

Research Article

Myricetin Possesses Potential Protective Effects on Diabetic Cardiomyopathy through Inhibiting $I\kappa B\alpha/NF\kappa B$ and Enhancing Nrf2/HO-1

Hai-han Liao,^{1,2,3} Jin-xiu Zhu,^{1,2,3} Hong Feng,⁴ Jian Ni,^{1,2,3} Nan Zhang,^{1,2,3} Si Chen,^{2,3} Huang-jun Liu,^{1,2,3} Zheng Yang,^{1,2,3} Wei Deng,^{1,2,3} and Qi-Zhu Tang^{1,2,3}

¹Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, China

²Cardiovascular Research Institute of Wuhan University, Wuhan, China

³Hubei Key Laboratory of Cardiology, Wuhan, China

⁴Department of Gerontology, Renmin Hospital of Wuhan University, Wuhan 430060, China

Correspondence should be addressed to Qi-Zhu Tang; qztang@whu.edu.cn

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Diabetic cardiomyopathy (DCM) is associated with a greater risk of mortality in patients with diabetes mellitus. Currently, no specific treatment has been suggested for DCM treatment. This study demonstrated that myricetin (M) attenuated DCM-associated cardiac injury in mice subjected to streptozotocin (SZT) and in neonatal rat cardiomyocytes (NRCM) challenged with high glucose. *In vivo* investigation demonstrated 6 months of M treatment (200 mg/kg/d) significantly alleviated cardiac hypertrophy, apoptosis, and interstitial fibrosis. Mechanically, M treatment significantly increased the activity of Nrf2/HO-1 pathway, strengthening antioxidative stress capacity evidenced by reversed activities of GPx and SOD, and decreased MDA production. M treatment also inhibited $I\kappa B\alpha/NF-\kappa B$ pathway, resulting in reduced secretion of inflammation cytokines including IL-1 β , TNF- α , and IL-6. Besides, the TGF β /Smad3 signaling was also blunted in DCM mice treated with M. These beneficial effects of M treatment protected cardiomyocytes from apoptosis as shown by decreased TUNEL-positive nucleus, c-caspase 3, and Bax. Similar effects of M treatment could be reproduced in NRCM treated with high glucose. Furthermore, through silencing Nrf2 in NRCM, we found that the regulation of $I\kappa B\alpha/NF\kappa B$ by M was independent on its function on Nrf2. Thus, we concluded that M possesses potential protective effects on DCM through inhibiting $I\kappa B\alpha/NF\kappa B$ and enhancing Nrf2/HO-1.

1. Introduction

Diabetic cardiomyopathy (DCM) is a major cardiac complication contributing to the increased morbidity and mortality of diabetic patients [1]. DCM is characterized by a series of structural and functional abnormalities including left ventricular dysfunction, cardiomyocyte apoptosis, and interstitial fibrosis, all of which are not resulting from coronary heart diseases, congenital heart diseases, or hypertension [1, 2]. Mechanically, multiple mechanisms have been involved in the pathogenesis of DCM, including oxidative stress, inflammation, disordered calcium handling,

mitochondrial dysfunction, impaired energy metabolism, and interstitial fibrosis [1, 3–5].

Oxidative stress and inflammatory response play the most important role in the occurrence and development of DCM [1, 3–5]. Reactive oxygen species (ROS) are responsible for cellular oxidative stress [4]. Normally, the synthesis and degradation of ROS are physiological and keep the dynamic balance dependent on cellular endogenous antioxidant factors [6]. However, under the conditions of DCM, the antioxidant factors, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and Nrf2, are decreased in cardiac tissue [6, 7]. Besides, sustained

accumulation of ROS contributes to activation of $\text{I}\kappa\text{B}\alpha/\text{NF}\kappa\text{B}$ (p65) pathway, which stimulates the expression of several proinflammatory, profibrotic and prohypertrophic genes including interleukin- (IL-) 1β , IL-6, and tumor necrosis factor- α (TNF- α) [6, 7]. In clinical practice, increased levels of C-reactive protein (CRP) and IL-6 have been suggested to predict the development of type 2 diabetes mellitus (DM) [8]. Based on these published investigations, antioxidative stress and anti-inflammation therapies have been suggested to be potential strategy for DCM.

Polyphenols have been famous for its various bioactivities such as antioxidant and anti-inflammation functions, free radical scavenging effect and regulating intracellular calcium function [9]. Myricetin (M), a polyphenol monomer, is commonly distributed in berries, vegetables, tea, and wine produced from various plants [10]. Emerging evidence has indicated that the consumption of M is significantly inversely associated with the fatal and nonfatal cardiovascular disease [11, 12]. After exposure of HepG2 cells to $20\ \mu\text{M}$ M for 9 h, microarray analysis revealed that 598 genes were changed between twofold and fourfold, while 2878 genes were changed between 1.5-fold and twofold [13]. The results definitely showed the multiple bioactivities of M. Of these changed genes, antioxidativ-related genes such as Nrf2 were significantly unregulated [13]. Recently, we have found that M significantly protected the mouse heart from lipopolysaccharide- (LPS-) induced injuries *in vivo* and *in vitro*, and the underlying mechanisms have been demonstrated to be associated with the inhibition of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ activity and attenuation of inflammatory cytokine secretion, such as IL- 1α , IL- 1β , TNF- α , and MCP-1 (unpublished data). However, the role and the underlying mechanism of M in DCM remain undefined. In this study, mouse DCM model was treated with M to observe its effects on DCM and explore the underling mechanisms.

2. Materials and Methods

2.1. Chemicals. M was purchased from Shanghai Winberb Medical S&T Development Co. Ltd. (Shanghai, China) with a purity >98% tested by high-performance liquid chromatography analysis.

2.2. Animals and Treatments. DCM model was established by intraperitoneal streptozotocin (STZ) injection at a dose of 50 mg/kg for 5 consecutive days. Control mice were injected with equal volume of citrate buffer. After one week of the final STZ injection, fasting blood glucose (FBG) was detected and $\text{FBG} > 26$ was defined as DM. During the next 6 months, mice were treated with M (100 mg/kg) at 8 am and 3 pm, respectively, with a total amount of 200 mg/kg/d or saline by gavage. Four groups were included as follows: control group (CON, $n = 12$), M treatment group (M, $n = 12$), STZ treatment group (STZ, $n = 16$), and STZ+M group ($n = 16$). All the experimental procedures were approved by the institutional guidelines of Animal Care and Use Committee of Renmin Hospital of Wuhan University.

2.3. Echocardiography. Cardiac functions were measured in our laboratory [14, 15]. Images of parasternal short axis were obtained at the mid-papillary muscle level under M mode. Left ventricle (LV) dimensions from five consecutive cardiac cycles were measured and averaged, including LV-end-systolic diameter (LVEDs), LV-end-diastolic diameter (LVEDd), end-diastolic interventricular septal thickness (IVSd), end-systolic interventricular thickness (IVSs), end-diastolic LV posterior wall thickness (LVPWd), and end-systolic LV posterior wall thickness (LVPWs). The fractional shortening (FS) and LV ejection fraction (EF) were calculated according to LVEDs and LVEDd.

2.4. Histological Analysis of Paraffin Section. The heart slides were obtained with methods previously described [14, 15]. Hematoxylin and eosin (HE) staining was performed to assess the cardiomyocyte cross-sectional area (CSA) and to observe the morphology of striated muscle. Sirius red in saturated picric acid (PSR) staining was used to determine interstitial fibrosis. We also determined collagen I and collagen III by immunohistochemistry method. Briefly, antigen retrieval was performed with citrate (pH=6) and 8% goat serum was used to block nonspecific binding sites. After incubation with primary antibody (collagen I (1:50), ABCAM, ab90395 and collagen III (1:50), Proteintech, 13548-1-A) at 4°C overnight, sections were washed with PBS for 3 min \times 5 times. Then, sections were incubated with anti-mouse or rabbit secondary antibodies labeled with horse radish peroxidase. After wash with PBS, staining was produced by DAB before counterstaining with hematoxylin. Negative control was performed by replacing primary antibody with PBS. Brownish area was interpreted as positive region. Image-Pro 6.0 (Media Cybernetics, Bethesda, MD, USA) was used to analyze images and calculate results.

2.5. Immunofluorescence Staining

2.5.1. Heart Section Staining. Hearts ($n = 6$) were arrested in 10% KCl solution and embedded in optimal cutting temperature compound for frozen sections at a thickness of $10\ \mu\text{m}$. Citrate (pH=6) was used for antigen repairing, and sections were blocked with 8% goat serum for 1 h. Sections were incubated with rabbit monoclonal antibodies against phosphorylated p65 (p-P65) (1:100) (CST, #3033) and Nrf2 (1:100) (Proteintech, 16396-1-AP) or mouse monoclonal antibody against cardiac troponin T (1:100) (ABCAM, ab8295) for overnight. The next day, sections were washed with PBS and then incubated with Alexa Fluor[®] 568 goat anti-rabbit IgG (H+L) (red) or Alexa Fluor 4888 goat anti-mouse IgG (H+L) antibody for 60 min at 37°C . Finally, after wash with PBS, Slow Fade Gold antifade reagent with DAPI was used for sealing the sections before observation and photography under fluorescence microscope. Image-Pro Plus 6.0 was used to analyze the pictures.

