Original Research

Epigallocatechin-3-gallate attenuates myocardial fibrosis in diabetic rats by activating autophagy

Qiang Jia[1,2], Rui Yang[3], Shomaila Mehmood[4] and Yan Li[5]

[1]Department of Physiology, Bengbu Medical College, Bengbu 233030, Anhui, China; [2]Key Laboratory of Cardiovascular and Cerebrovascular Diseases, Bengbu Medical College, Bengbu 233030, Anhui, China; [3]School of Life Sciences, Hefei Normal University, Hefei 230601, Anhui, China; [4]School of Life Sciences, Anhui University, Hefei 230601, Anhui, China; [5]Clinical College, Bengbu Medical College, Bengbu 233030, Anhui, China
Corresponding author: Rui Yang. Email: xzxyr@sina.com

Impact Statement

Diabetic cardiomyopathy (DCM) is a serious cardiovascular complication of diabetes. Myocardial fibrosis is a fundamental characteristic of most cardiac pathologies in DCM. Accumulating evidence has manifested that defective autophagy promotes myocardial fibrosis. Epigallocatechin-3-gallate (EGCG), one of the most abundant catechins in green tea, exhibits anti-fibrotic properties in diverse tissues, but how EGCG exerted the anti-fibrotic effect on DCM remains unclear. The current work, for the first time, revealed that EGCG enhanced cardiac contractile function and attenuated myocardial fibrosis in type 2 diabetes. The mechanism may mainly involve the activation of autophagy via modulation of the adenosine 5'-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway, and then downregulation of the transforming growth factor (TGF)-β/matrix metalloproteases (MMPs) signaling pathway.

Abstract

Epigallocatechin-3-gallate (EGCG) possesses anti-fibrotic potential in diverse tissues; however, the molecular mechanisms underlying the impacts of EGCG on diabetes-induced myocardial fibrosis remain unclear. This present study aimed to unravel the anti-fibrotic effects of EGCG on the heart in type 2 diabetic rats and investigate its molecular mechanisms. Rats were randomly assigned to the following four groups: Normal (NOR), diabetic cardiomyopathy (DCM), DCM + 40 mg/kg EGCG, and DCM + 80 mg/kg EGCG groups. After 8 weeks of EGCG treatment, fasting blood glucose, left ventricular hemodynamic indices, heart index, and myocardial injury-related parameters were measured. Hematoxylin and eosin staining and Sirius Red staining were used to evaluate myocardial pathological alterations and collagen accumulation. The contents of myocardial hydroxyproline, collagen-I, collagen-III, transforming growth factor (TGF)-β1, matrix metalloprotease (MMP)-2, and MMP-9 were measured. The gene expression levels of myocardial TGF-β1, MMP-2, and MMP-9 were detected. Autophagic regulators, including adenosine 5'-monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), and autophagic markers, including microtubule-associated protein-1 light chain 3 and Beclin1 were estimated. The results indicated that diabetes significantly decreased cardiac contractile function and aggravated myocardial hypertrophy and injury. Furthermore, diabetes repressed the activation of autophagy in myocardial tissue and promoted cardiac fibrosis. Following ingestion with different doses of EGCG, myocardial contractile dysfunction, hypertrophy and injury were ameliorated; myocardial autophagy was activated, and myocardial fibrosis was alleviated in the EGCG treatment groups. In conclusion, these findings suggested that EGCG could attenuate cardiac fibrosis in type 2 diabetic rats, and its underlying mechanisms associated with activation of autophagy via modulation of the AMPK/mTOR pathway and then repression of the TGF-β/MMPs pathway.

Keywords: Epigallocatechin-3-gallate, diabetes mellitus, rat, myocardial fibrosis, autophagy, AMPK/mTOR signaling pathway

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Introduction

Diabetic cardiomyopathy (DCM) is a serious cardiovascular complication of diabetes, which significantly contributes to increased mortality among diabetic patients worldwide. DCM is defined by cardiac contractile dysfunction in patients with diabetes independent of the coexistence of hypertension, coronary artery disease, or valvular heart disease.[1] In recent years, myocardial fibrosis, which is a fundamental characteristic of most cardiac pathologies, is a dynamic process associated markedly with the progression of DCM.[2] The healthy heart comprises multiple cell types, such as cardiomyocytes, endothelial cells, and cardiac fibroblasts. Cardiac interstitial fibrosis is caused by increased fibroblasts and excessive deposition of extracellular matrix (ECM) constituents, such as collagen-I, collagen-III, and fibronectin.[3] Cardiac ECM deposition is primarily due to its overgeneration and reduced degradation and is often regulated by

the transforming growth factor (TGF)-β/matrix metalloproteases (MMPs) signaling pathway.[4] Ultimately, excess deposition of ECM components increases the stiffness of the heart and may even aggravate ventricle contractile dysfunction, eventually leading to heart failure.[5]

Macroautophagy (hereafter called autophagy) is a lysosome-dependent bulk degradation mechanism that is essential for degradation and recycling intracellular misfolded or aggregated proteins and damaged organelles to restore cellular homeostasis.[6] Microtubule-associated protein-1 light chain 3 (LC3) and beclin1 are key autophagic molecules, which are essential for autophagosome formation.[7] A growing body of evidence has manifested that defective autophagy dramatically promotes myocardial interstitial fibrosis and heart failure progression and aggravates ventricular dysfunction in some cardiomyopathies, including doxorubicin-induced cardiomyopathy and cardiac proteinopathy.[8,9] In addition, activation of autophagy could inhibit the TGF-β signaling pathway to alleviate tissue fibrosis.[10] Furthermore, the adenosine 5′-monophosphate-activated protein kinase (AMPK), which is considered to be a prime energy sensor, modulates many cellular metabolic processes, including autophagy, in the eukaryotic cells.[11] In diabetic patients, sustained hyperglycemia reduces the number of glucose transporters in the cardiomyocytes, which impacts the transport of glucose into the myocardial cells, resulting in insufficient cardiac energy metabolism.[12] Energy or nutrient deprivation promotes the phosphorylation of AMPK, resulting in the repression of the mammalian target of rapamycin (mTOR). mTOR protein, which is an essential part of the mTOR complex, can repress the activation of autophagy.[13] Under high blood glucose, the AMPK was phosphorylated and inactivated, and then mTOR was phosphorylated and activated, eventually leading to the repression of autophagy in type 2 diabetic hearts.[14]

