. No. 23200, ThermoFisher Scientific).

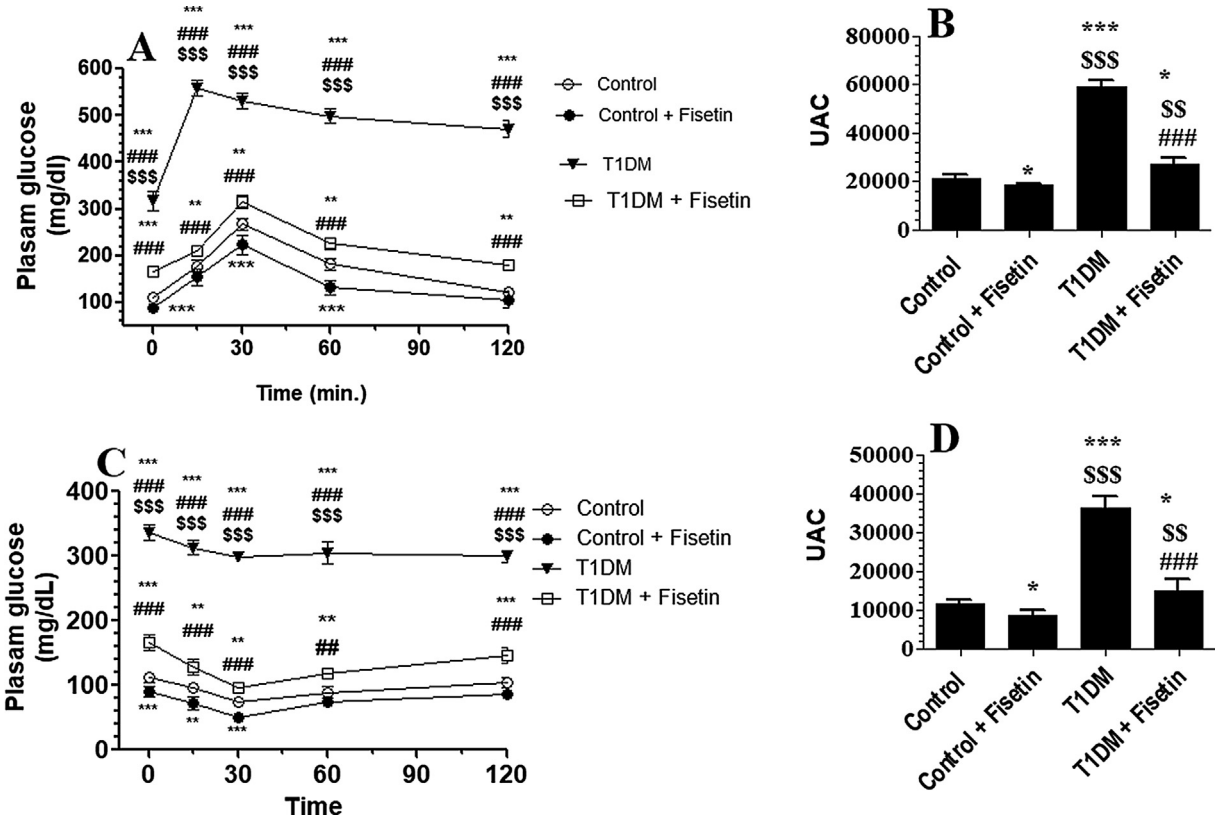

Fig. 1. Plasma glucose and its calculated area under the curve (UAC) after the oral glucose tolerance test (OGTT) (A & B) or intraperitoneal insulin tolerance test (IPITT) (C & D). Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. *, **, ***: vs. control at $p < 0.05$, 0.01, and 0.001, respectively. ##, ###: vs. Control + Fisetin at $p < 0.05$, and 0.001, respectively. $$$: vs. T1DM at $p < 0.001$.

2.8.5. Western blotting

Homogenates from LVs and cells were prepared in 5X SDS-PAGE loading buffer (Cat. No. MBS176755, MyBioSource, CA, USA). During western blotting, all washing, and dilution of antibodies were done using tris-buffered saline (TBS)-tween 20 (TBST) buffer. Proteins (40 μg/sample) were separated on an SDS page (various percentages, 8–15%) and electrically transferred on nitrocellulose membranes. Membranes were blocked with milk solution, washed, and incubated with the precise primary antibody (dilutions 1:500–1000): PKR (Cat. No. 3072), phospho-PKR (Thr446) (Cat. No. 3075), p-eIF-2α (Ser51) (Cat. No. 3398), JNK (Cat. No 9252,), p-JNK (cat. No. 9255), NF-κB p65 (Cat. No. 6956), p38 MAPK (Cat. No. 8960), p-p38 MAPK (Thr180/Tyr182), p53 (Cat. No.), Cleaved Caspase-3 (Asp175) (Cat. No. 9664), Lamin A (Cat. No.86846, 74 kDa), β-actin (Cat. No. 3700), MetAP2 (p67) Antibody (Cat. No. 11833), p58IPK (Cat. No. 2940) (Cell Signaling Technology, USA), and PACT (Cat. No. sc-377103), peroxisome proliferator-activated receptor-α (PPAR-α) (Cat. No. sc-398394) (Santa Cruz Biotechnology, USA). Membranes were then washed again and incubated with the corresponding 2nd antibodies. All antibodies were incubated at room temperature for 2 h with rotation. Membranes were stripped up to 3 times, in which the phosphorylated proteins were detected first. Bands were visualized using a price ECL substrate (Cat. No. 32109, ThrmoFisher Scientific). All bands were scanned and analyzed using the LI-COR C-DiGit scanner (USA). Normalization between the stripped gels was done using an internally known sample.

2.8.6. Microscopic studies

For morphological analysis, staining with hematoxylin and eosin (H&E) was used. For the evaluation of the cardiac fibrosis, Masson's trichrome (MT) staining was used. In both cases, parts of the LVs of all groups were first fixed in 10% buffered formalin for 20 h. Next, the fixed tissues were dehydrated in ethanol (70–100%), cleared in xylene, and embedded in paraffin wax. Then, all tissues were sectioned at a thickness of 5 μM, stained with the proper staining, and examined under a light microscope (Nikon Eclipse, model ME600). For the transmission electron microscope (TEM) to determine the ultrastructural changes, small parts of the LVs of all groups were fixed for 20 h in glutaraldehyde solution and then fixed in osmium tetraoxide-cacodylate buffer. The tissues were then dehydrated in ethanol and embedded in Spurr's resin. Finally, all LVs parts were stained with uranyl acetate, lead citrate stains, and examined under the TEM (model HF3300, Hitachi). All mages were examined by an external observer.

2.9. Statistical analysis

All analysis was done using the GraphPad Prism software (version 8). The degree of significance ($P < 0.05$) was calculated using two-way ANOVA and Tukey's post hock test. Data were presented as mean ± SD.

3. Results

3.1. Fisetin improves ventricular function and lowers glucose levels and tolerance in the control and T1DM-diabetic rats

The final body and heart weights were significantly reduced in the T1DM-diabetic rats as compared to control non-diabetic rats (Table 1). Concomitantly, a significant increase in the values of LVSP, dp/dt_{max}, dp/dt_{min}, as well as plasma levels insulin levels

Table 1
Changes in final body and heart weights, cardiac hemodynamics, and plasma glucose and insulin levels in all experimental groups.

	Control	Control + Fisetin	T1DM	T1DM + Fisetin
Final body wights (g)	372 ± 12.5	377 ± 15.8	278 ± 9.5$^{***###}$	338 ± 11.3$^{*#\$\$\$}$
Heart weight (g)	1498 ± 36.5	1515 ± 37.2	1122 ± 24.3$^{***###}$	1408 ± 18.6$^{*##\$\$\$}$
LVSP (mmHg)	102.2 ± 8.3	118.5 ± 9.1*	75.6 ± 7.9$^{***###}$	89.4 ± 6.5$^{*##\$\$\$}$
LVEDP (mmHg)	3.8 ± 0.67	4.1 ± 0.73	8.78 ± 1.1$^{***###}$	4.9 ± 0.71$^{*#\$\$\$}$
dp/dt_{max} (mmHg)	5786 ± 234	6765 ± 343*	3344 ± 287$^{***###}$	4983 ± 252$^{*#\$\$\$}$
dp/dt_{min} (mmHg)	4983 ± 198	5411 ± 276*	2321 ± 119$^{***###}$	4114 ± 235$^{*#\$\$\$}$
Plasma glucose (mg/dl)	112 ± 5.3	90.3 ± 4.2*	317 ± 21.2$^{***###}$	167 ± 6.4$^{***###\$\$\$}$
Plasma insulin (μlU/ml)	3.17 ± 0.17	3.19 ± 0.11	1.17 ± 0.08$^{***###}$	2.24 ± 0.17$^{**##\$\$\$}$
HOMA-IRI	0.87 ± 0.03	0.71 ± 0.03*	0.94 ± 0.08$^{*###}$	0.51 ± 0.03$^{***###\$\$\$}$

Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. *, ***: vs. control at p < 0.05 and 0.001, respectively. #, ##, ###: vs. Control + Fisetin at p < 0.05, 0.01, and 0.001, respectively. $$$: vs. T1DM at p < 0.001.

with a significant reduction in fasting plasma glucose levels and values of LVEDP were observed in the T1DM-diabetic rats as compared to non-diabetic rats (Table1). T1DM-induced rats also showed higher plasma glucose levels during the OGTT and IPITT at all measured intervals (15, 30, 60, 120 min) with higher AUCs as compared to non-diabetic rats (Fig. 1). However, T1DM + Fise