2.5.2. Neonatal Rat Cardiomyocyte (NRCM) Staining. NRCM were prepared in our laboratory following the method as described previously [15]. NRCM were treated with high glucose or M for 36 h. The culture medium was discarded, cell climbing was washed with PBS, and then the NRCM were

TABLE 1: Primers used in this study.

Target	Forward	Reverse
Mice-GAPDH	TCATCAACGGGAAGCCCATC	CTCGTGGTTCACACCCATCA
Mice-ANP	ACCTGCTAGACCACCTGGAG	CCTTGGCTGTATCTTCGGTACCGG
Mice-BNP	GAGGTCACCTATCTCTGG	GCCATTTCTCCGACTTTTCTC
Mice-fibronectin	CCGGTGGCTGTGAGTCAGA	CCGTTCCCACTGCTGATTTATC
Mice-CTGF	TGTGTGATGAGCCCAAGGAC	AGTTGGCTCGCATCATAGTTG
Mice-IL1 β	TGGTACATCAGCCCGAAC	GTCAGCTGGATAGCGACA
Mice-IL6	GTCAGCTGGATAGCGACA	GAAGCACAGGAGCAGGT GTAGA
NQO1	GGTGAGCTGAAGGACTCGAA	CAATATCTGGGCTCAGGCGT
Mice-TNF α	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT
SOD1	GGAACCATCCACTTCGAGCA	CCCATGCTGGCCTTCAGTTA
SOD2	GAACAATCTCAACGCCACCG	GCTGAAGAGCGACCTGAGTT
Rat-GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
Rat-IL1 β	CCGTGGACCTTCCAGGATGA	GGGAACGTCACACACCAGCA
Rat-IL6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
Rat-TNF α	CATCTTCTCAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC

fixed with 4% paraformaldehyde for 15 min at room temperature. 0.5% Triton X-100 was used for increasing membrane permeability after PBS rinse and then cell climbing was rinsed for 5 min \times 3 times. Cardiac troponin (ABCAM, ab8295) and Nrf2 (Proteintech, 16396-1-AP) were diluted at a concentration of 1 : 100 and added into 24-well plate at 4°C overnight. The following steps were performed as the above description.

2.6. Western Blot. Total protein was extracted and transferred to PVDF membrane as previously described [15]. The next day, blots were incubated with secondary antibody labeled with horse radish peroxidase for 1 h. ECL working solution was added for protein blot scanning. Image Lab 5.2.1 was used to analyze the blots. The primary antibodies used in this study were listed as follows: Nrf2 (1 : 1000) (Proteintech, 16396-1-AP); p65 (p-P65) (1 : 1000) (CST, #3033); T-p65 (1 : 1000) (CST, #8242); p-I κ B α (1 : 1000) (ABCAM, ab-133462) (heme oxygenase 1 (HO-1) (1 : 1000) (ABCAM, ab-13243); 4-hydroxynonenal (4-HNE) (1 : 1000) (ABCAM, ab-46545); Bax (1 : 1000) (CST, #2722); c-caspase 3 (1 : 1000) (CST, #9661); p-Smad3 (1 : 1000) (CST, #8760); T-Smad3 (1 : 100) (CST, SC101154); TGF- β 1; and GAPDH (1 : 1000) (CST, #2118).

2.7. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). Total RNA and cDNA were prepared according to the previous description [14, 15]. We used 20 μ l reactions according to the standard protocol of the manufacturer and ran the cycle ((95°C/5 min + 45 \times (95°C/10 sec + 60°C/10 sec + 72/10s) + 95°C/5 sec + 60°C/1 min + 97°C/0.11 sec) + 40°C/10 min). The results were analyzed with the $2^{-\Delta\Delta C_t}$ method and normalized to GAPDH gene expression. The primers used in this study were presented in Table 1.

2.8. Tdt-Mediated dUTP Nick-End-Labeling (TUNEL) Assay. TUNEL staining was performed by a commercial kit (Millipore, Billerica, MA, USA) according to the

manufacturer's instruction. The frozen sections of the mouse heart were used for TUNEL staining.

2.9. Measurements of MDA Level and Activity of GPx and SOD. The malondialdehyde (MDA), glutathione peroxidase (GPx) assay kit (S 0056), and the total superoxide dismutase (SOD) assay kit with WST-8 (S0101) were purchased from Beyotime Co. (Nantong, China). The experiments were performed in fresh cardiac tissue according to the indication of the manufacturer.

2.10. Neonatal Rat Cardiomyocytes. Neonatal rat cardiomyocytes (NRCM) were prepared according to protocol in our laboratory [15]. NRCM were seeded at a density of 100,000/24 multiwell or 500,000/6 multiwell. Brdu was used to inhibit the proliferation of fibroblast. Cells were designed into four groups: control group (CON), M treatment group (40 μ M), high glucose (HG) group (40 μ M), and HG + M group. After 72 h treatment, cells from 6 wells were harvested for apoptosis-associated protein detection.

2.11. Silencing of Nrf2 in NRCM. siRNA (rat) targeting at Nrf2 (sc-156128) and control siRNAs (sc-37007) were purchased from Santa Cruz Biotechnology Inc. (California, USA). The experiments were performed according to the siRNA transfection protocol (http://datasheets.scbt.com/protocols/siRNA_protocol.pdf). After 24 h transfection, the cells were treated with high glucose for 36 h or LPS (1 μ g/ml, 24 h); then, the cells were harvested for protein and mRNA detection.

2.12. Statistical Analysis. All values were presented as the mean \pm SEM. SPSS 19.0 for Windows was used for analysis. One-way ANOVA test followed by post hoc Tukey test was performed for the data analysis. A *P* value < 0.05 (two-tailed) was considered significant.

TABLE 2: Effects of myricetin on blood glucose, heart weight (HW), body weight (BW), and lung weight (LW). Data were presented as means \pm SEM; * $P < 0.05$, compared with CON or M group, and # $P < 0.05$, compared with STZ group.

	CON	M	STZ	STZ + M
<i>n</i>	12	12	16	16
Blood glucose (mM)	6.3 \pm 1.23	6.5 \pm 1.47	27.1 \pm 2.33*	25.3 \pm 3.45#
BW (g)	30.64 \pm 1.90	29.74 \pm 1.41	21.95 \pm 2.34*	26.56 \pm 2.41#
HW (mg)	135 \pm 5.31	129.4 \pm 3.52	109.46 \pm 3.30*	120.07 \pm 2.62#
LW (mg)	148.25 \pm 6.70	151.4 \pm 8.73	144.4 \pm 8.68	148.6 \pm 12.21
HW/BW	4.40 \pm 0.31	4.35 \pm 0.18	4.99 \pm 0.34	4.53 \pm 0.27
LW/BW	4.84 \pm 0.33	4.98 \pm 0.43	6.60 \pm 0.64*	5.61 \pm 0.41#

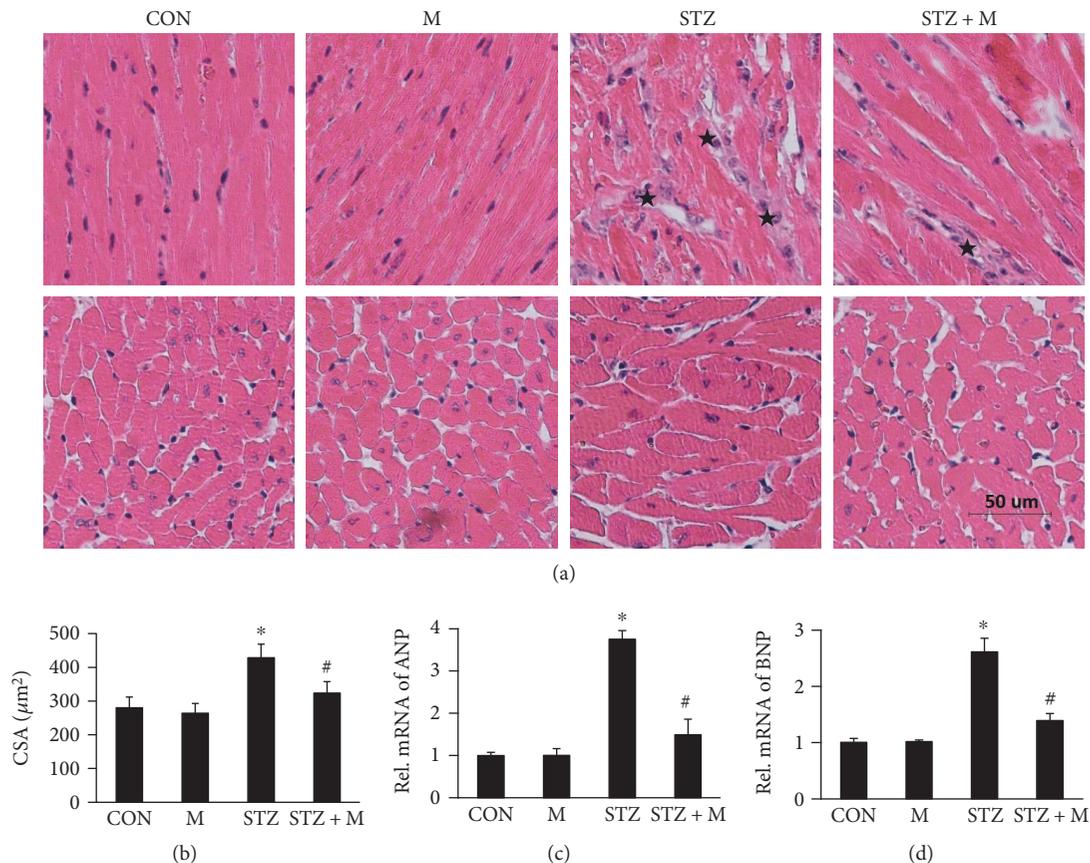


FIGURE 1: Myricetin prevented the mouse heart from STZ-induced remodeling. (a) Hematoxylin and eosin (HE) staining presented the morphological changes of myocardial tissue, black stars showed the disorder of striated muscle and interstitial fibrosis ($n = 8$). (b) The cross-sectional area of cardiomyocytes (CSA) ($n = 8$). (c) and (d) The biomarkers of cardiac remodeling, atrial natriuretic peptide (ANP), and B-type natriuretic peptide (BNP) ($n = 4$); data were presented as means \pm SEM. * $P < 0.05$ versus CON or M and # $P < 0.05$ versus STZ group.