Epigallocatechin-3-gallate (EGCG) is one of the most abundant catechins derived from green tea, which is the more popular beverage in China.[15] It has been reported that EGCG has diverse health benefits, including anti-cancer, antioxidative, anti-inflammatory, and anti-fibrotic properties.[16,17] A previous study has demonstrated that EGCG can ameliorate aging-induced cardiac hypertrophy and fibrosis, thereby improving cardiac function in rats.[18] Another study reported that EGCG could stimulate autophagy by enhancing LC3 and Beclin1 expression in high glucose-induced retinal Müller cells.[19] Moreover, EGCG is well known as an activator of AMPK in eukaryotic cells.[20] Kim *et al*.[21] indicated that EGCG promoted autophagy by activating the AMPK pathway in vascular endothelial cells. However, in type 2 diabetic myocardial tissue, whether EGCG mediated anti-fibrotic property is associated with the activation of autophagy via regulating the AMPK/mTOR pathway remains poorly determined. Hence, the current study was carried out to unravel the protective impact of EGCG on the myocardium in type 2 diabetic rats and detect its molecular mechanism.

Materials and methods

Experimental animals

Male healthy Sprague-Dawley rats 6–7 weeks old and weighed 160–200 g were acquired from the Experimental Animal Center of Anhui Medical University (Hefei, China). Rats were given free access to a normal diet and tap water and were housed in a monitored laboratory environment: alternating 12 h light and 12 h dark cycle, the temperature of 22 °C –24°C, and the relative humidity of 50%–60%. All animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care and Ethics Committee of Bengbu Medical College.

Experimental protocol

The rats were assigned at random into 4 experimental groups: (1) normal (NOR) group; (2) DCM group; (3) diabetic rats received 40 mg/kg EGCG (LEG) group; (4) diabetic rats received 80 mg/kg EGCG (HEG) group, each group consisted of 6 rats. The rats assigned to the NOR group received a standard laboratory rodent diet, while the rats assigned to the other three groups received a high-fat diet (HFD, consisting of 15% white sugar, 72.5% standard rodent food, 0.5% pig bile salt, 10% lard, and 2% cholesterol) throughout the whole experimental period. After 4 weeks of HFD treatment, these rats were fed with HFD, fasted overnight and were injected intraperitoneally with streptozotocin at a dose of 30 mg/kg twice a week, while the rats allocated in the NOR group fasted and were injected with the equal volume of citrate buffer. At the end of the fifth week (72 h after the last injection), the blood from the tail veins was obtained to measure the fasting blood glucose (FBG) using a handheld glucometer (Johnson, USA). The overnight-fasted rat with FBG > 11.1 mmol/L was considered type 2 diabetic. Hereafter, rats in the EGCG treatment groups were intragastrically administered daily with 40 mg/kg and 80 mg/kg EGCG (purity ⩾ 95%, Sigma-Aldrich) freshly dissolved in 0.9% saline solution for 8 weeks, respectively.[22] Rats in the NOR and DCM groups were daily gavaged with a similar volume of 0.9% saline solution over the same time.

Left ventricular function measurements

The left ventricular function-related indices were assessed according to our previously reported method.[23] Briefly, the body weight (BW) of rat was measured and injected intraperitoneally with 2% pentobarbital sodium at a dose of 45 mg/kg to induce anesthesia. The left ventricle was intubated through the right carotid artery of the rat and connected to the pressure transducer. Left ventricular function-related indices were acquired using the Med-Lab Biological Recording and Processing System (Medease Technology, Nanjing, China) following stabilization for 10 min. Subsequently, left ventricular systolic pressure (LVSP), end-diastolic pressure (LVEDP), and maximal rise/fall rates ($\pm dp/dt_{max}$) were determined.

Detection of blood glucose and heart index

Following ventricular hemodynamic indices measurement, the FBG was detected using the glucometer. The blood of the rat was collected to obtain the plasma, and then the rats were sacrificed. Next, the hearts were rapidly excised and weighed (heart weight, HW), and then the heart index (HW/BW) was calculated.

Histomorphological evaluation and immunohistochemical staining

One part of the fresh left ventricle of the heart was fixed for 24 h in a solution of 10% neutral formalin, embedded in a paraffin block, and subsequently cut into slices at a thickness of 4 μm. These slices were dewaxed and stained with hematoxylin and eosin (H&E) and Sirius Red reagents according to the protocols, respectively. Myocardial structural abnormalities were imaged using a light microscope. The deposition of collagen was examined using a polarized light microscope. The results of the fibrotic region in Sirius Red stained myocardial slices observed under the light microscope were determined by Image-Pro Plus (IPP) software. Five regions of each myocardial slice were selected at random, and the average of these slices was taken for analysis.

Immunohistochemical staining was carried out in strict accordance with our previously described method.[23] Briefly, the paraffin-embedded myocardial tissue sample was sectioned serially to 5 μm thickness and fixed on glass slides. After deparaffinization and antigen retrieval, these slices were incubated with anti-LC3 (1:300) and anti-Beclin1 (1:50) primary antibodies (ProteinTech Group, Wuhan, China), respectively. Subsequently, these myocardial slices were probed with a secondary antibody (1:500, ProteinTech Group). The immunohistochemical results were imaged using the light microscope. Five regions of each myocardial slice were selected randomly, and the mean optical density of positively stained cells was analyzed by IPP software.

Determination of plasma and cardiac indices

The levels of creatine kinase isoenzyme MB (CK-MB) and cardiac troponin I (cTnI) in the plasma were detected using corresponding assay kits (Jiancheng Bioengineering Institute, Nanjing, China). The hydroxyproline content in myocardial tissue was measured as per the manufacturer's protocol.[24] The concentrations of collagen-I, collagen-III, TGF-β1, MMP-2, and MMP-9 were measured in heart homogenates as per the instruction protocols of corresponding ELISA kits (Cusabio Biotechnology, Wuhan, China).