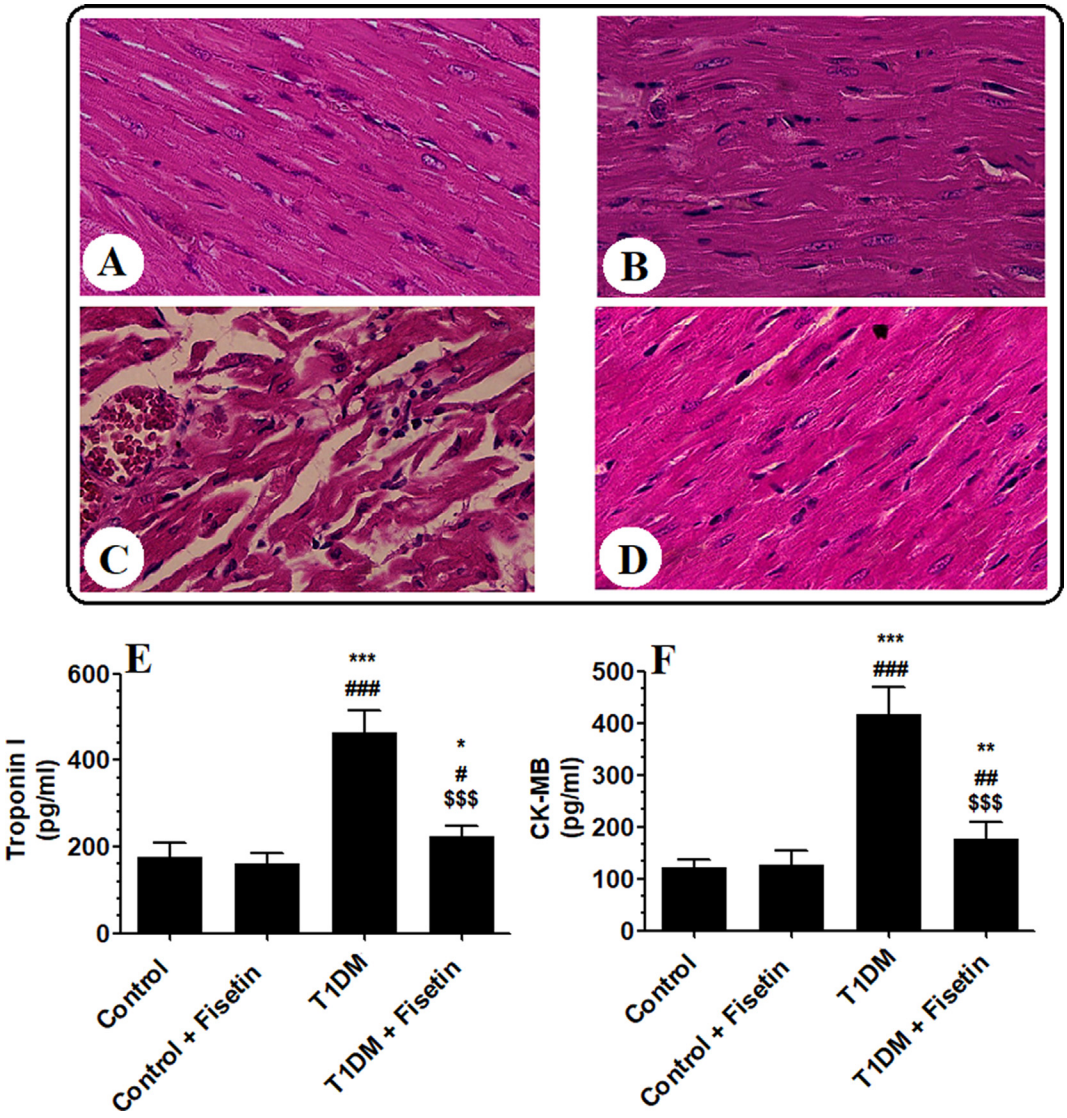

Fig. 2. Cardiac histological alterations (A-D) and serum levels of Troponin-I and Creatinine kinase-MB (CKMB) (F) in all experimental groups. ***A and B:*** were taken from control and control + Fisetin-treated hearts and showed normal myofibrillar structure with normal striations and branches (long arrow) and continuity with normal nuclei located centrally. ***C:*** was taken from a T1DM-treated rat (T1DM) and showed damage in myofibrils (short arrow), loss of striations, congestion (short arrow), and presence of inflammatory cells (arrowhead). ***D:*** was taken from a T1DM + Fisetin-treated rat and showed almost normal structure like the control hearts. ***For E and F:*** Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 6/group. *, **, ***: vs. control at p < 0.05, 0.01, and 0.001, respectively. #, ##, ###: vs. Control + Fisetin at p < 0.05, 0.01, and 0.001, respectively. $$$: vs. T1DM at p < 0.001.

tin-treated rats showed significantly reduced fasting glucose levels and LVEDP, a significant increase in LVSP, dp/dt_{max}, dp/dt_{min}, and insulin levels, and a significant reduction in glucose levels (and AUCs) at all measured intervals during OGTT and IPITT as compared to the T1DM-diabetic rats (Table 1 **and** Fig. 1**).** The final body and heart weights were also significantly increased in this group of rats (Table 1 **and** Fig. 1**).** Of note, a significant decrease in the levels of fasting plasma glucose and values of HOMA-IRI and LVSP were seen in the control + Fisetin-treated rats as compared to vehicle administered control rats (Table 1**).** Also, plasma glucose levels measured at 15, 30, and 60 min during the OGTT and IPITT were significantly decreased in the Fisetin + control-treated rats as compared to the vehicle administered control (Fig. 1**).**

3.2. Fisetin improves the histological and ultrastructural features and inhibits oxidative stress in the LVs of T1DM-diabetic rats

Normal histological and ultrastructural features were observed in the LVs tissue obtained from either the control or T1DM-diabetic rats (Fig. 2**A-D and** Fig. 3**A-D).** As compared to control non-diabetic rats, Fisetin + control rats showed non-significantly different serum levels of CK-MB or Troponin-I and LV levels of ROS, MDA, SOD, or GSH (Fig. 2**E&F and** Fig. 3**E-H).** However, the hearts obtained from T1DM rats showed severe myofibrillar damage with damaged nuclei, mitochondria, blood vessels. They also showed some congestion and increase the infiltration of inflammatory cells (Fig. 2**A-D and** Fig. 3**A-D).** Besides, circulatory levels of Troponin-I and CK-MB, as well as LV levels of ROS, MDA were significantly higher but the LV levels of GSH and SOD were significantly lower in T1DM-diabetic rats as compared to non-diabetic rats (Fig. 2**E&F and** Fig. 3**E-H).** On the other hand, normal and cardiac architectures with intact nuclei, mitochondria, blood vessels, and myofibrils with were seen in the LVs of the T1DM + Fisetin as compared to T1DM-diabetic rats administered the vehicle (Fig. 2A-D and Fig. 3A-D). Nonetheless, LV levels of ROS and MDA were significantly decreased and levels of SOD and GSH have significantly increased the LVs of T1DM + Fisetin as compared to T1DM-diabetic rats (Fig. 2**E&F and** Fig. 3**E-H).**

3.3. Fisetin diminishes cardia fibrosis in the LVs of the T1DM-diabetic rats and suppresses markers of inflammation in the LV of both the control and T1DM-diabetic rats

A normal amount of collagen fibers with non-significant protein levels TGF-β1 and collagenaseA1 were seen in the LVs of Control + Fisetin-treated rats as compared to non-diabetic rats administered