3. Results

3.1. Myricetin Prevented STZ-Induced Pathogenic Changes and Dysfunction of Mouse Heart. As shown in Table 2, STZ significantly increased blood glucose and M treatment slightly decreased the blood glucose of diabetic mice. The body weight (BW) and heart weight (HW) of STZ group decreased compared with CON group (Table 2). M

treatment significantly prevented the reduction of BW and HW of diabetic mice (Table 2). Although HW and BW in STZ group decreased, the ratio of HW/BW was significantly unregulated in STZ group but markedly decreased by M treatment (Table 2).

As shown in Figure 1(a), STZ treatment caused disordered myocardium and enlarged cardiomyocytes, which was obviously attenuated by M treatment (Figures 1(a)

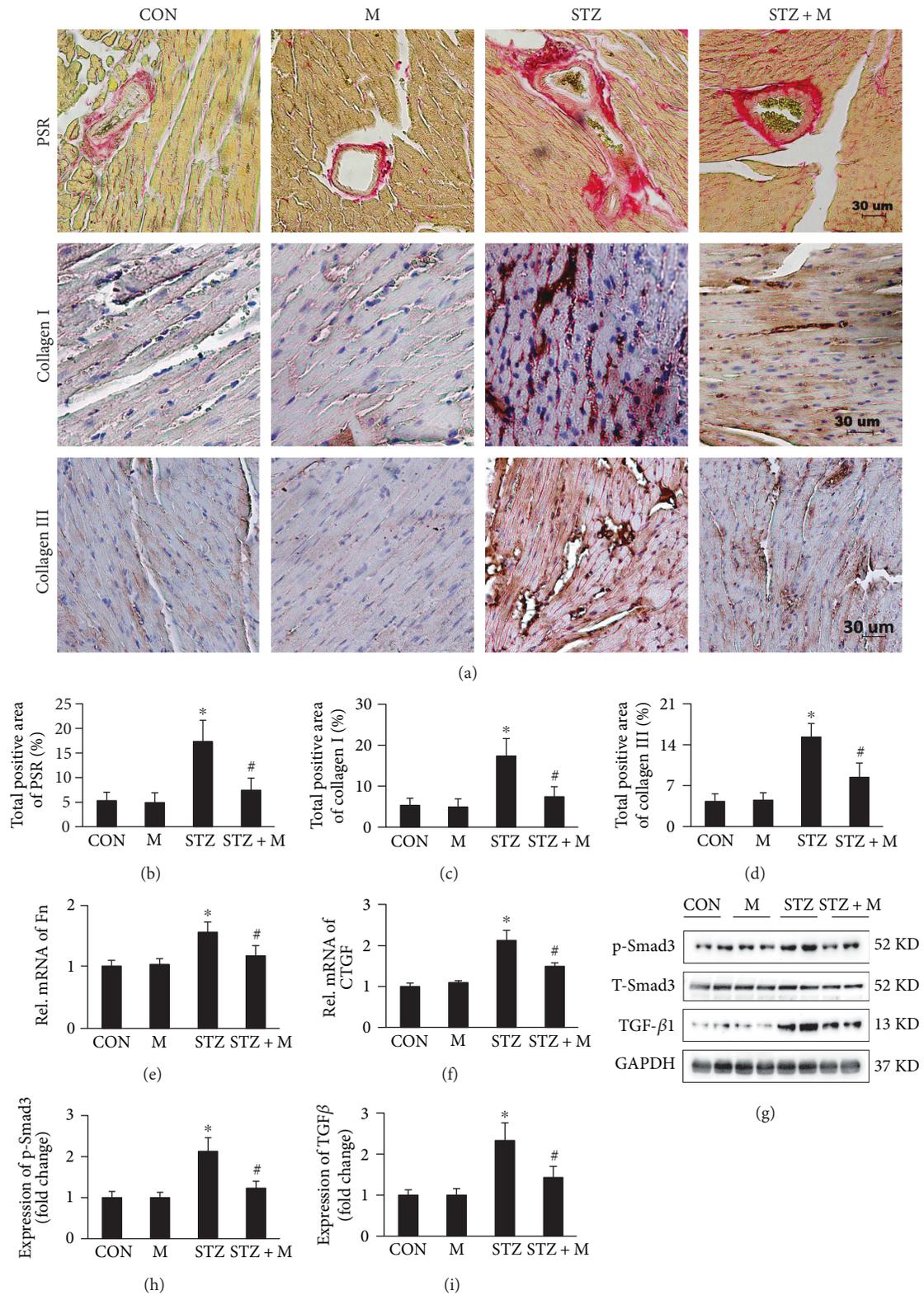


FIGURE 2: Myricetin inhibited the cardiac fibrosis of DCM induced by treatment of STZ. (a) Histological sections of mouse hearts stained by picosirius red (PSR) for fibrosis and immunohistochemistry for collagen I/III ($n = 8$). (b) The calculation of PSR positive area. (c) and (d) The calculation of collagen I/III positive area ($n = 8$). (e) and (f) The relative expression of biomarkers of cardiac fibrosis, fibronectin (Fn), and connective tissue growth factor (CTGF) ($n = 4$). (g) Representative blots of transforming growth factor (TGFβ1), T-Smad3, p-Smad3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ($n = 6$). (h) and (i) Histogram indicated the expression changes among different groups ($n = 6$). * $P < 0.05$ as compared with the corresponding CON or M group. # $P < 0.05$ as compared with STZ group, GAPDH was selected as the internal reference, and all of the proteins were normalized to GAPDH before quantitative statistics.

TABLE 3: Effects of myricetin on echocardiographic parameters. LVEDs: left ventricle-end-systolic diameter; LVEDd: LV-end-diastolic diameter; IVSd: end-diastolic interventricular septal thickness; IVSs: end-systolic interventricular thickness; LVPWd: end-diastolic LV posterior wall thickness; LVPWs: end-systolic LV posterior wall thickness; FS: fractional shortening; EF: LV ejection fraction. Data were presented as means \pm SEM; * $P < 0.05$ compared with CON or M group; # $P < 0.05$ compared with STZ group.

	CON	M	STZ	STZ + M
<i>n</i>	12	12	16	16
HR	530.81 \pm 20.39	547.30 \pm 22.88	514.91 \pm 19.43	536.62 \pm 29.74
LVEDd	3.79 \pm 0.086	3.81 \pm 0.089	3.65 \pm 0.102*	3.71 \pm 0.181#
LVEDs	1.86 \pm 0.070	1.89 \pm 0.058	2.33 \pm 0.081*	2.06 \pm 0.104#
IVSd	0.73 \pm 0.046	0.73 \pm 0.035	0.66 \pm 0.037*	0.70 \pm 0.043#
IVSs	1.22 \pm 0.098	1.20 \pm 0.071	0.94 \pm 0.010*	1.03 \pm 0.067
LVPWd	0.71 \pm 0.057	0.68 \pm 0.138	0.67 \pm 0.052	0.71 \pm 0.043
LVPWs	1.29 \pm 0.070	1.25 \pm 0.085	1.09 \pm 0.095*	1.18 \pm 0.093#
EF	82.11 \pm 0.679	81.49 \pm 0.877	66.72 \pm 4.513*	76.54 \pm 2.39#
FS	49.93 \pm 1.59	50.39 \pm 1.14	36.13 \pm 3.26*	44.37 \pm 1.67#