Real-time polymerase chain reaction (RT-PCR) assay

Total RNA was prepared on ice from each myocardial tissue sample using a Beyozol reagent (Beyotime Biotechnology, Shanghai, China). Reverse transcription was performed in strict accordance with the protocol of the PrimeScript real-time reagent kit (Takara, Dalian, China). RT-PCR was quantified using a TB Green Premix Ex TaqII kit (Takara). The sequences of used primers were as follows: TGF-β1 (Forward: CCAAGGAGACGGAATACAGG, Reverse: ATG AGGAGCAGGAAGGGTC), the amplified length was 156 bp; MMP-2 (Forward: CCAAGAACTTCCGACTATCCAATGA, Reverse: CAGTGTAGGCGTGGGTCCAGTA), the amplified length was 107 bp; MMP-9 (Forward: ATCTCTTCTAGAG ACTAGGAAGGAG, Reverse: CAAGCTGATTGGTTC GAGTAGC), the amplified length was 130 bp; β-actin (Forward: CGTAAAGACCTCTATGCCAACA, Reverse: AGCCACCAATCCACACAGAG), the amplified length was 163 bp, which was used as an internal control. The results of RT-PCR were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Part of the myocardial tissue sample was homogenized using an NP-40 Lysis Buffer containing protease and phosphatase inhibitor cocktail (Beyotime Biotechnology) to extract total protein. The 50 μg protein lysate was loaded onto 12% SDS-PAGE (Biosharp Biotechnology, Hefei, China) and then transferred onto PVDF membranes (Merck Millipore, USA). After blocking for 2 h in the mixed solution of tris-buffered saline, Tween-20, and 5% skim milk powder (TBST), all the membranes were probed overnight with the corresponding primary antibodies directly against p-AMPK, AMPK, p-mTOR, mTOR (all 1:1000 and purchased from Cell Signaling Technology), and β-actin (1:2000, ProteinTech Group) at 4°C. Following 4 rinses with TBST, the secondary antibody (1:4000) was subsequently used to incubate with the above membranes for 1 h at room temperature. After 4 rinses with TBST, these membranes were incubated with BeyoECL Moon solutions (Beyotime Biotechnology) and scanned using the ChemiDoc XRS chemiluminescence imaging system (Bio-Rad Laboratories, USA). The gray value of the band was determined by Quantity One software, and β-actin was used to normalize protein expression levels.

Statistical analysis

Data were represented as the mean ± standard deviation of the mean. All statistical analysis was conducted using GraphPad Prism software. One-way analysis of variance was used for the statistical analysis. Significant effects were obtained using the Newman-Keuls test. Statistically, significance was set at a P-value of less than 0.05.

Results

Effects of EGCG on left ventricular contractile function

The left ventricular contractile indices were measured to assess the cardiac function in rodents. As shown in Figure 1, sustained hyperglycemia markedly reduced LVSP and $\pm dp/dt_{max}$ and elevated LVEDP ($P < 0.01$) in the DCM group compared with the NOR group. After treatment of diabetic rats with 40 mg/kg and 80 mg/kg EGCG, LVSP and $\pm dp/dt_{max}$ were markedly increased ($P < 0.05$, $P < 0.01$), whereas LVEDP was reduced ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups. These results indicated that EGCG improved left ventricular contractility in type 2 diabetic rats.

Effects of EGCG on FBG, BW, HW, heart index, CK-MB, and cTnI

As shown in Figure 2, BW was decreased ($P < 0.01$), whereas the levels of FBG, HW, heart index, CK-MB, and cTnI were significantly increased ($P < 0.01$) in the DCM group compared with the NOR group. These results suggested that sustained high blood glucose caused myocardial hypertrophy and injury in rats. Following treatment with a low and high dose of EGCG, compared with the DCM group, there were