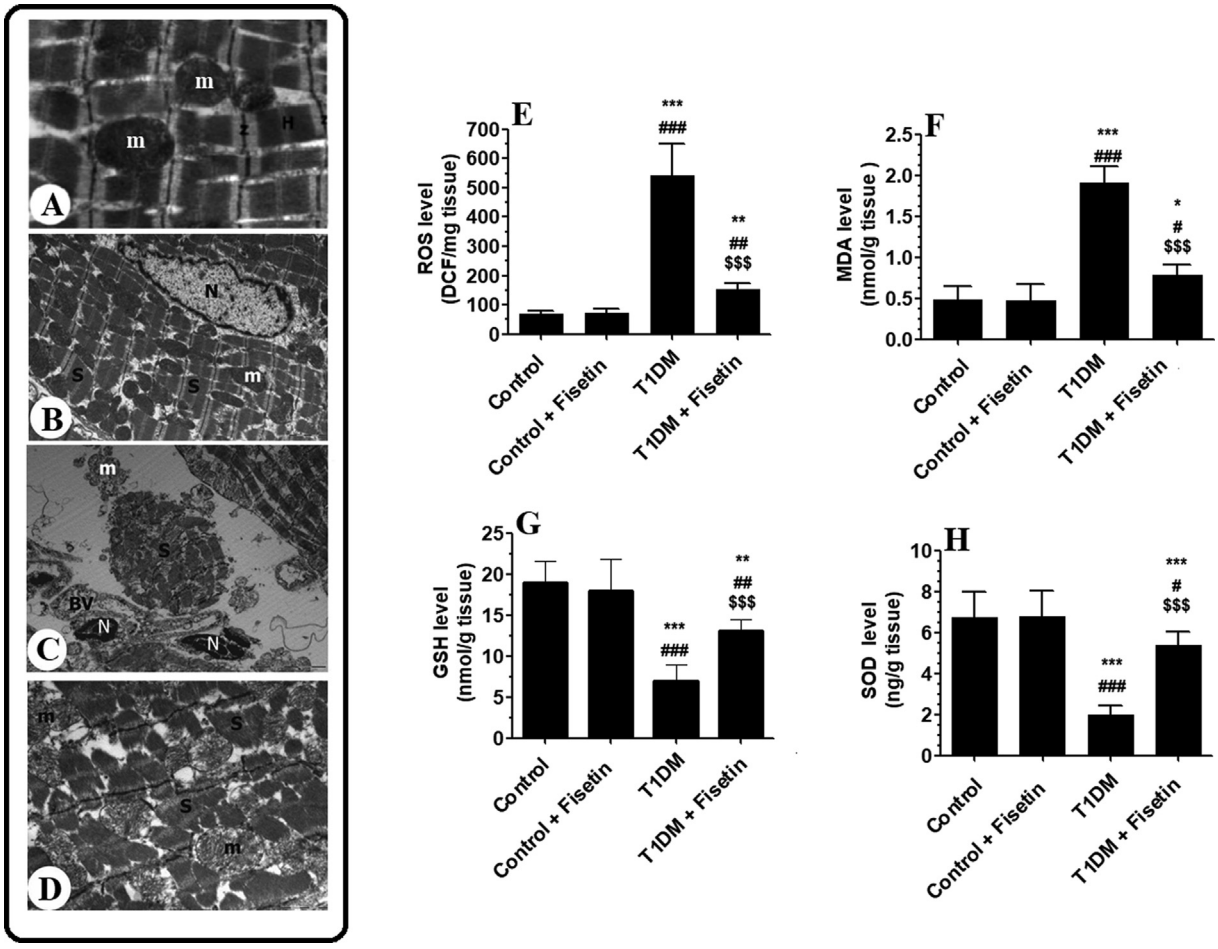

Fig. 3. Cardiac ultrastructural changes (A-D) and cardiac levels of reactive oxygen species (ROS) (E), malondialdehyde (MDA) (F), reduced glutathione (GSH) (G) and superoxide dismutase (SOD) (H) in all experimental groups. ***A and B:*** were taken from control and control + Fisetin-treated hearts and showed normal myofibrillar structure (S), in longitudinal section, well-identified Z-line, and H bands with intact mitochondria (m) and nucleus (N). ***C:*** was taken from a T1DM rat and showed a reduction in muscle fiber mass with severe degeneration of myofibrils (S), abnormal nucleus (N), and degenerated blood vessels (BV). ***D:*** was taken from a T1DM + Fisetin-treated rat and showed improvement in the structure of the myofibrils and mitochondria. ***In E-H:*** Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 6/group. *, **, ***: vs. control at $p < 0.01$, 0.05, and 0.001, respectively. #, ##, ###: vs. Control + Fisetin at $p < 0.01$, 0.05, and 0.001, respectively. $$$: vs. T1DM at $p < 0.001$.

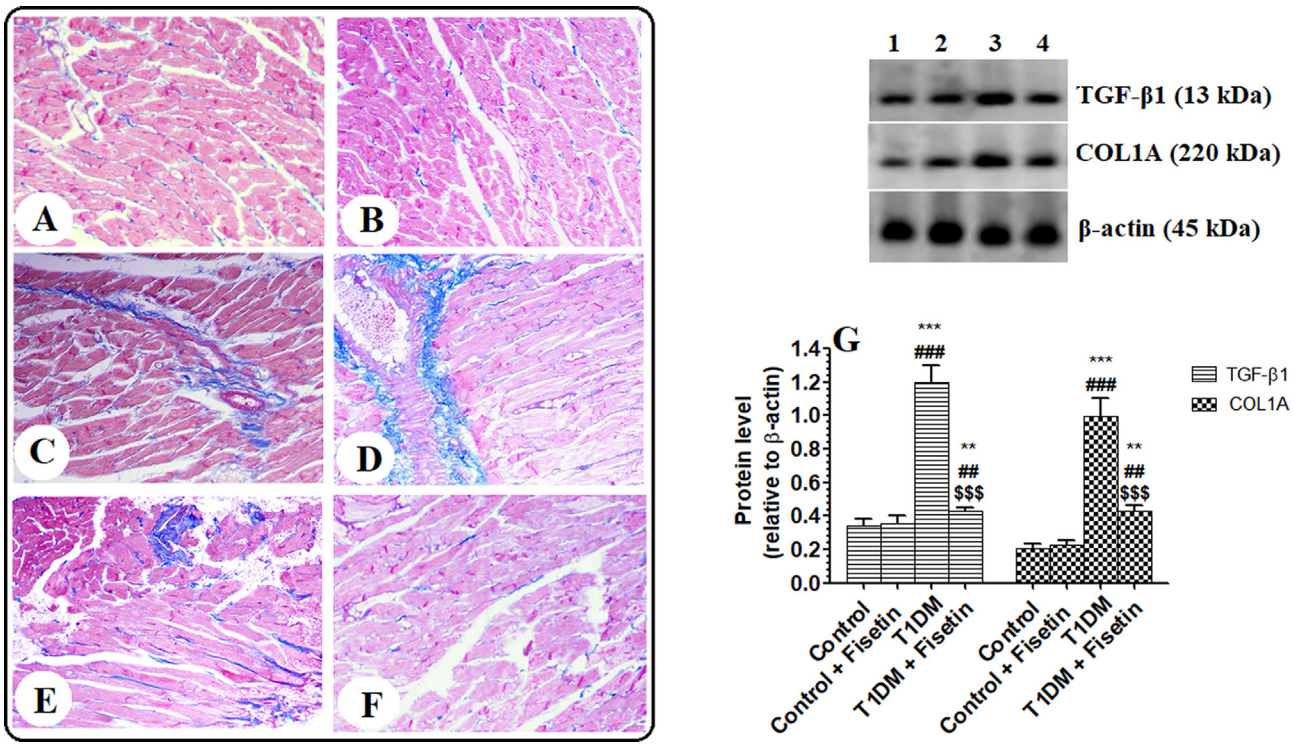

Fig. 4. Collagen deposition (A-F) and protein levels of transforming growth factor-β-1 (TGF-β1) and collagenase 1A (COL1A) (G) in the hearts of all experimental groups. ***A and B:*** were taken from control and control + Fisetin-treated hearts and showed few filaments of collagen (blue color) (long arrow. ***C-F:*** were taken from T1DM-treated rats (T1DM) and showed increased collagen deposition between the myofibrils (periventricular) and around the blood vessels (perivascular). ***D:*** was taken form a T1DM + Fisetin-treated rat and showed almost normal collagen deposition like the control hearts. ***In G:*** Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 6/group. **, ***: vs. control (lane 1) at p < 0.01, and 0.001, respectively. ###: vs. Control + Fisetin (lane 2) at p < 0 0.001. $$ $: vs. T1DM (lane 3) at p < 0.001. Lane 4: T1DM + Fisetin. Lane 4: T1DM + Fisetin.

the vehicle (Fig. 4**A-G**). The LVs of this group of rats also showed a significant decrease in the levels of TNF-α and IL-6 and nuclear activity and protein levels of NF-κB as compared to control rats administered the vehicle (Fig. 5**A-D**). on the other hand, increased deposition of collagen with higher LV levels of TNF-α and IL-6, as well as protein levels of TGF-β1, collagenaseA1, and nuclear activity and protein levels of NF-κB were observed in the LVs of T1DM-induced rats as compared to control rats (Fig. 4**A-G and** Fig. 5**A-D**). The collagen deposition and all the levels of all these inflammatory and fibrotic markers were significantly reduced in the LVs of T1DM + Fisetin as compared to T1DM-diabetic rats (Fig. 4**A-G and** Fig. 5**A-D**).

3.4. Fisetin suppresses FAs oxidation and stimulates glucose oxidation in the LVs of both the control and T1DM-diabetic rats

The protein Activities of PPAR-α and the activity of MCAD were significantly higher but the activities of PDH and PFK were significantly lower in the LVs of T1DM-diabetic rats as compared to non-diabetic rats (Fig. 6**A-D**). On the contrary, the activity of MCAD and protein levels of PPAR-α were significantly reduced and the activities of PDH and PFK were significantly increased in the LVs of both the control + Fiestin and T1DM + Fisetin as compared to control and T1DM-diabetic rats, respectively (Fig. 6**A-D**).

3.5. Fisetin inhibits PKR activity by upregulating MetAP2 (p67) in the LV of the control and T1DM-diabetic rats

No significant variations in the total protein levels of PKR and eIF-2 were seen with any treatment. However, higher activity of PKR and protein levels of p-PKR (Thr^{446}) and p-eIf-2 (ser^{51}) were observed in the LVs of T1DM-diabetic rats as compared to non-diabetic rats (Fig. 7**A-C**). These alterations were associated with higher protein levels of PACT and a concomitant reduction in protein levels of MetAP2 (p67) and p58IPK (Fig. 8**A**). However, reduced activity of PKR and protein levels of p-PKR (Thr^{446}), p-eIf-2 (ser^{51}), PACT with a significant increase in protein levels of MetAP2 and p58IPK were seen in the LVs of T1DM + Fisetin-treated rats as compared to T1DM-diabetic rats administered the vehicle (Fig. 7**A-C and** Fig. 8**A**). Of note, higher protein levels of MetAP2 (p67) with reduced protein levels of p-PKR (Thr^{446}) and p-eIf-2 (ser^{51}) were observed in the LVs of control + Fiestin-treated rats as compared to control non-diabetic rats administered the vehicle (Fig. 7**A-C and** Fig. 8**A**).