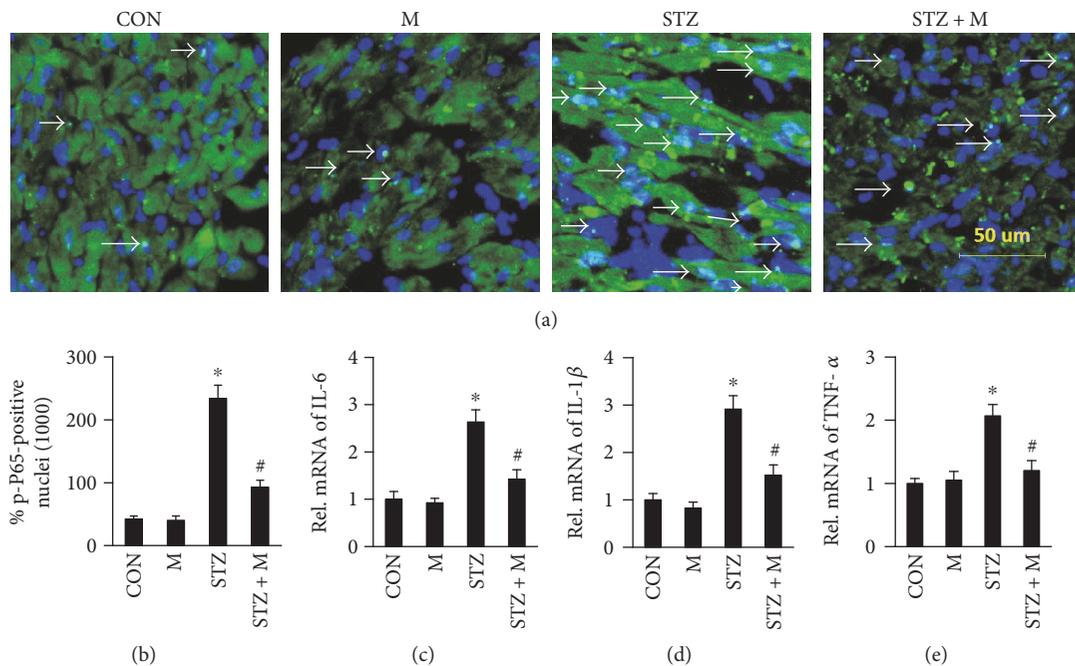


FIGURE 3: Myricetin inhibited the nuclear translocation of p-P65 and secretion of inflammatory cytokines. (a) Frozen section for the detection of p-P65 by immunofluorescence, white arrows showed the merging of p-P65 and nuclei ($n = 6$). (b) The calculation of p-P65-positive nuclei among different groups ($n = 6$). (c), (d), and (e) The relative mRNA expression of interleukin 6 (IL-6) and IL-1 β and tumor necrosis factor (TNF- α) ($n = 4$). GAPDH was selected as the internal reference, all of the mRNA was normalized to GAPDH before relative quantitative statistics. Data were presented as means \pm SEM. * $P < 0.05$ versus CON or M and # $P < 0.05$ versus STZ.

and 1(b)). Our study demonstrated that ANP and BNP were significantly upregulated in the STZ group, but downregulated in the STZ + M group (Figures 1(c) and 1(d)).

PSR staining displayed that STZ treatment markedly caused interstitial and perivascular fibrosis, which could be mitigated by M treatment (Figures 2(a) and 2(b)). The attenuation of cardiac fibrosis by M treatment was further validated by detecting collagens I and III through immunohistochemical staining (Figures 2(a), 2(c), and 2(d)). The mRNA expression of cardiac fibrosis biomarkers were also examined, and the result showed that STZ treatment promoted the mRNA expression of fibronectin (Fn) and

CTGF, which were prevented by M treatment (Figures 2(e) and 2(f)). In the development of cardiac fibrosis, TGF β 1/Smad3 signaling pathway plays a key role. Our study showed that M treatment effectively blocked TGF β 1/Smad3 signaling pathway, which was evidently activated by STZ treatment (Figures 2(g), 2(h), and 2(i)).

The prominent feature of DCM is the impaired systolic and diastolic functions. As shown in Table 3, STZ treatment caused severe cardiac dysfunctions evidenced by decreased LVEDd, IVSd, IVSs, EF, and FS and increased LVEDs compared with CON or M group, and all of these parameters could be partially normalized by M treatment (Table 3).

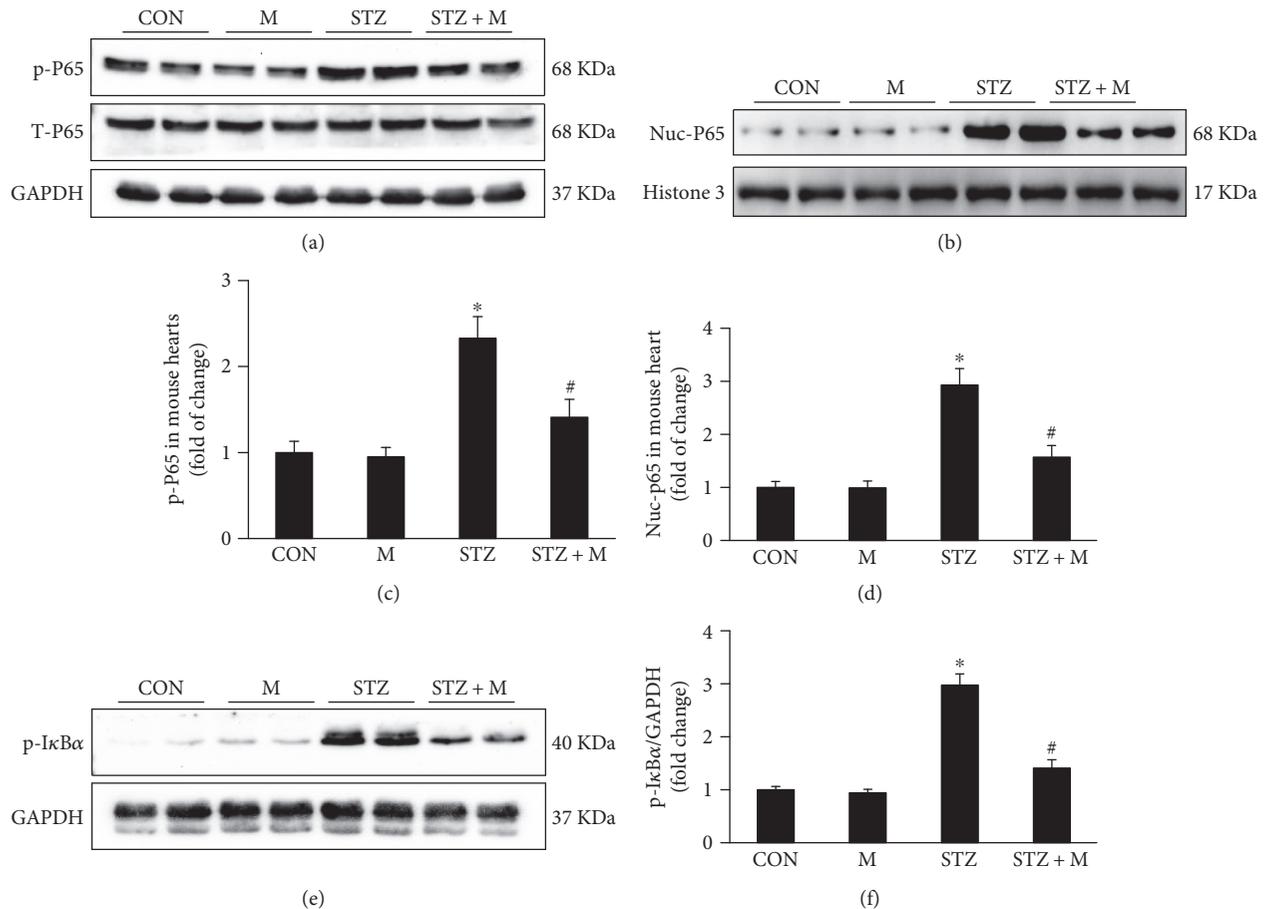


FIGURE 4: Myricetin inhibited $\text{I}\kappa\text{B}\alpha/\text{P65}$ signaling pathway. (a) Representative blots of p-P65, T-P65, GAPDH, Nuc-P65, histone 3, and p-I $\kappa\text{B}\alpha$ ($n = 6$). (b) Histogram showed the quantitative statistics of the Western blots ($n = 6$); phosphorylation (p), total (T), nuclei (Nuc), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); GAPDH and histone 3 were selected for internal reference, the phosphorylated and total protein were normalized to GAPDH, and the nuclei protein was normalized to histone 3. Data were presented as means \pm SEM. * $P < 0.05$ versus CON or M and # $P < 0.05$ versus STZ group.

3.2. Myricetin Suppressed STZ-Induced NF- κB Activation In Vivo. NF- $\kappa\text{B}/\text{p65}$ has been well known for its regulation of several inflammatory cytokines by translocation into the nucleus after phosphorylation. We detected the accumulation of phosphorylation p65 (p-P65) in diabetic hearts (Figures 3(a) and 3(b) and Figure S1 in Supplementary Material available online at <https://doi.org/10.1155/2017/8370593>), which could be blocked by M treatment (Figures 3(a) and 3(b) and Figure S1). The translocation of p65 induced the overexpression of IL-1 β , IL-6, and TNF- α in the heart of diabetic mice, while M treatment blunted the overexpression of these cytokines at mRNA level (Figures 3(c), 3(d), and 3(e)). Western blot indicated that STZ promoted the phosphorylation and nuclear accumulation of p65, and M treatment mostly reversed this change (Figures 4(a), 4(b), 4(c), and 4(d)). Besides, the phosphorylation of $\text{I}\kappa\text{B}\alpha$ (p-I $\kappa\text{B}\alpha$) induced by STZ was also inhibited by M treatment (Figures 4(e) and 4(f)).