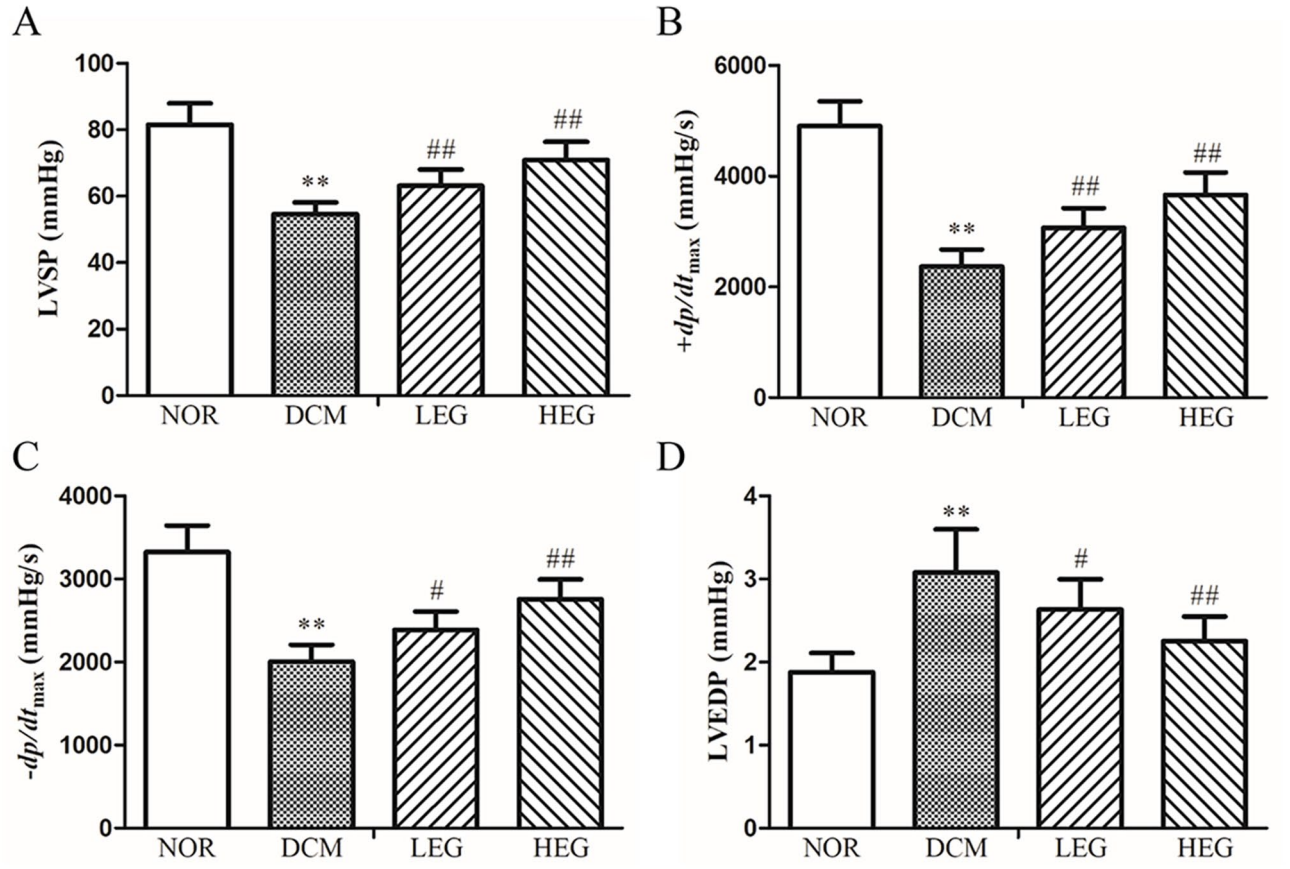

Figure 1. Effects of EGCG on left ventricular hemodynamic indices in the four groups. (A) LVSP. (B) + dp/dt_{max}. (C) -dp/dt_{max}. (D) LVEDP. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group.

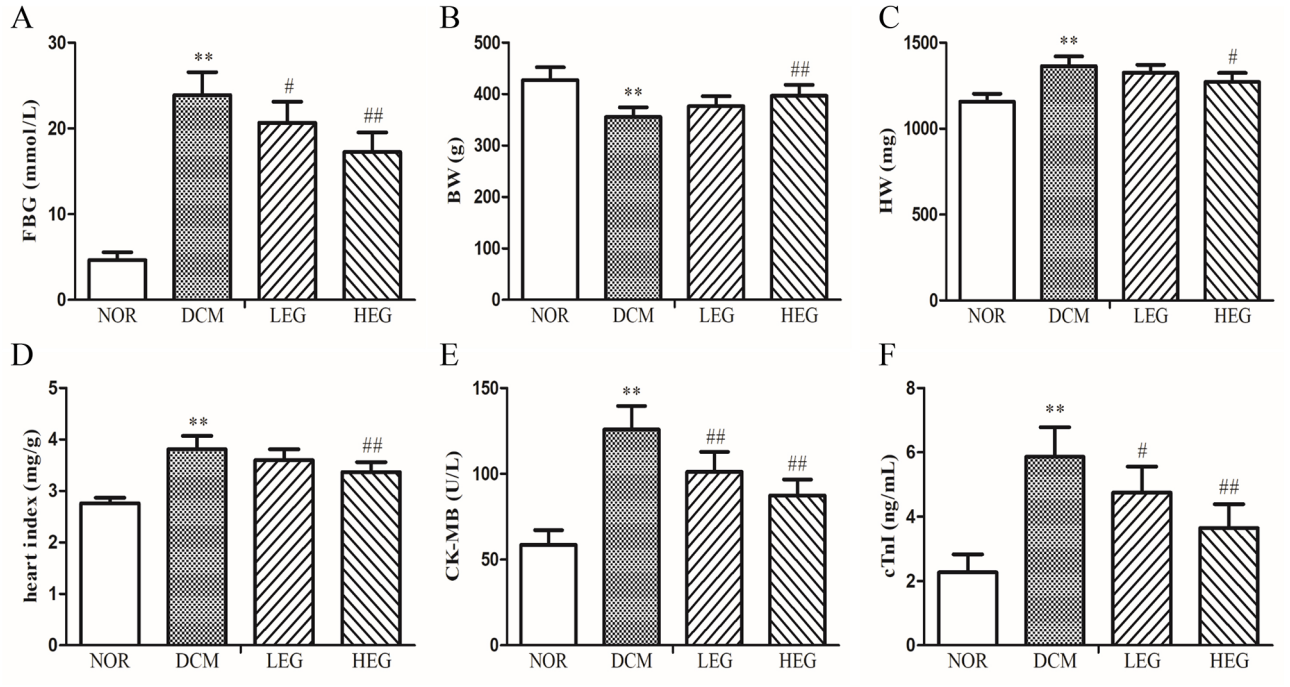

Figure 2. Effects of EGCG on (A) FBG, (B) BW, (C) HW, (D) heart index, (E) CK-MB, and (F) cTnI in the four groups. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group.

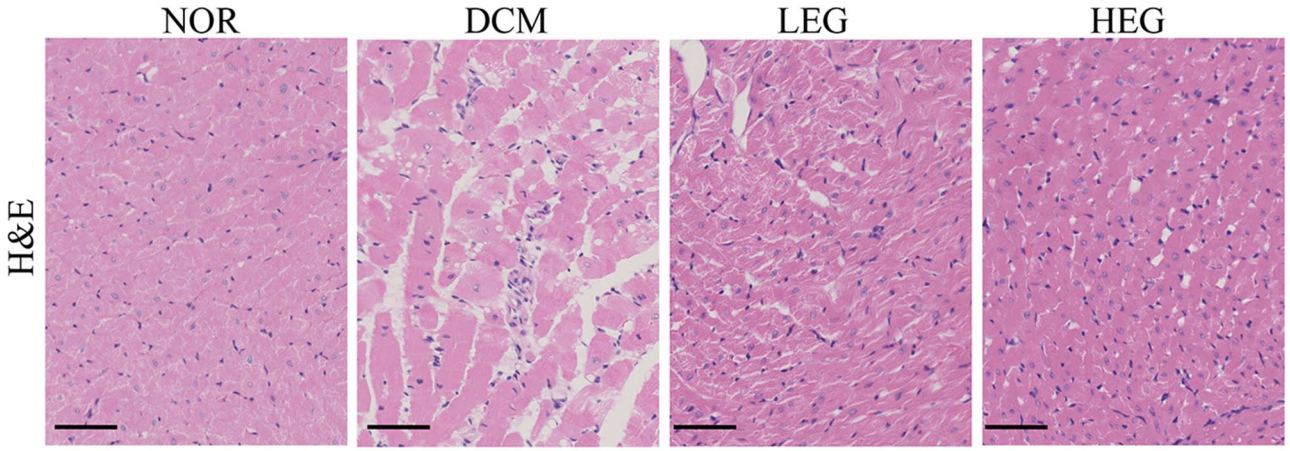

Figure 3. Effects of EGCG on histological alteration in myocardial tissues. Scale bar: 50 μm. (A color version of this figure is available in the online journal.)

no statistical differences in the BW, HW, and heart index in the LEG group; the levels of FBG, CK-MB, and cTnI were decreased ($P < 0.05$, $P < 0.01$) in the LEG group. In the HEG group, BW was increased ($P < 0.01$), whereas the other five indices were decreased markedly ($P < 0.05$, $P < 0.01$). These results indicated that EGCG alleviated high blood glucose and myocardial hypertrophic alterations and injury.