3.6. Fisetin inhibits major apoptotic pathways in the LVs of T1DM-induced rats

No significant variation in the protein levels of p38 and JNK were seen in all groups of rats with any treatment (Fig. 9**A-D**). However, there was a significant increase in the levels of p-JNK in the LVs of the control + Fisetin-treated rats as compared to control rats administered the vehicle (Fig. 9**A**). On the other hand, higher protein levels of p-JNK, p53, p-MAPK (Thr^{180}/Tyr^{182}), and caspase-3 were seen in the LVs of T1DM-diabetic rats as compared to control non-diabetic rats, all of which were significantly reduced in the hearts of T1DM + Fisetin-treated rats (Fig. 9**A-D**).

3.7. Fisetin increases cell survival and inhibits apoptosis in HG-treated cells by upregulation of MetAP2 and inhibiting PKR

A significant reduction in cell survival, an increase in the levels of ssDNA, increased protein levels of p-PKR (Thr^{446}), and downregulation of MetAP2 HG-cultured cells showed as compared to LG-

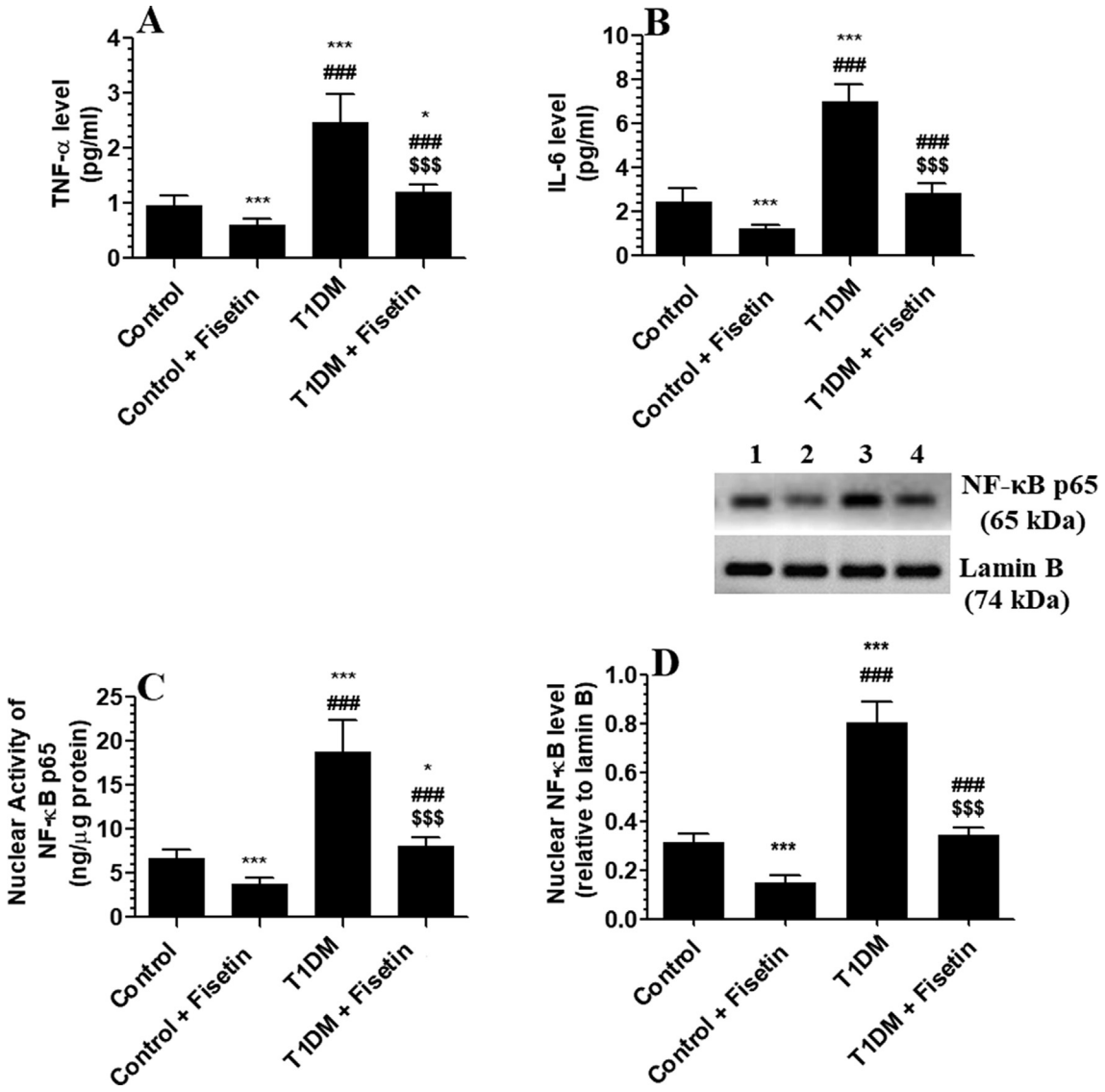

Fig. 5. Levels of tumor necrosis factor-alpha (TNF-α) (A), interleukin-6 (IL-6) (B), and nuclear activity and protein level of nuclear factor kappa Beta (NF-κB) (C and D, respectively) in the heart of hearts of all experimental groups. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. *, ***: vs. control (lane 1) at p < 0.05 and 0.001, respectively. ###: vs. Control + Fisetin (lane 2) at p < 0 0.001. $$$: vs. T1DM (lane 3) at p < 0.001. Lane 4: T1DM + Fisetin.

cultured cells (Fig. 10**A-D**). Fisetin significantly increased cell survival, suppressed the levels of ssDNA and the phosphorylation of PKR (Thr^{446}), and increased the protein levels of MetAP2, in a dose-dependent manner in HG-treated cells as compared to HG control cells (Fig. 10**A-D**). Besides, Fisetin didn't affect cell survival or levels of ssDNA but significantly increased protein levels in LG-treated cells (Fig. 11**A-C**). However, Fisetin failed to alter the expression of p-PKR (Thr^{446}) in both LG and HG-treated cells which were exposed to siRNA silencing of MetAP2 as compared to their control cells (Fig. 11**A-C**).

4. Discussion

Previous studies have shown that Fistien can protect against DCM in diabetic rodents by suppression of oxidative stress, inflammation, and apoptosis (Althunibat et al., 2019). However, the mechanisms behind this remain largely elusive. In this study, we continued this area of research and showed that the cardioprotective effect of Fisetin is mediated by several-interconnected mechanisms including hypoglycemic and insulin-sensitizing effects, improving cardiac glucose oxidation through downregulation of PPARα, and suppression of PKR activity mediated by upregulation of MetAP2 (P67). A detailed mechanism of protection is provided in the graphical abstract (Fig. 12).

Overproduction of ROS from the mitochondria is the major cause of DCM, which leads to cardiac damage and LV dysfunction by inducing oxidative stress, inflammation, and fibrosis, and apoptosis (Jia et al., 2018; Ritchie and Dale Abel, 2020). In this study, we initially validated our animal DCM model in T1DM-treated rats by various cardiac-related biochemical and structural changes. Diabetic rats showed a reduction in body and heart weights with impaired systolic and diastolic functions. Such a decrease in rats' body weights could be explained by the increased lipolysis from the adipose tissue. However, the reduction of heart weights and impaired LV function could be attributed to the reduction in cardiac protein synthesis and increased apoptosis. Also, higher circulatory levels cardiac markers (i.e Troponin-I and CKMB) with