3.3. Myricetin Enhanced the Expression and Nuclei Translocation of Nrf2. In the hearts of diabetic mice, expression of Nrf2 was significantly downregulated as detected by

immunofluorescence (Figure 5(a) and Figure S2) and Western blot (Figures 5(b) and 5(c)). Nuclear translocation of Nrf2 was little detected in CON and DCM groups (Figures 5(a), 5(d), and 5(e) and Figure S2), and treatment with M contributed to obvious nuclear translocation of Nrf2 (Figures 5(a), 5(d), and 5(e) and Figure S2). The nuclear translocation of Nrf2 increased downstream gene expression, such as HO-1, NQO1, SOD1, and SOD2 (Figures 6(a), 6(b), 6(c), 6(d), and 6(e)), all of which were downregulated in STZ group and upregulated after M treatment (Figures 6(a), 6(b), 6(c), 6(d), and 6(e)). The oxidative stress protein 4-HNE increased in DCM group and increased after M treatment (Figures 6(f) and 6(g)). Meanwhile, the enzymatic activity of SOD and GPx was decreased in DCM and could be reversed by M treatment (Table 4), while MDA level showed a similar change.

3.4. Myricetin Blocked Cardiomyocyte Apoptosis. In this study, cardiomyocyte apoptosis was estimated by TUNEL staining. DCM presented marked cardiomyocyte apoptosis in the mouse heart compared with CON and M groups (Figures 7(a) and 7(b) and Figure S3), while M treatment

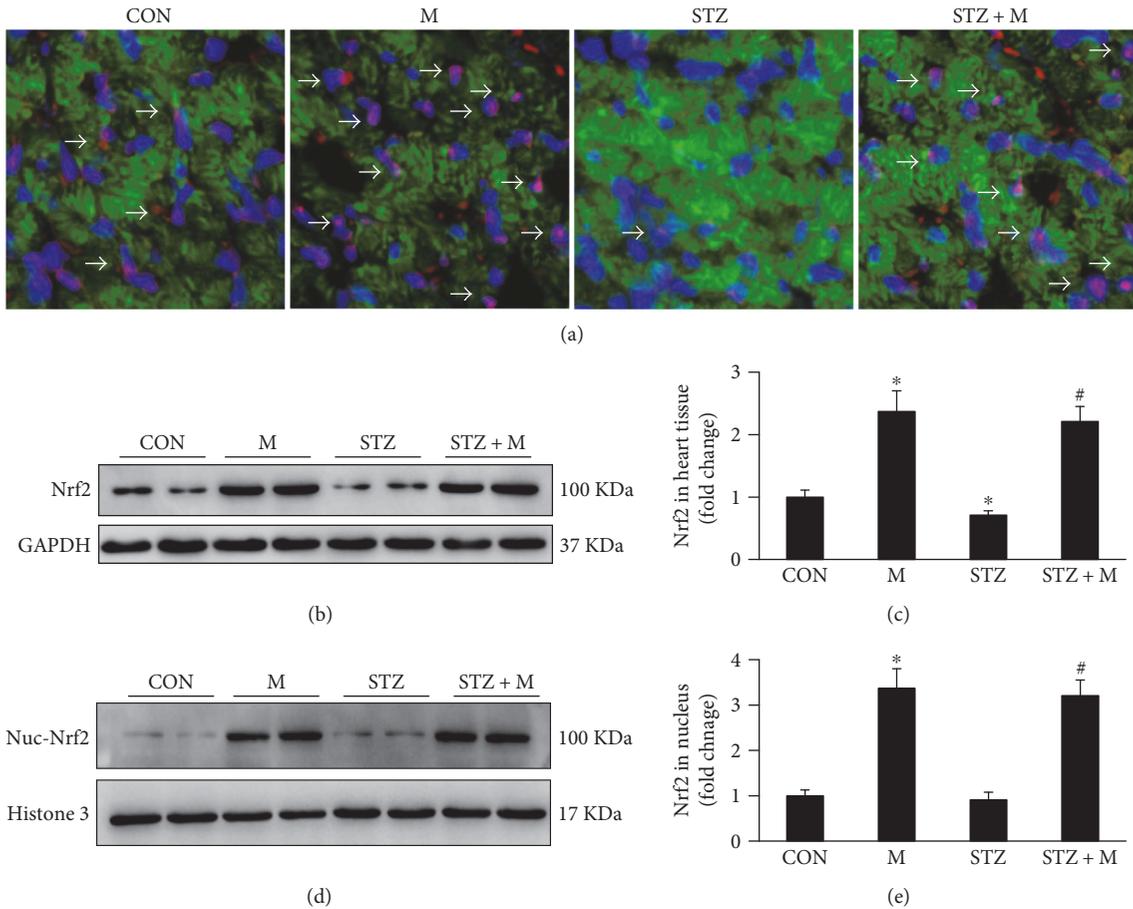


FIGURE 5: Myricetin promoted the expression and nuclei translocation of Nrf2. (a) Frozen section for detecting the expression and location of Nrf2 by immunofluorescence ($n=6$). (b) and (c) Representative blots and histogram of total expression of Nrf2 in the indicated groups ($n=6$). (d) and (e) Representative blots and histogram of Nrf2 expression in the nuclei ($n=6$); GAPDH and histone 3 were selected for internal reference, the total protein were normalized to GAPDH, and the nuclei protein was normalized to histone 3. Data were presented as means \pm SEM. * $P < 0.05$ versus CON or M and # $P < 0.05$ versus STZ group.

attenuated the apoptotic rate of cardiomyocytes significantly (Figures 7(a) and 7(b) and Figure S3). Concomitantly, proapoptosis proteins (c-caspase 3 and Bax) were upregulated in STZ group and significantly downregulated after M treatment (Figures 7(c) and 7(d)).

3.5. Myricetin Enhanced Nrf2 Expression and Nuclei Translocation in NRCM. In *in vitro* experiments, we observed M treatment promoted the expression and translocation of Nrf2 in NRCM at baseline (Figures 8(a), 8(b), 8(c), and 8(d) and Figure S4). High glucose treatment significantly downregulated the expression and nuclei translocation of Nrf2, and M treatment blocked this change (Figures 8(a), 8(b), 8(c), and 8(d) and Figure S4). The downstream gene of Nrf2, HO-1, was also decreased by treatment of high glucose but increased by adding of M (Figures 8(b) and 8(e)).

3.6. Myricetin Inhibited p65 Nuclei Translocation and Apoptosis in NRCM. We further found that high glucose stimulated the phosphorylation and nuclei translocation of p65 in NRCM. Treatment with high glucose for 24 h caused the hyperphosphorylation and nuclei translocation of p65, which could be blocked by treatment of M (Figures 9(a),

9(c), and 9(d)). Treatment with high glucose for 72 h could cause the apoptosis of NECM evidenced by overexpression of apoptotic proteins Bax and c-caspase 3, both of which could be inhibited by treatment with M (Figures 9(b), 9(e), and 9(f)).

3.7. Myricetin Inhibited $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ Pathway Independent of Enhancing Expression of Nrf2. Nrf2 has been suggested to be involved in the inhibition of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ pathway. We explored if there is a relationship between Nrf2 enhancement and $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ inhibition by M treatment. After silencing Nrf2 with siRNA (Figures 10(a) and 10(b)), high glucose significantly increased the phosphorylation of $\text{I}\kappa\text{B}\alpha$ and $\text{NF-}\kappa\text{B}$ compared with the group without Nrf2 silencing (Figures 10(a), 10(c), and 10(d)). After silencing of Nrf2 by siRNA in NRCM, M treatment could also significantly depress the activity of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ pathway (Figures 10(a), 10(c), and 10(d)). We further tested the inflammatory response of NRCM challenged with LPS after Nrf2 silencing, and the results also demonstrated that silencing Nrf2 further augmented the production of IL-1 β , IL-6, and TNF- α (Figures 11(a), 11(b), and 11(c)). The