Effects of EGCG on myocardial histological changes

In H&E staining of the heart tissue sample, the cross-sectional shape of the myocardial fibers was arranged regularly, and the inflammatory cells were rare in the NOR group. In the DCM group, the myocardial fibers cross-section's cell size was increased, suggesting that cardiomyocytes were hypertrophic, and the inflammatory cells were infiltrated obviously. After supplementation with EGCG, the cardiomyocyte hypertrophy was ameliorated, and the infiltration of inflammatory cells was reduced in the LEG and HEG groups (Figure 3).

Effects of EGCG on myocardial collagen deposition

Sirius Red staining reagents are mainly composed of Sirius Red solution and Mayer Hematoxylin solution. Sirius Red is a strongly acidic anionic dye, which is easy to combine with the basic group in collagen. Under a light microscope, the collagen fibers are stained red, and cell nuclei are stained blue; under a polarized light microscope, the collagen fibers produce obvious birefringence, showing different colors. In Sirius Red staining of the heart tissue sample, the collagen fibers were stained red under a light microscope (Figure 4(A)); under a polarized light microscope, the type I collagen was stained red, and the type III collagen was stained green (Figure 4(B)). The results suggested that sustained hyperglycemia caused the overaccumulation of collagen fibers in the heart tissue, especially type I collagen. Different doses of EGCG supplement markedly downregulated the deposition of collagen fibers in the diabetic myocardial tissue, especially in the HEG group.

The levels of fibrosis area in the Sirius Red stained section, hydroxyproline, collagen-I, and collagen-III (Figure 4(C) to (F)) unraveled significant increases ($P < 0.01$) in collagen generation and accumulation in the hearts of the DCM group in contrast to those in the NOR group. After supplementation with a low and high dose of EGCG, the levels of fibrosis area, hydroxyproline, collagen-I, and collagen-III were decreased ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups, indicating that the generation and accumulation of myocardial collagen fibers reduced in the EGCG treatment groups. These findings suggested that EGCG exhibited an anti-fibrotic effect on myocardial tissue in diabetic rats.

Effects of EGCG on myocardial LC3 and Beclin1 expression

As shown in Figure 5, the numbers of myocardial LC3 and Beclin1 positively stained cells were reduced ($P < 0.01$) in the DCM group compared with the NOR group. Following treatment with different doses of EGCG, the numbers of myocardial LC3 and Beclin1 positively stained cells were elevated ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups. These results unraveled that EGCG could upregulate autophagic markers in diabetic hearts.

Effects of EGCG on myocardial TGF-β1/MMPs pathway

As shown in Figure 6(A) to (C), compared with the NOR group, the mRNA expression level of myocardial TGF-β1 was elevated ($P < 0.01$), whereas the mRNA expression levels of MMP-2 and MMP-9 were reduced ($P < 0.01$) in the DCM group. Following ingestion with EGCG, the mRNA expression levels of TGF-β1 were reduced ($P < 0.05$, $P < 0.01$), whereas the mRNA expression levels of MMP-2 and MMP-9 were elevated ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups. The corresponding ELISA results (Figure 6(D) to (F)) showed that compared with the NOR group, the protein content of myocardial TGF-β1 was elevated ($P < 0.01$), whereas the protein contents of MMP-2 and MMP-9 were reduced ($P < 0.01$) in the DCM group. Following ingestion with EGCG, the protein contents of TGF-β1 were reduced