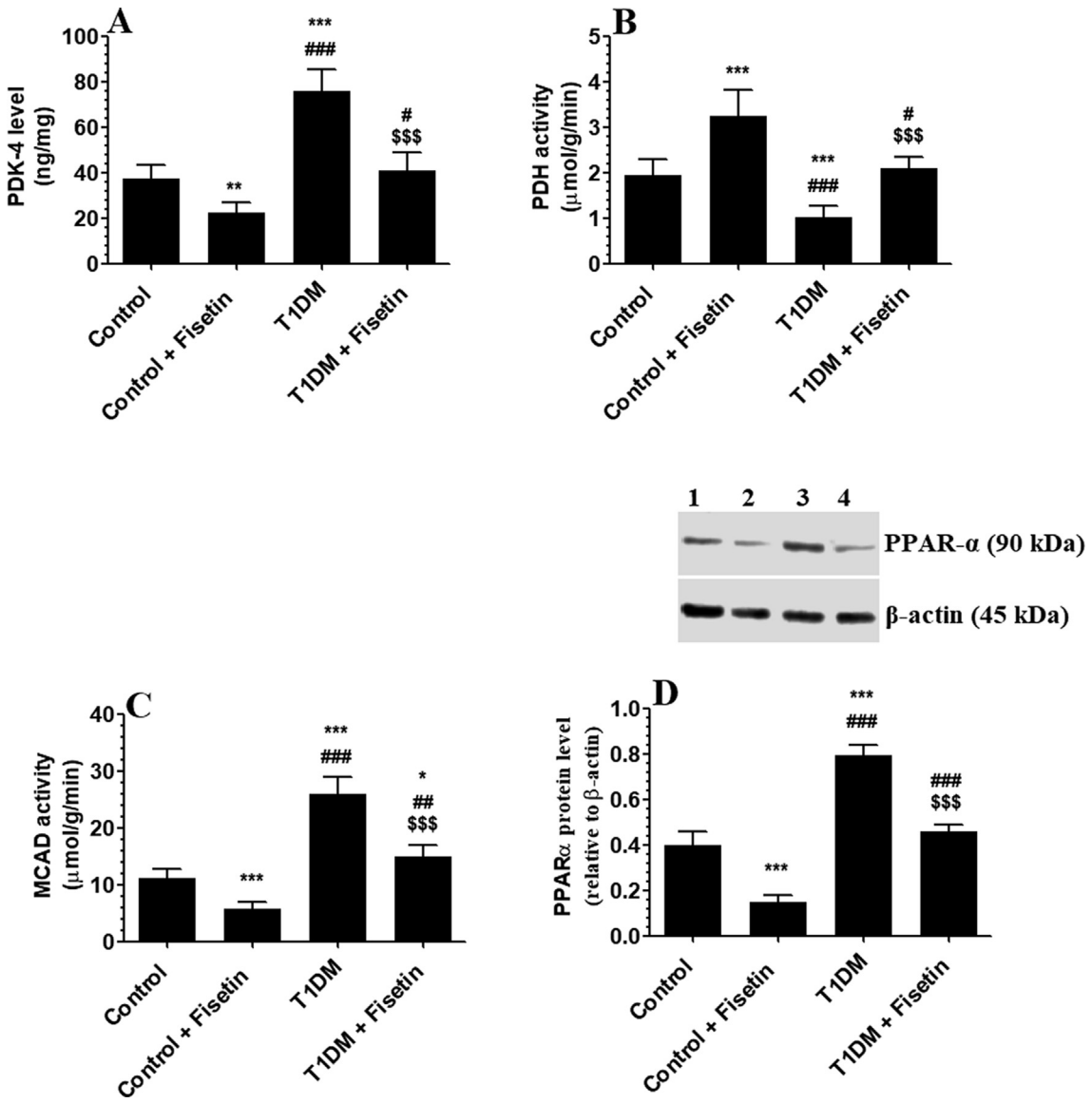

Fig. 6. Activity of pyruvate dehydrogenase kinase-4 (PDK-4) (A), levels of pyruvate dehydrogenase (B), the activity of medium-chain acyl-coenzyme A dehydrogenase (MCAD) (C), and protein levels of peroxisome proliferator-activated receptor-α (PPAR-α) in the left ventricles of all experimental groups. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. *, ***: vs. control (lane 1) at p < 0.05 and 0.001, respectively. #, ##, ###: vs. Control + Fisetin (lane 2) at p < 0.05, 0.01, and 0 0.001. $$$: vs. T1DM (lane 3) at p < 0.001. Lane 4: T1DM + Fisetin.

severe myofibril and mitochondria damage and higher cardiac levels of ROS, inflammatory cytokines, and activity NF-κB with a parallel decrease in cardiac endogenous antioxidants (GSH and SOD) were observed in T1DM-diabetic rats. However, Fisetin reversed the LV structure and prevented all these events in the treated diabetic rats. This is not a novel finding and just confirmatory to many previous results which have shown a potent cardioprotective effect of Fisetin in several animal models including DM, ischemia/reperfusion (I/R) injury, and doxorubicin (DOX)-induced cardiac damage, mainly mediated by suppressing oxidative stress, inflammation and apoptosis (Shanmugam et al., 2018; Dong et al., 2018; Ma et al., 2019; Althunibat et al., 2019; Rodius et al., 2020).

Besides, Fisetin significantly reduced glucose levels in both the control and diabetic rats but only and significantly increased insulin levels in diabetic rats. Similar findings were also reported in diabetic rats (not in the control rats) as demonstrated by Althunibat et al., (2019), who referred to the findings of others to explain these results. Fisetin can increase insulin levels by suppressing pancreatic β-cells apoptosis in T1DM-treated rats (Prasath et al., 2013). Also, Fisetin can inhibit hepatic gluconeogenesis by downregulating key enzymes such as PEPCK and G6Pase (Prasath et al., 2014). However, the reduction in HOMA-IR in the control rats, along with the improvement in OGTT and IPITT in both the control and diabetic rats suggests that Fisestin can stimulate insulin peripheral and central sensitivity (i.e adipose tissue, muscles, heart, and liver). Such hypoglycemic and insulin-sensitizing/releasing effects could participate significantly in the observed cardioprotective effect of Fisetin.

On the other hand, the heart is a highly dynamic organ that depends on both FAs (70%) and glucose (10–30%) metabolism as an energy source (Lopaschuk et al., 2010). Such a low level of glucose oxidation was shown to be essential for normal contractility (Lopaschuk et al., 2010). However, during DM, the heart becomes more dependent on FAs oxidation which blunt glucose oxidation (Randle cycle). This leads to lipotoxicity, steatosis, mitochondria

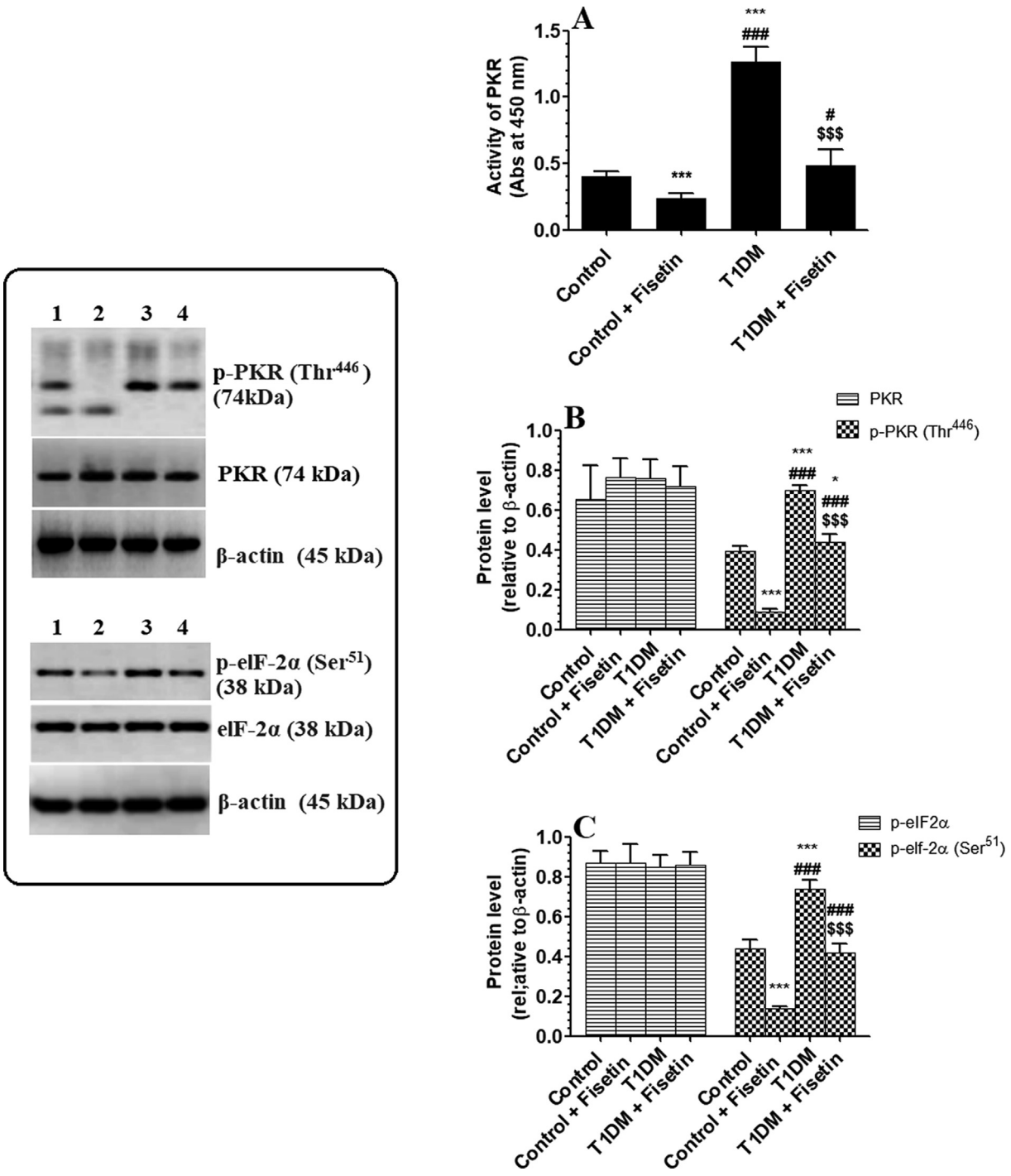

Fig. 7. Activity of protein kinase R (PKR) (A), as well as protein levels of PKR/phospho-PKR (B) and Eukaryotic initiation factor 2 (elf-2)/p-eIF2 (C) in the heart of hearts of all experimental groups. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. *, ***: vs. control (lane 1) at $p < 0.01$ and 0.001, respectively. #, ###: vs. Control + Fisetin (lane 2) at $p < 0.05$ and 0.001, respectively. $$$: vs. T1DM (lane 3) at $p < 0.001$. Lane 4: T1DM + Fisetin.

damage, and further generation of ROS (Rodriguesand, 1992; Rodrigues et al., 1995; McGavock et al., 2007; Lopaschuk et al., 2010 Winhofer et al., 2012; Jia et al., 2018; Ritchie and Dale Abel, 2020). In most cells, including the heart, the enzymes phosphofructokinase (PFK) and Pyruvate dehydrogenase (PDH) are the major enzymes that regulate the cellular glycolysis process and stimulate the citric acid cycle, respectively (Hue and Taegtmeyer, 2009). In diabetic hearts, the activity of PFK is allosterically inhibited by the high citrate levels (Da Silva et al., 2012). Likewise, the diabetic cardiac activity of PDH kinase (PFK) is inhibited by the higher activity of PDH kinase (PDK4) which is activated in response to high levels of β-oxidation and accumulation of acetyl CoA/CoA, NADH/ NAD, and ATP/ADP (Wieland et al., 1971; Kuo et al., 1985; Stanley et al., 1997). This was also confirmed in the diabetic hearts of this study where the higher activity of medium-chain acyl-coenzyme A dehydrogenase (MCAD), a mitochondrial fatty