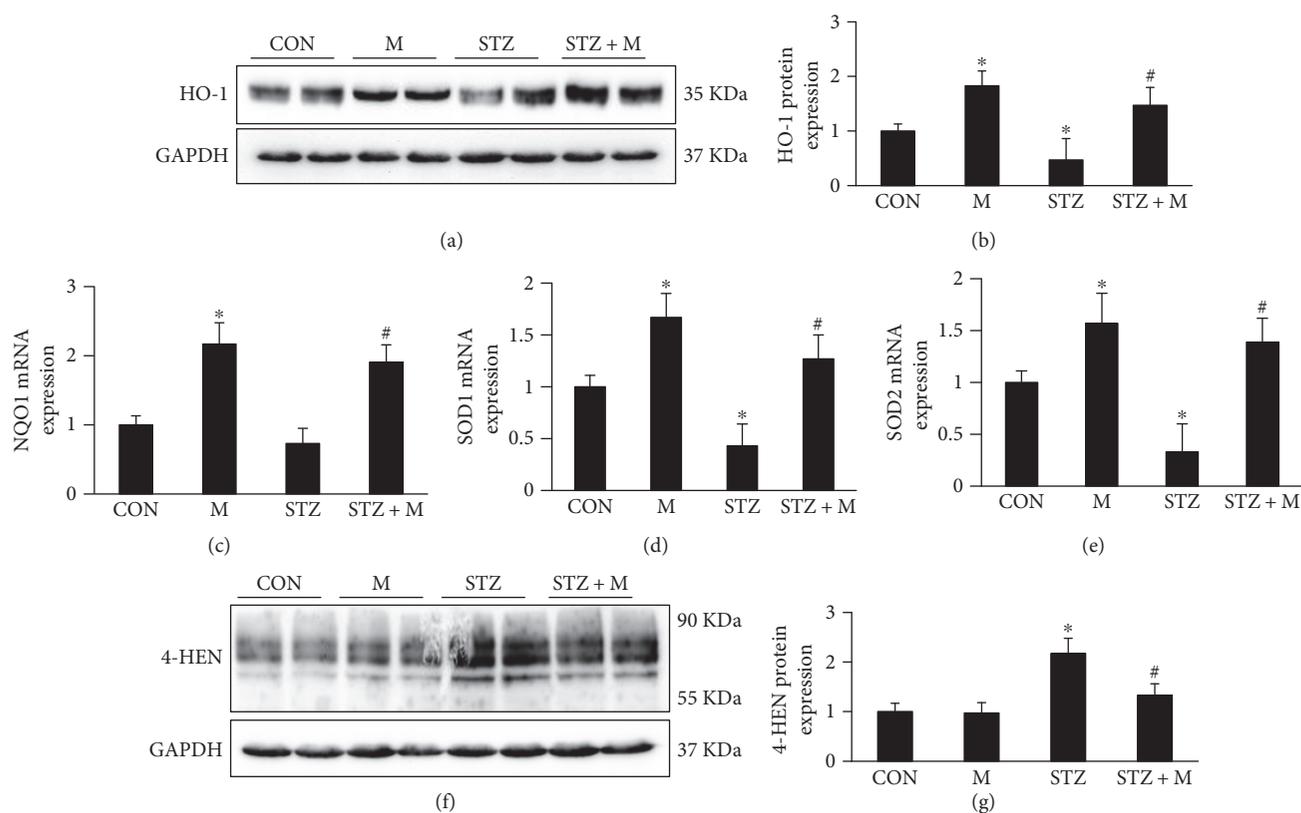


FIGURE 6: Myricetin promoted the production of Nrf2 downstream genes and inhibited the expression of 4-HEN *in vivo*. (a) Representative blots of heme oxygenase 1 (HO-1) and GAPDH ($n = 6$). (b) Histogram presented the calculation of Western blots of HO-1 ($n = 6$). (c), (d), and (e) The relative mRNA expression of NQO1, SOD1, and SOD2 ($n = 4$). (f) Representative blots showed the expression of 4-hydroxynonenal (4-HEN). (g) Histogram presented the calculation of Western blots of 4-HEN; all of the proteins and mRNA expression were normalized to GAPDH before relative quantitative analysis. Data were presented as means \pm SEM. * $P < 0.05$ versus CON and # $P < 0.05$ as compared with STZ group.

TABLE 4: The determination of MDA, SOD, and GPx. MDA: malondialdehyde (MDA); GPx: glutathione peroxidase; SOD: superoxide dismutase. Data were presented as means \pm SEM; * $P < 0.05$ compared with CON or M group; # $P < 0.05$ compared with STZ group.

	CON	M	STZ	STZ + M
SOD (U/mg protein)	1.721 \pm 0.095	2.124 \pm 0.0204	0.93 \pm 0.048*	1.689 \pm 0.024#
GPx (nmol/mg protein)	232.39 \pm 14.05	254.35 \pm 24.29	132.41 \pm 33.88*	202.47 \pm 25.26#
MDA (nmol/mg protein)	402.67 \pm 41.25	385.18 \pm 55.09	1076.63 \pm 21.68*	574.20 \pm 90.33#

production of these inflammatory cytokines could also be significantly inhibited after the silencing of Nrf2 (Figures 11(a), 11(b), and 11(c)).

4. Discussion

Consistent with previous reports, DM induced by STZ promoted oxidative stress and inflammation in cardiac tissue, resulting in cardiac fibrosis and cardiomyocyte apoptosis followed by cardiac dysfunctions. Pharmacologically, M treatment effectively enhanced Nrf2 expression and translocation into the nucleus and accordingly promoted the transcription of genes encoding antioxidant enzymes such as HO-1, SOD, and GPx. Besides, our study also demonstrated that M treatment inhibited $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling,

leading to the decreased secretion of $\text{IL-1}\beta$, IL-6 , and $\text{TNF-}\alpha$. To explore the relationship between M regulation of Nrf2 and of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling, siRNA was used to silence Nrf2 expression in NRCM. After silencing of Nrf2, the $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling pathway was further activated compared with the group without silencing. Interestingly, M treatment could still markedly decrease the activity of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ signaling pathway after the silencing of Nrf2. These results indicated that M exerts antioxidative stress and anti-inflammation effects through different regulating mechanisms, which finally protected the heart from diabetic mellitus.

Hyperglycemia, hyperlipidemia, autoimmunity, microvascular rarefaction, and accumulation of advanced glycation end-products (AGEs) are five main metabolic abnormalities

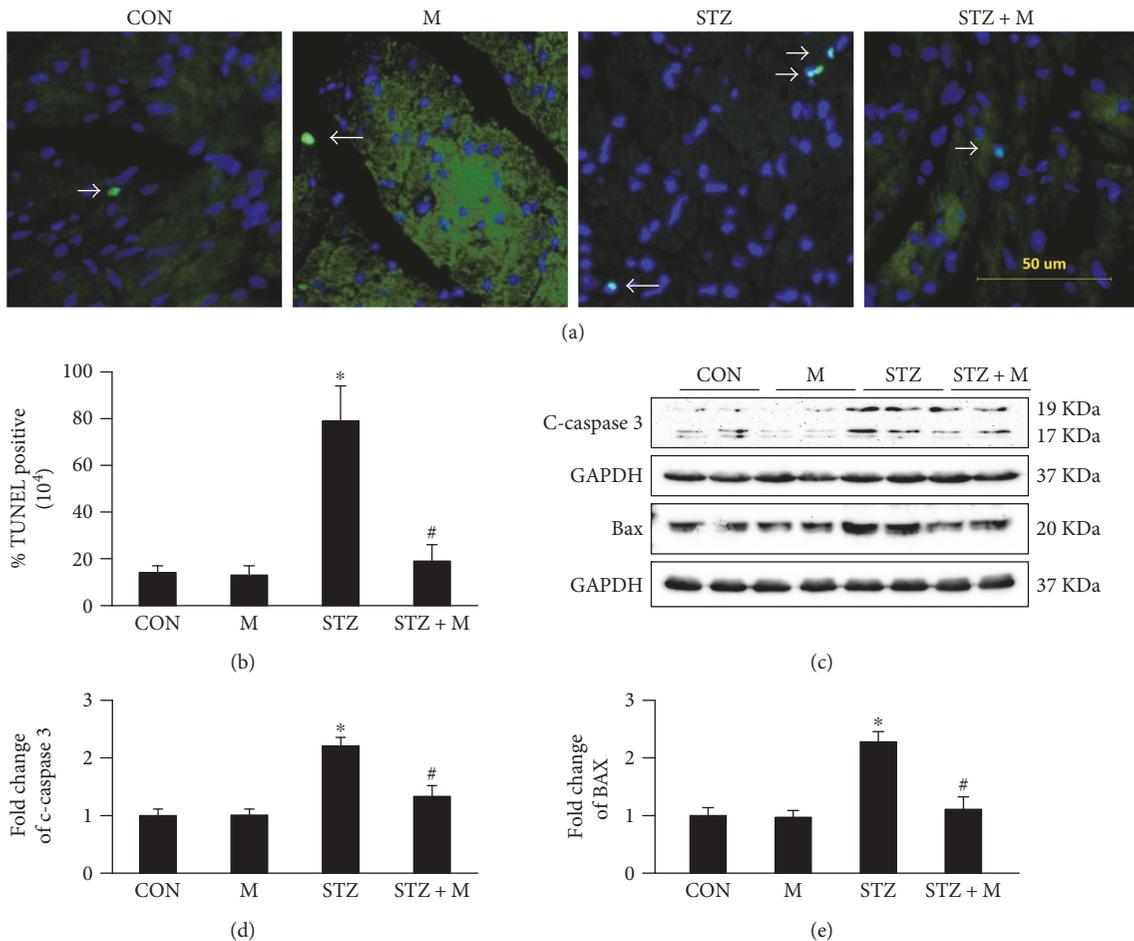


FIGURE 7: Myricetin attenuated STZ-induced apoptosis *in vivo*. (a) TUNEL staining of frozen section, white arrows showed the TUNEL-positive nuclei ($n = 6$). (b) The calculation of TUNEL-positive nuclei among different groups ($n = 6$). (c) Representative blots of c-caspase 3 and Bax ($n = 6$). (d) and (e) Histogram presented the fold change of c-caspase 3 and Bax; all of the proteins were normalized to GAPDH before relative quantitative analysis. Data were presented as means \pm SD. * $P < 0.05$ as compared with CON and # $P < 0.05$ as compared with STZ group.

in DCM [2]. These abnormalities stimulate overproduction of ROS and nitrogen species (RNS) and lead to oxidative stress [2–5, 16]. ROS or RNS are able to exhaust endogenous antioxidants such as SOD and GPx and cause the accumulation of pro-oxidative stress products such as MDA and 4-HEN in the heart. Our study and lots of previous investigations have showed consistent results regarding this point. To defend against the sustained oxidative stress injuries, cells have evolved endogenous antioxidative mechanisms, one of which is the potent redox-sensitive transcription factor Nrf2 [7]. At the early stage, high glucose stimulates a slight upregulation of Nrf2 to resist oxidative stress in DCM. However, as further development of DCM, expression of Nrf2 was significantly downregulated in mouse model [17, 18]. The downregulation of Nrf2 was also observed in the heart of patients with chronic DM [17]. In this study, we have demonstrated that myricetin treatment increased the expression and nuclear translocation of Nrf2 *in vitro* and *in vivo* and thus protected cardiomyocytes from oxidative damage and apoptosis caused by STZ and high glucose. Polyphenols have been suggested to show cytoprotective effects by

disrupting the combination of Nrf2 with Keap1 and promoting the nuclear translocation of Nrf2 [19]. Several dietary polyphenols, including kaempferol, naringenin, quercetin, resveratrol, apigenin, lutein [19, 20], and M, have been demonstrated to possess the ability to upregulate the expression and nuclear translocation of Nrf2. These imply that polyphenolic compounds share a common molecular structure for regulating Nrf2 activity.