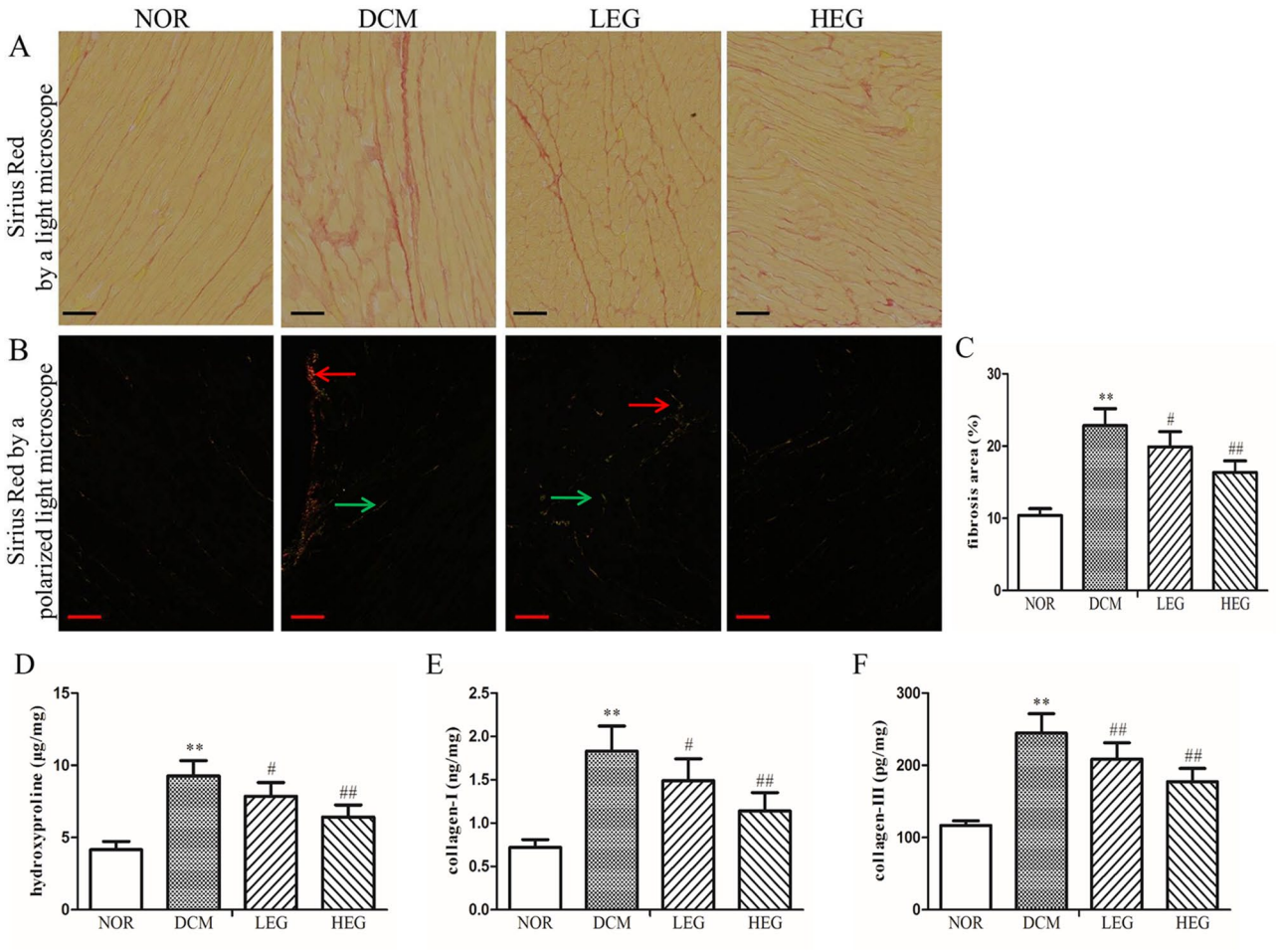

Figure 4. Effects of EGCG on collagen deposition in myocardial tissues. (A) Representative Sirius Red staining of myocardial tissues under a light microscope. Scale bar: 50 μm. (B) Representative Sirius Red staining of myocardial tissues under a polarized light microscope. Red arrow refers to the collagen-I and green arrow refers to the collagen-III. Scale bar: 50 μm. (C) fibrosis area in Sirius Red stained section. (D) hydroxyproline. (E) collagen-I. (F) collagen-III. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group. (A color version of this figure is available in the online journal.)

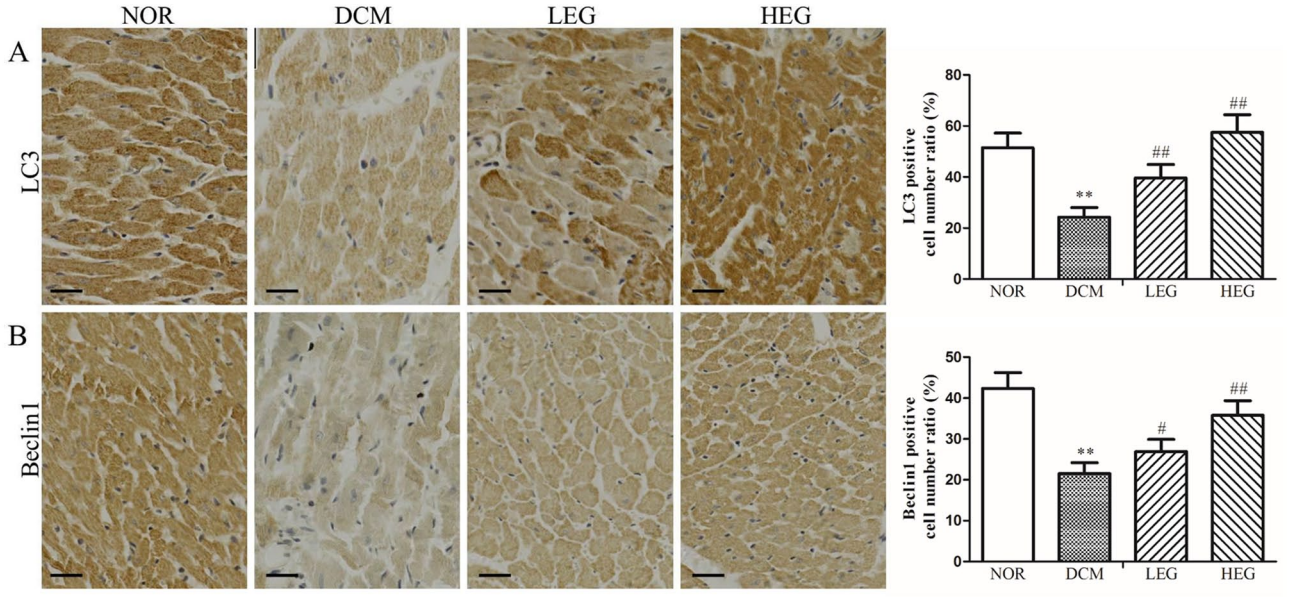

Figure 5. Effects of EGCG on the protein expression levels of LC3 and Beclin1 in myocardial tissues. (A) Representative IHC result for LC3. Scale bar: 25 μm. (B) Representative IHC result for Beclin1. Scale bar: 25 μm. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group. (A color version of this figure is available in the online journal.)