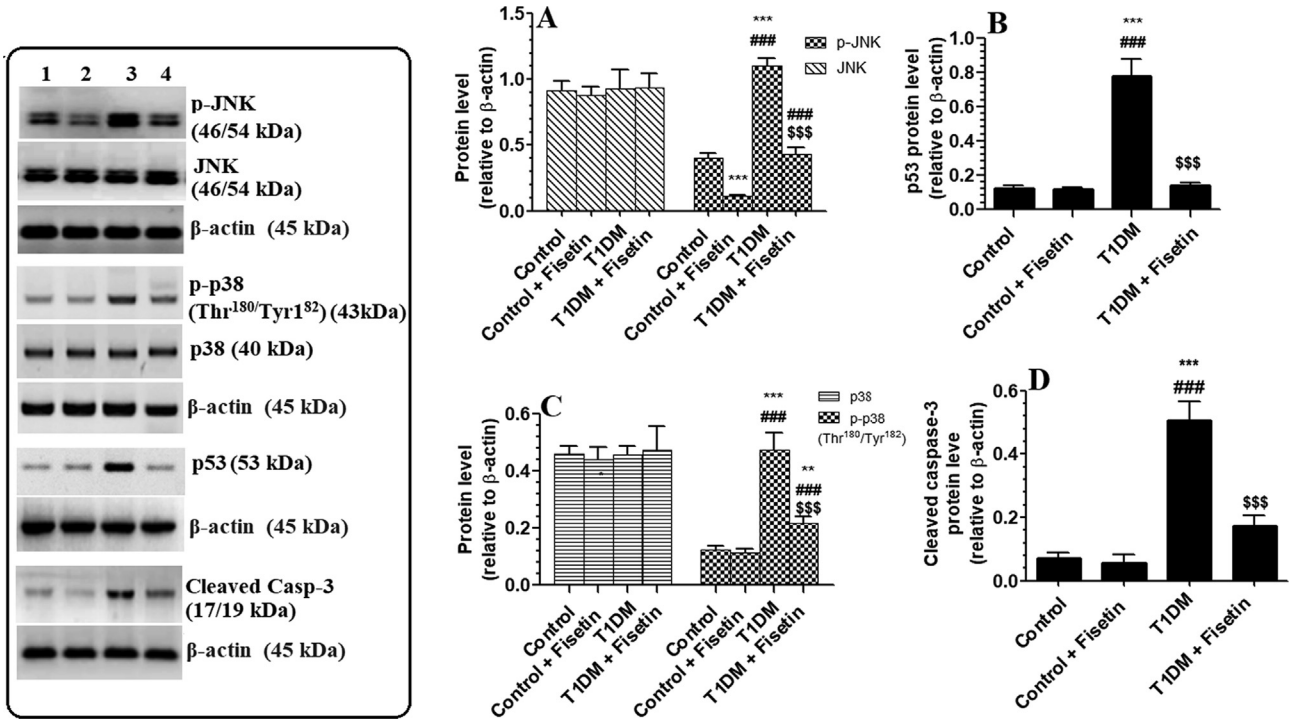

Fig. 8. Protein levels of protein kinase R activating protein (PACT), p58IPK, and MetAP2 (p67) in the heart of all experimental groups. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tykey's *t* test as post-hoc for n = 12/group. ***: vs. control (lane 1) at p < 0 0.001. ###: vs. Control + Fisetin (lane 2) at p < 0 0.001. $$$: vs. T1DM (lane 3) at p < 0.001. Lane 4: T1DM + Fisetin.

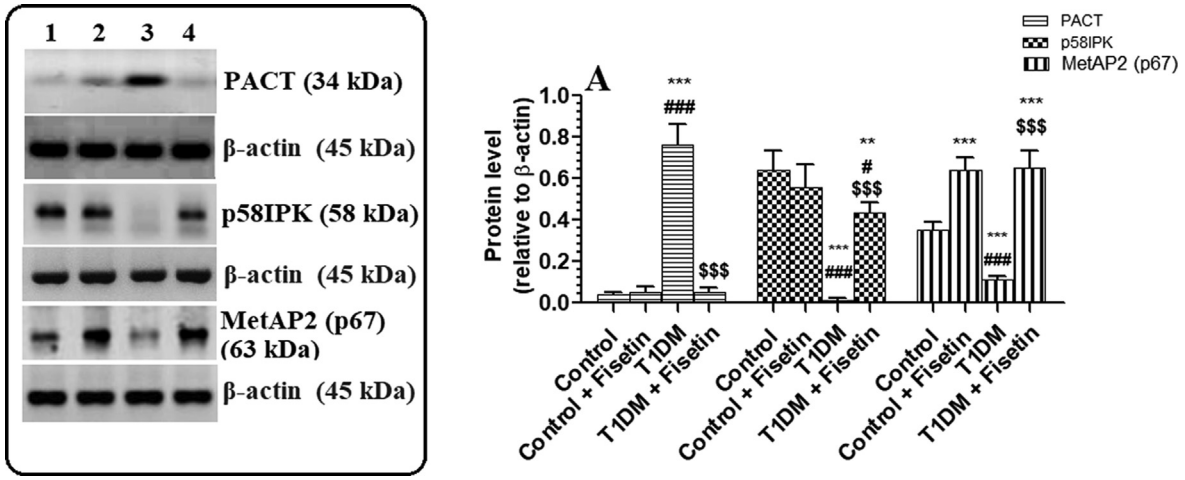

Fig. 9. Protein levels of NH2 -terminal Jun kinase (JNK)/phospho-JNK (A), p53 (B), p38 mitogen-activated protein kinase (p38 MAPK)/phospho-p38 MAPK (Thr^{180}/Tyr^{182}) (C), and cleaved caspase-3 (D) in the hearts of all experimental groups. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for n = 12/group. ***: vs. control (lane 1) at p < 0 0.001. ###: vs. Control + Fisetin (lane 2) at p < 0 0.001. $$$: vs. T1DM (lane 3) at p < 0.001. Lane 4: T1DM + Fisetin.

acid β-oxidation enzyme, and PDK4 and lower activities of PDH and PFK were observed, thus confirming a cardiac shift toward FAs metabolism.

However, Fisetin increased the activation of PDH and PFK and suppressed the activities of PDK4 and MCAD in the hearts of both the control and diabetic hearts. These data suggest that Fisetin favors cardiac glucose oxidation and suppresses FAs metabolism. This could explain the higher contractility in the heart of the control hearts. To reveal the precise mechanisms, we have targeted the expression of PPAR-α, a major transcription factor responsible for the cardiac uptake of FAs (Finck, 2004; Yin et al., 2019; Wu et al., 2018). Besides, overexpression of PPAR-α inhibited the uptake of Ca2 + by the sarcoplasmic reticulum, induced IR, and reduced systolic dysfunction (Finck et al., 2002; Ferré, 2004; Park et al., 2005). As expected, the levels of PPARα were significantly higher in the LVs of diabetic animals and were reduced after Fisetin treatment. Surprisingly, Fisetin also downregulated PPAR-α in the LVs of control animals. Based on this observation, we can strongly argue that Fisetin can improve the cardiac function and afford cardioprotection in both the control and diabetic hearts by suppression of FAs oxidation mediated by downregulation of PPAR-α.

Nonetheless, ROS, as an initiator, inflammation, fibrosis, and apoptosis collaborate in a positive-feedback loop in the diabetic heart and end up with cardiac damage (Watanabe et al., 2010;