Studies have also revealed that the activation of Nrf2 pathway could attenuate inflammation-associated pathogenesis. To systematically understand the role of Nrf2 in inflammation, Thimmulappa et al. [21] examined the gene expression profiles with Nrf2^{-/-} and Nrf2^{+/+} mice after a nonlethal LPS injection. Microarray analyses indicated enhanced expression of inflammation-associated genes in Nrf2^{-/-} mice, including IL-1 α , IL-6, IL-12 β , CSF2, and TNF- α [21]. Further investigation revealed that the activity of NF- κ B was much higher in Nrf2^{-/-} mice than in their wild-type counterparts [21]. In LPS-stimulated RAW264.7 macrophages, M treatment suppressed the production of NO, iNOS, TNF- α , IL-6, and IL-12 by depressing the activity

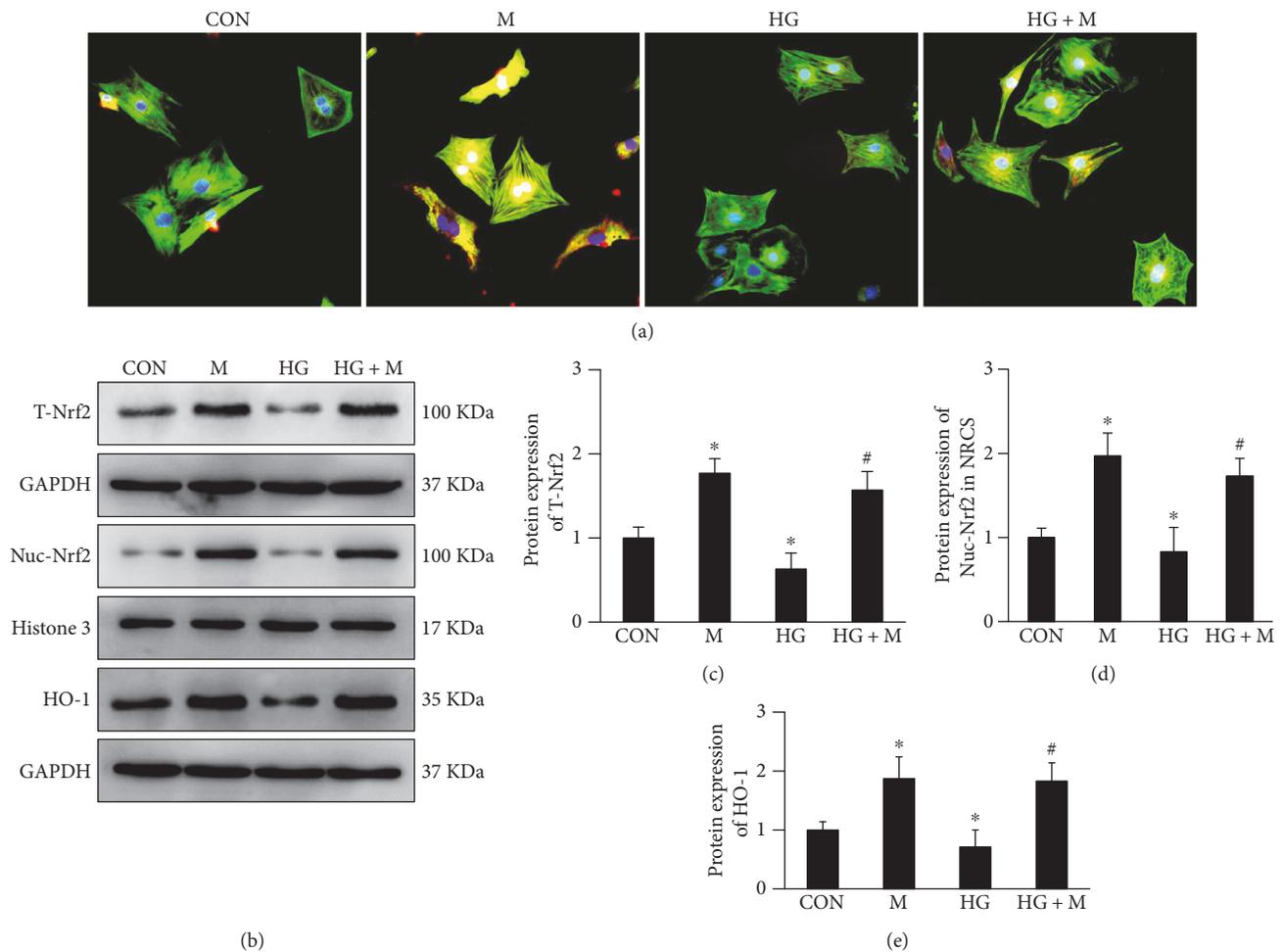


FIGURE 8: Myricetin regulated the expression of Nrf2 and HO-1 in neonatal rat cardiomyocytes (NRCM). (a) Myricetin enhanced the expression and nuclei translocation of Nrf2 after 36 h treatment with or without high glucose. (b) Representative blots showed the expression of Nrf2 and HO-1. (c), (d), and (e) Histogram showed the fold change of T-Nrf2, Nuc-Nrf2, and HO-1; all of the proteins were normalized to GAPDH or histone 3 before relative quantitative analysis, and all experiments were repeated 3 times independently. Data were presented as means \pm SD. * $P < 0.05$ as compared with CON and # $P < 0.05$ as compared with HG group.

of NF- κ B and the degradation of I κ B- α via promoting Nrf2/HO-1 pathway [22]. Our study indicated that silencing of Nrf2 could further activate the phosphorylation of I κ B- α /NF- κ B/p65 signaling in NRCM after incubation with high glucose for 72 h. We also detected further overexpression of IL-1 β , IL-6, and TNF- α at mRNA level after Nrf2 silencing by stimulating with LPS for 16 h. These results were coincident with previous reports that Nrf2 could inhibit I κ B- α /NF- κ B/p65 signaling. However, we found that M treatment could still significantly inhibit the phosphorylation of I κ B- α /NF- κ B/p65 signaling in NRCM exposed to high glucose and inhibited the production of IL-1 β , IL-6, and TNF- α in NRCM exposed to LPS after silencing of Nrf2. These finds indicated that M could protect cardiomyocytes from inflammatory response by a mechanism independent on Nrf2 activity. As a matter of fact, polyphenol-rich propolis extracts have been demonstrated to possess anti-inflammatory properties through regulation of I κ B- α /NF- κ B/p65 pathway by specifically delaying the ubiquitination of TRAF 6 [23]. Many other studies have also suggested that dietary

polyphenols have many targets to attenuate inflammation, including but not limited to STAT, PI3K, and COX [24]. The exact target of M against inflammation is being explored in our laboratory.