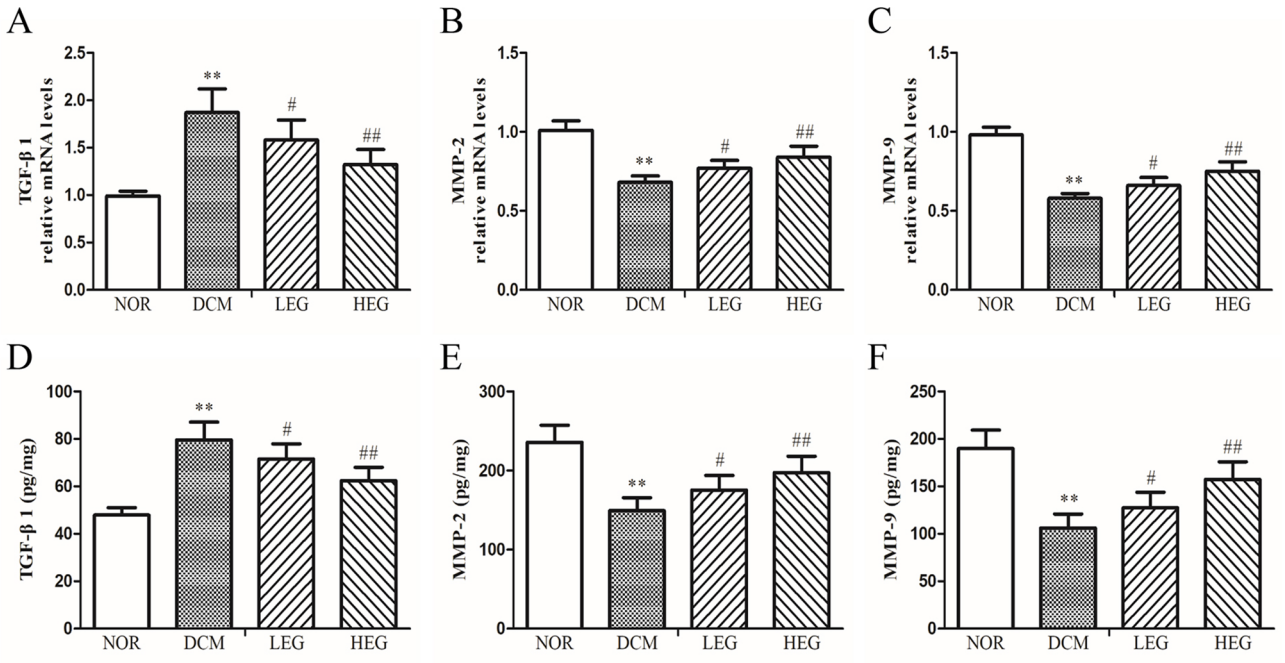

Figure 6. Effects of EGCG on the TGF-β1/MMPs pathway in myocardial tissues. (A-C) Relative mRNA levels of TGF-β1, MMP-2, and MMP-9. β-actin was used as a loading control. (D-F) The protein contents of TGF-β1, MMP-2, and MMP-9. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group.

($P < 0.05$, $P < 0.01$), whereas the protein contents of MMP-2 and MMP-9 were elevated ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups. These results revealed that EGCG could downregulate myocardial TGF-β1 expression and enhance the MMPs expression in diabetic rats.

Effects of EGCG on the myocardial AMPK/mTOR signaling pathway

As shown in Figure 7, there were no obvious impacts on the AMPK and mTOR protein levels in the four groups. Conversely, the expression level of myocardial p-AMPK was reduced ($P < 0.01$), whereas the level of p-mTOR was elevated ($P < 0.01$) in the DCM group compared with the NOR group. These findings revealed that the AMPK/mTOR signaling pathway was repressed in the diabetic heart. Following supplementation with different doses of EGCG, the levels of myocardial p-AMPK were increased ($P < 0.05$, $P < 0.01$), whereas the levels of p-mTOR were reduced ($P < 0.05$, $P < 0.01$) in the LEG and HEG groups. These results suggested that EGCG regulated AMPK/mTOR signaling pathway to activate autophagy in type 2 diabetic hearts.

Discussion

DCM, one of the most common cardiovascular complications of diabetes mellitus, is a critical factor in heart failure in diabetic patients. DCM implies the existence of cardiac injury, abnormal cardiac structure, and abnormal cardiac performance, which is characterized by the development of cardiomyocyte hypertrophy, apoptosis, and fibrosis.[25] CK-MB and cTnI have specificity for myocardial injury and are routinely used for clinical diagnosis of cardiomyopathy.[26] Upon myocardial injury, the number of cardiomyocytes decreases, whereas the number of myocardial fibroblasts increases, leading to an increase in myocardial ECM protein secretion, including collagen-I and collagen-III. Myocardial fibrosis is a crucial histological hallmark of DCM, which is characterized by the overgeneration and reduced degradation of ECM components. As a result, excessive deposition of ECM components increased the stiffness of the heart and then contributed to a decrease in cardiac systolic and diastolic function.[27] Our current work established an animal model of type 2 diabetes by treating the rats with an HFD combined with twice injections of low doses of streptozotocin. The results revealed that cardiac dysfunction of the rat was aggravated and BW was reduced, while the FBG, HW, heart index, CK-MB, and cTnI were markedly elevated, which indicated that myocardial injury, hypertrophy, and cardiac contractile dysfunction existed in the type 2 diabetic rat. Subsequently, the experimental results of Sirius Red staining, myocardial hydroxyproline, and collagens revealed that type 2 diabetes enhanced the synthesis and deposition of collagen fibers in the hearts of the DCM group, especially type I collagen. These results demonstrated that cardiac fibrosis was exacerbated in type 2 diabetic rats.

EGCG, one of the most abundant catechins present in green tea, is famous for its antioxidative, anti-inflammatory, and anti-apoptotic properties in various tissues and cells.[28,29] Emerging evidence has demonstrated that EGCG exhibits anti-fibrotic properties in diverse tissues in mammals, such as kidneys, liver, and pulmonary.[30,31,32] Othman *et al.*[33] reported that EGCG exerted a protective effect against DCM through alleviating cardiac oxidative stress, inflammation, apoptosis, and fibrosis in type 2 diabetic rats. Our present study indicated that following intervention with 40 mg/kg