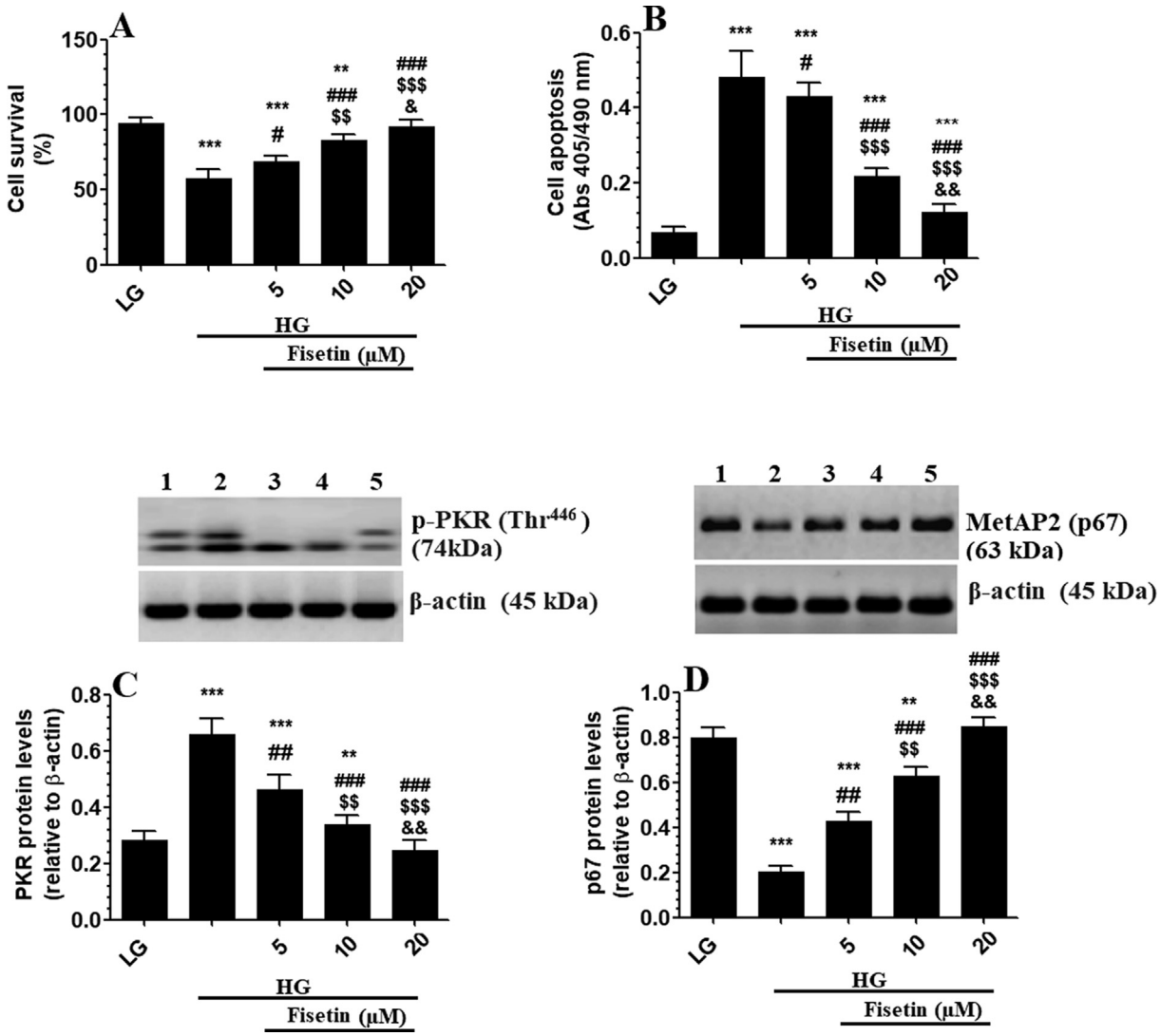

Fig. 10. Cell survival (A) and levels of single-stranded DNA (ssDNA) (Cell apoptosis) (B) as well as protein levels of protein kinase R (C) and MetAP2 (p67) (D) in cultured cardiomyocytes of all treatments. Cells were the heart of all experimental groups. Cells ($1X10^4$) were cultured in a humified atmosphere in DMEM (Dulbecco's modified Eagle's medium) in either low/high glucose media (5 μM or 25 μM glucose, respectively). In HG conditions, some cells were co-incubated with an increasing concentration of Fisetin (5, 10, 20 μM). Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for three trials each in duplicate for each treatment. **, ***: vs. LG-treated cells (lane 1) at $p < 0.01$ and 0 0.001, respectively. #, ##, ##: vs. HG-treated cells (lane 2) at $p < 0.05$, 0.01, and 0 0.001, respectively. $$, $$$: vs. HG + Fiestin (5 μM) (lane 3) at $p < 0.01$ and 0.001, respectively. &, &&: vs. HG + Fiestin (10 μM) (lane 4) at $p < 0.05$ and 0 0.001, respectively. Lane 5: HG + Fisetin (20 μM).

Ritchie and Dale Abel, 2020). Hyperglycemia, ROS, AGE-mediated signaling, and inflammatory cytokines trigger cardiac fibrosis by activating TGF-β1/Smad 2/3 pathway (Jia et al., 2018; Ritchie and Dale Abel, 2020). Besides, hyperglycemia, ROS, FFA, and impaired insulin signal can directly induce cardiac inflammation by activating NLRP3 inflammasome and subsequent activation of NF-κβ (Pal et al., 2017; Jia et al., 2018). Besides, hyperglycemia, ROS, and inflammatory cytokines can activate numerous death pathways in the diabetic hearts including JNK and p38 MAPK, as well as p53 (Watanabe et al., 2010). Oxidative stress, fibrosis, inflammation, as well as activation of all these apoptotic pathways were all seen in the LVs of diabetic rats of this study. However, Fisetin reduced the generation of ROS, suppressed TGF-β1, p53, and p38 MAPK, reduced collagen synthesis and deposition, and prevented the activation of NF-κB in the LVs of the T1DM-diabetic rats. These data suggest that Fisetin cardioprotective effect is mediated by antioxidant, anti-inflammatory, anti-fibrotic, and anti-apoptotic effects.

Although all these protective effects could be attributed to Fisetin hypoglycemic and insulin-releasing/sensitizing effects, as well as to improving cardiac energy metabolism and suppressing FAs oxidation, the data from the hearts of control rats may suggest an alternative story. In these hearts, Fisetin significantly suppressed the activation of JNK and NF-κB without altering the levels of ROS nor antioxidants. Therefore, it seems reasonable that Fisetin has potent independent potent anti-inflammatory and anti-apoptotic effects, thus suggesting that the antioxidant and anti-fibrotic effects of Fisetin are secondary events. In support, Fisetin downregulated the mRNA of TNF-α and prevented the nuclear activation of NF-κP p65 in lipopolysaccharide (LPS)-stimulated macrophage-like cells (Kim et al., 2015a). Besides, Fisetin also prevented epithelial cell damage and inflammation induced by IL-1β by suppressing NF-κB (Peng et al., 2018). In addition, Fisetin extracted from various plants showed potent anti-inflammatory effects (Kim et al., 2015b). Furthermore, Fisetin significantly prevented inflammation and the activation of NF-κB in the heart of

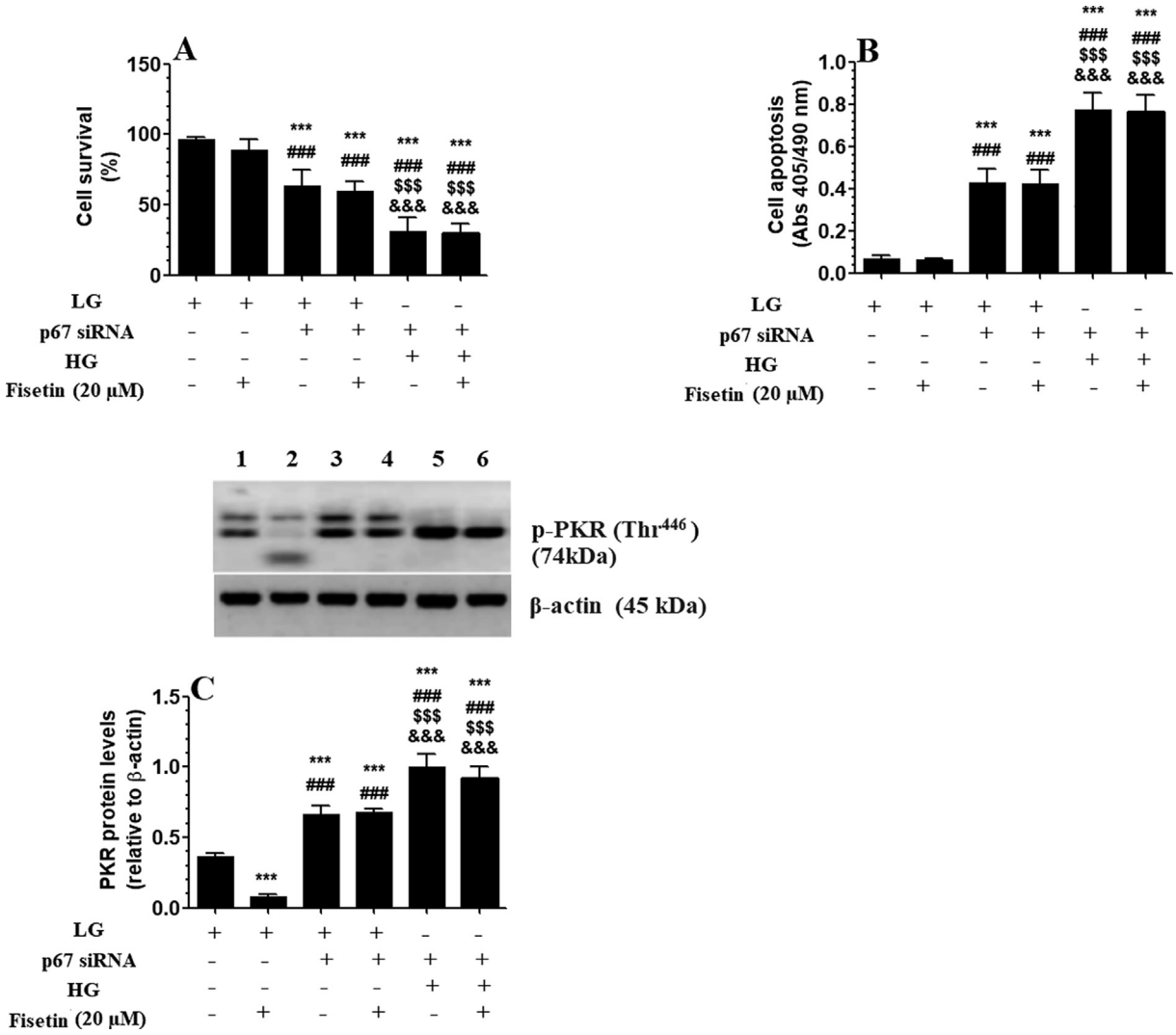

Fig. 11. Cell survival (A) and levels of single-stranded DNA (ssDNA) (Cell apoptosis) (B) as well as protein levels of protein kinase R (C) in cultured cardiomyocytes of all treatments. Cells ($1X10^4$) were cultured in a humified atmosphere in DMEM (Dulbecco's modified Eagle's medium) in either low/high glucose media (5 μM or 25 μM glucose, respectively). In HG conditions, some cells were co-incubated with 20 μM Fisetin. Data are presented as mean ± SD. Data were analyzed by two-way ANOVA followed by Tukey's *t*-test as post-hoc for three trials each in duplicate for each treatment. ***: vs. LG-treated cells (lane 1) at p < 0 0.001. ###: vs. LG + Fisetin-teated cells (lane 2) at p < 0 0.001, $$$: vs. LG + SiRN p67-treated cells (lane 3) at p < 0.001. &, &&: vs. HG + SiRN p67-treated cells at p < 0.05 and 0.01, respectively. Lane 5: HG + SiRN p67 + Fiestin (20 μM).