Extensive evidences have shown that myocardial fibrosis exists in both type I and type II DM [25]. In a study examining left ventricular endomyocardial biopsies from diabetes patients without coronary disease, authors have documented the increased collagen level with reduced LVEF [26]. In another study, aortic stenosis patients with diabetes showed severe myocardial stiffness and increased myocardial collagen content [27]. In this study, we also demonstrated that STZ induced cardiac injuries through increase of cardiac fibrosis and accumulation of collagen I/III in the heart. Chronic stimuli of high glucose have attributed to the overproduction of inflammatory cytokines [28], reactive oxygen species (ROS) [29], and neurohumoral factors [30], all of which have been suggested to be closely related with the severity and pathogeny of diabetes-induced cardiac fibrosis. These pathologic factors could activate the TGF β /Smad-

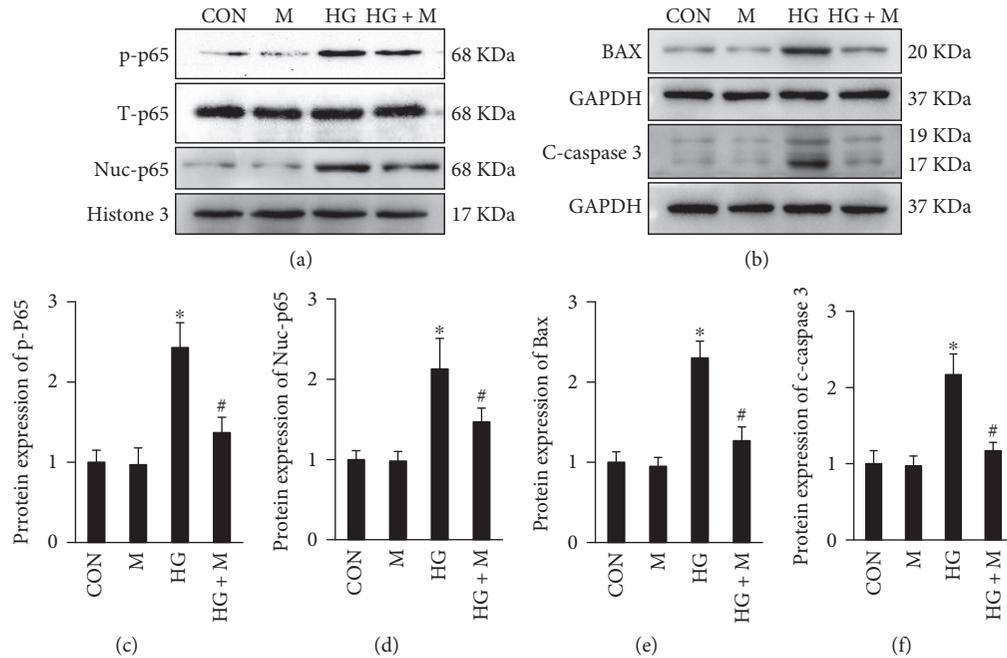


FIGURE 9: Myricetin blocked nuclei translocation of p65 and apoptosis in NRCM. (a) After 36 h of high glucose treatment, p65 were phosphorylated and translocated into the nuclei. (b) After 72 h of high glucose treatment, apoptosis-associated proteins (Bax and c-caspase 3) were accumulated in NRCM, which could be blocked by treatment of myricetin. (c), (d), (e), and (f) Histogram showed the fold change of p-p65, Nuc-p65, Bax, and c-caspase 3; all of the proteins were normalized to GAPDH or histone 3 before relative quantitative statistics, and all experiments were repeated 3 times independently. Data were presented as means \pm SD. * $P < 0.05$ as compared with CON or M group and # $P < 0.05$ as compared with HG group.

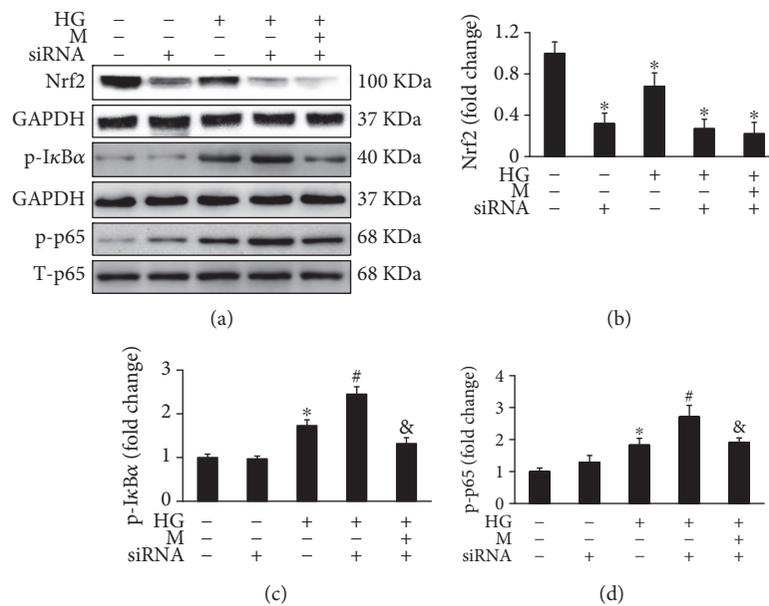


FIGURE 10: Myricetin inhibited activity of IκBα/NF-κB pathway independent on Nrf2 in high glucose-treated NRCM. (a) NRCM were transfected with siRNA for Nrf2 for 24 h, followed by treatment with high glucose (HG) or M for another 36 h; the total protein were extracted for detecting the expression of Nrf2, p-IκBα, and p-P65. (b), (c), and (d) Histogram showed the fold change of Nrf2, p-P65, and p-IκBα; all of proteins were normalized to GAPDH before relative quantitative analysis, and all experiments were repeated 3 times independently. Data were presented as means \pm SD. * $P < 0.05$ as compared with CON, # $P < 0.05$ as compared with CON or siRNA group, and &P < 0.05 as compared with LPS + siRNA group.

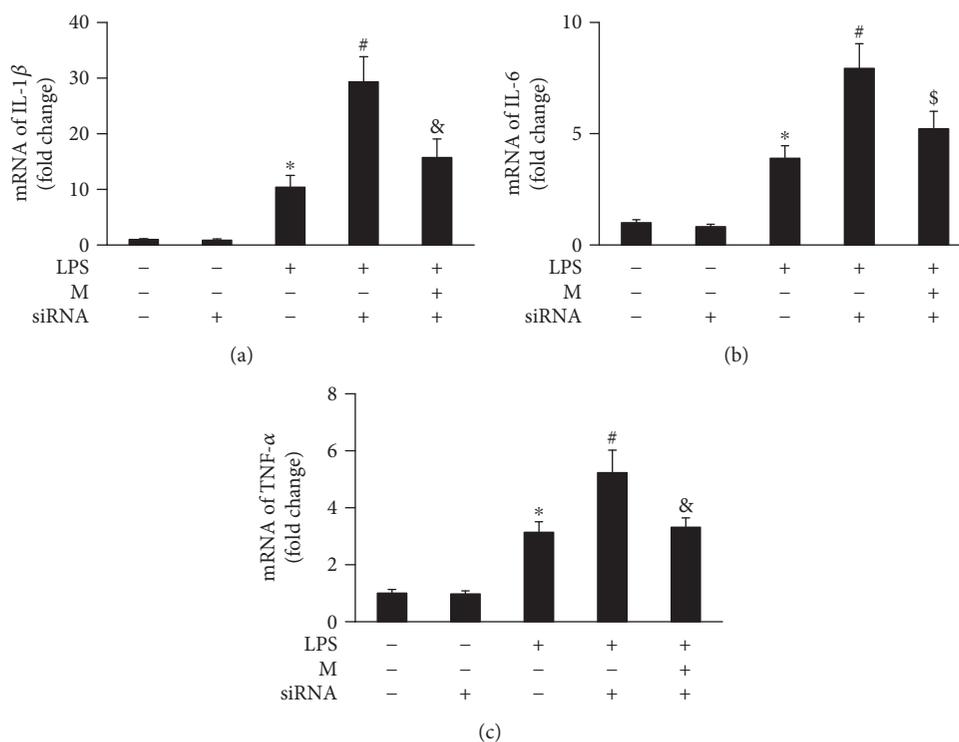


FIGURE 11: Myricetin decreased the expression of downstream inflammatory cytokines of $\text{I}\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$ pathway independent on Nrf2 in lipopolysaccharide- (LPS-) treated NRCM. (a), (b), and (c) NRCM were transfected with siRNA of Nrf2 for 24 h, followed by treatment with LPS ($1\ \mu\text{g}/\text{ml}$) or M ($40\ \mu\text{mol}$) for another 24 h; RT-PCR was performed to detect the expression of IL-1 β , IL-6, and TNF- α ; all of the mRNA expressions were normalized to GAPDH before relative quantitative analysis, and all experiments were repeated 3 times independently. Data were presented as means \pm SD. * $P < 0.05$ as compared with CON, # $P < 0.05$ as compared with CON or siRNA group, and & $P < 0.05$ as compared with LPS + siRNA group.

dependent or TGF β /Smad-independent signaling pathways of cardiac fibrosis. TGF β /Smad3 pathway has been proved to be the most important signaling pathway in the process of cardiac fibrosis [31]. Experiments using genetic mouse model demonstrated that global loss of Smad3 significantly decreased cardiac fibrosis and increased myocardial compliance [31]. In this study, we documented the effective inhibition of TGF β /Smad3 signaling pathway by M treatment. Of course, it still needs more investigations to answer whether M treatment could directly target at TGF β /Smad3 signaling pathway to reduce cardiac fibrosis.

5. Conclusion

In this study, we definitely suggested that M could improve cardiac injuries in DCM. The underlying mechanisms are at least partly associated with inhibiting $\text{I}\kappa\text{B-}\alpha/\text{NF-}\kappa\text{B}/\text{p}65$ and TGF β /Smad signaling and enhancing the expression of Nrf2, which accordingly alleviate oxidative stress, inflammation, apoptosis, and fibrosis. Our data exhibited that M may be a hopeful therapy or adjuvant drug for DCM. However, it remains to explore its multiple bioactivities, for the reason that microarray analysis has revealed that M treatment could significantly affect a total of 1104 genes [13]. In the next, we are intending to investigate the exact mechanism of M in regulating $\text{I}\kappa\text{B-}\alpha/\text{NF-}\kappa\text{B}$ signaling.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Hai-han Liao and Jin-xiu Zhu contributed equally to this work.

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