T1DM-diabetic rats, as well as in HG-treated monocytes. On the note, NF-κB and JNK are positively cross-talked with each other where each one can activate the other. Hence, it could be possible that Fisetin and through suppression of NF-κB reduced the activation of JNK. However, the opposite could be also correct.

Although these data dissipate the direct antioxidant potential of Fisetin, the precise mechanism by which Fisetin affords its potent anti-inflammatory and anti-apoptotic effects remain largely unknown. Therefore, in this study, we have targeted PKR given its key role as a metabolic mediator in DM and its crucial roles in the induction of oxidative stress, inflammation, and apoptosis (Gal-Ben-Ari et al., 2019). Also, PKR activity is significantly increased in diabetic and failing hearts, as well as in cardiomyocytes under hyperglycemia (Udumula et al., 2016, 2018; Kalra and Dhar, 2017; Mangali et al., 2019). Under stress, ROS, inflammatory cytokines, and ER stress can induce rapid phosphorylation and activation of PKR at Thr^{446} and Thr^{451} by activating PACT (Gal-Ben-Ari et al., 2019, Gil and Esteban, 2000). Once activated PKR can induce cell oxidative stress, inflammation, and apoptosis by activating NLRP-3 inflammasome (NF-κB & inflammatory cytokines) and several apoptotic pathways including p53, p38 MAPK, and JNK this study (Mangali et al., 2019; Gal-Ben-Ari et al., 2019). Besides, PKR can suppress protein synthesis and induce cell death by suppressing (phosphorylation) of eIf-2 at ser51 (Mangali et al., 2019; Gal-Ben-Ari et al., 2019). However, the resting cell inhibits PKR by expressing p58IPK and (MetAP2) (p67) (Gil and Esteban, 2000), as a pro-oxidant, pro-inflammatory.

In the same line with the above-mentioned studies, an increase in the activity (phosphorylation) of PKR was shown in the hearts of T1DM-diabetic rats and HG-cultured cardiomyocytes of this study. These data confirm that hyperglycemia is an independent factor to activate PKR, which could occur through increasing ROS and inflammatory cytokines. Such an increase in PKR activity could also explain the significant increase in cardiac phosphorylation (inhibition) of eIF-2, which further explains the reduction in diabetic heart weights, impaired LV function, and possibly apoptosis. Also, the increase in PKR could explain the sustained increase in the activity NF-κB, p53, JNK, and p38 MAPK. It could be also possible that the activation of PKR induces peripheral and cardiac IR, which significantly participated in the development of DCM, as, discussed

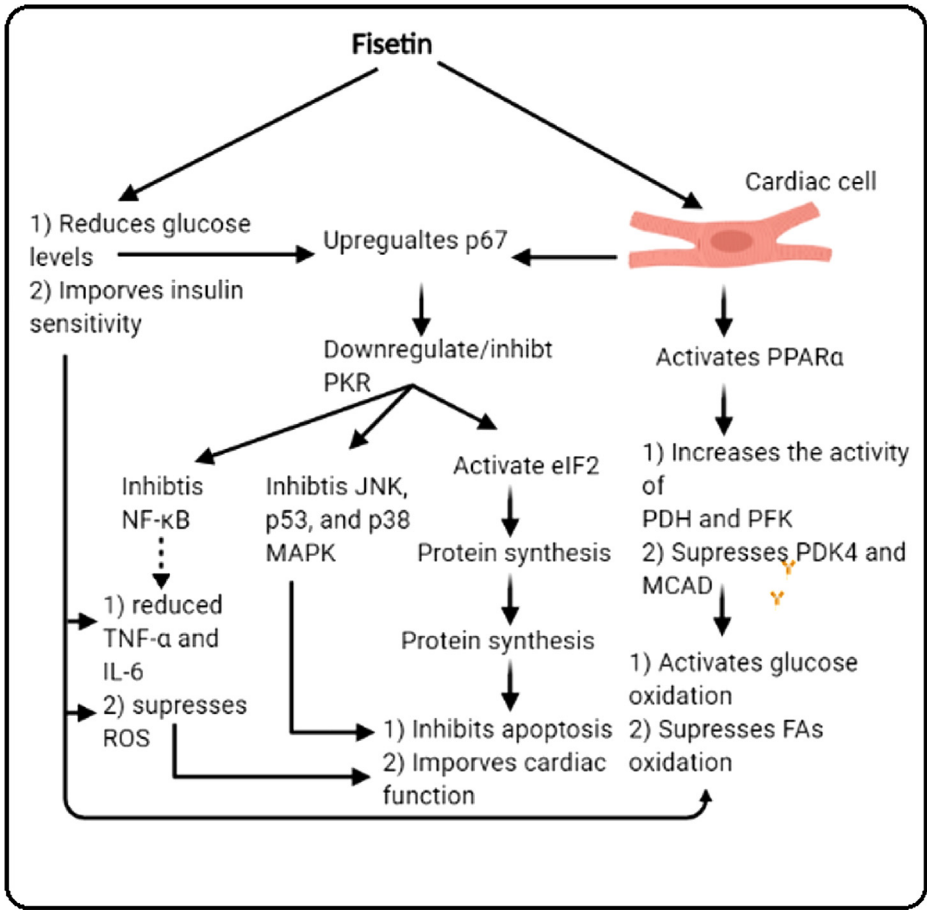

Fig. 12. A graphical abstract presenting the possible mechanism of action of Fisetin to prevent diabetic cardiomyopathy in rats. These effects are shown in the left ventricles (LVs) of both the control and streptozotocin-induced type1 diabetes mellitus (T1DM). the anti-oxidant and anti-apoptotic effect of Fisetin is mediated by acting through different mechanisms including 1) upregulation of cardiac expression of peroxisome proliferator-activated receptor-α (PPAR-α) which leads to shifting the cardiac metabolism toward glucose oxidation mediated by the activation of pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK) and suppression of pyruvate dehydrogenase kinase-4 (PDK-4) and medium-chain acyl-coenzyme A dehydrogenase (MCAD), 2) activating of p67 which in turn inhibit protein kinase-3. The suppression of PKR results in potent antioxidant, anti-inflammatory, and anti-apoptotic through inactivation of JNK, p53, and P38 mAPK, preventing inflammation by and NF-κB; and 3) stimulating peripheral insulin sensitivity which leads to a hypoglycemic effect, which may mediate all these events.

before. These effects were also associated with higher protein levels of PACT and downregulation of p58IPK and MetAP2 (p67).

The most interesting observation in this study is that our data are the first to show that Fisetin can regulate the activity of PKR by upregulation of p67. Indeed, higher levels of p67 with reduced phosphorylation of PKR were significantly observed in the hearts of both the control and diabetic rats. Besides, Fisetin, in a dose–response manner increased the protein levels of p67 and suppressed the phosphorylation of PKR in the cultured cells. These effects were associated with blunting cell death in cultured cells. To confirm these observations, we have silenced p67 and repeated the experiments. Interestingly, Fisetin was unable to prevent the activation of PKR and cell death in both LG and HG cultured cells lacking p67. Accordingly, we confirmed that the anti-inflammatory, antioxidant, and anti-apoptotic effect of Fisetin is mediated by suppression of PKR mediated by the upregulation of p67.

Despite these findings, our study still has some limitations. Most importantly, although numerous pathways were shown to be regulated by Fisting including those related to FAs oxidation, inflammation, ROS, etc. which were attributed, at least by the attenuation in the expression of P67/PKR, further studies are needed to confirm these effects and remained observational based on the data in our hands. Besides, the precise mechanisms by which Fisetin upregulates p67 needed further investigation especially at the transcriptional and post-translational levels. Also, although our data provide direct evidence that Fisetin suppresses the activation of PKR through upregulation of p67 as demonstrated in vitro, more evidence using p67 knockout animals are crucial to confirm these data, given that difference between the two systems.

5. Conclusion

The data of this study reveal an important mechanism for the cardioprotective effect of Fisetin against DCM in T1DM-induced rats. In this regard, Fisetin can preserve cardiac function and prevent further damage by 1) hypoglycemic and insulin-sensitizing/releasing effect, 2) improving cardiac glucose metabolism and suppression of FAs oxidation and 3) anti-inflammatory, anti-fibrotic, and anti-apoptotic effect mediated by upregulation of p67-induced suppression of PKR. However, it seems that the antioxidant effect of Fisetin is secondary to all these mechanisms.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contribution

J.Z.A., N.A.A., and A.M.A. designed the experimental procedures. A.F.E. and A.M.A. supervised animal treatment and tissue collection. M.N.B. and R.I.A. contributed equally to designing laboratory experimental works and statistical analysis. All authors wrote and reviewed the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.12.003.

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