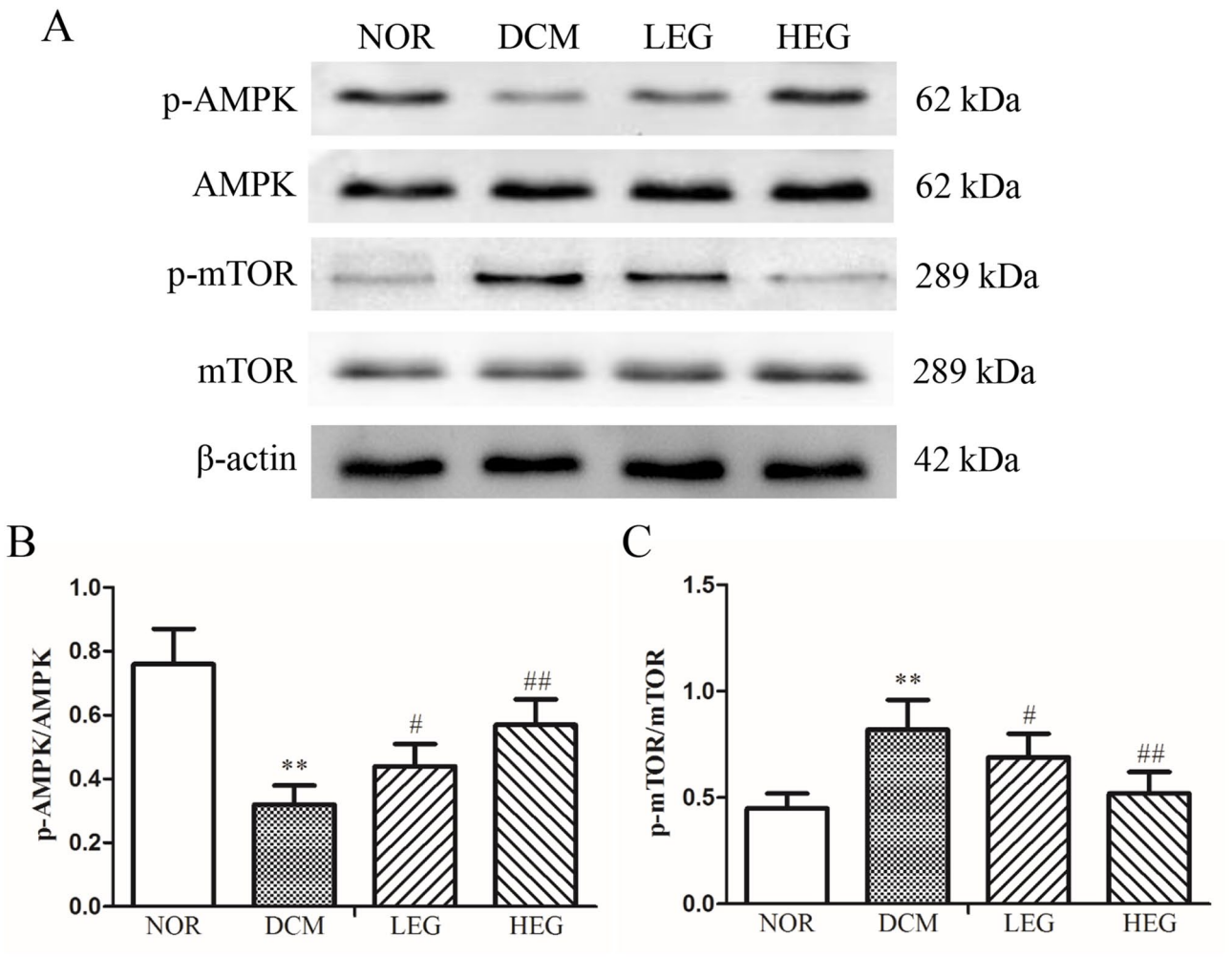

Figure 7. Effects of EGCG on the AMPK/mTOR autophagic pathway in myocardial tissues. (A) Representative western blotting showing p-AMPKα, AMPKα, p-mTOR, and mTOR alterations. (B) Quantitative analyses of p-AMPKα/AMPKα. (C) Quantitative analyses of p-mTOR/mTOR. Values, mean ± SD; $n = 6$; $^{**}P < 0.01$ vs the NOR group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the DCM group.

and 80 mg/kg EGCG, the cardiac contractile function was improved, whereas cardiac injury, hypertrophy, and fibrosis were ameliorated in the EGCG treatment groups compared with the type 2 diabetic rats, especially in the high dose of EGCG group, which further demonstrated that EGCG had an anti-fibrotic effect on the diabetic hearts; however, its precise molecular mechanism has not been completely elucidated. Thus, in the current study, we hoped to elucidate the underlying mechanism of the anti-fibrotic effects of EGCG on DCM in type 2 diabetic rats.

Autophagy is a highly regulated dynamic process that swallows the damaged organelles and misfolded proteins and enfolds them into autophagosomes and then fuses with lysosomes to form autolysosomes which degrade their enclosed contents. LC3 participates in autophagosome maturation and autophagosome-lysosome fusion in mammals.[34] Beclin1 is a prime part of the autophagy core complex, which serves a function in mediating the localization of other autophagic proteins to pre-autophagosome.[35] Therefore, LC3 and Beclin1 are widely regarded as autophagic markers. Autophagy is commonly controlled by the mTOR that can phosphorylate the unc-51 like autophagy activating kinase 1. In eukaryotic cells, insufficient energy generation represses the mTOR pathway via activating AMPK, and the latter is known as a cellular energy sensor and regulates diverse pathophysiological mechanisms, including apoptosis, autophagy, and protein synthesis.[36] Thus, AMPK has recently been recognized as a key activator of autophagy in the heart. Xie *et al*.[37] found that activation of AMPK inhibited the mTOR pathway and restored cardiac autophagy in DCM. Therefore, the AMPK/mTOR pathway is an important upstream regulatory pathway of autophagy. The activation of autophagy has been considered to provide an important beneficial role in DCM.[38] Our present study indicated that autophagy and autophagic activator were inhibited in type 2 diabetic hearts and was manifested by decreased expression of LC3, Beclin1, and p-AMPK and increased expression of the p-mTOR.

Recent research has proposed that EGCG is a recognized agonist of AMPK and can regulate the phosphorylation of AMPK.[39] EGCG promoted the generation of autophagosomes and enhanced autophagy by regulating the phosphorylation of AMPK in vascular endothelial cells.[21] Our experimental data also showed that following treatment with different doses of EGCG, the levels of p-AMPK, LC3, and Beclin1 were increased, whereas the level of p-mTOR was decreased in the LEG and HEG groups, which indicated that EGCG increased

p-AMPK and decreased p-mTOR expression, regulated the upstream signals of autophagy, thereby activating autophagy in the diabetic heart.

Autophagy plays a beneficial role in attenuating the excessive accumulation of ECM in the hearts.[40] To mitigate cardiac fibrosis, it is essential to degrade ECM proteins that are excessively synthesized in the heart, such as collagen. First, collagen fibers are degraded into small segments by protease degradation systems, including MMPs, and then macrophages can swallow and decompose these collagen segments. For instance, during the recovery process of lung fibrosis, autophagy is known to be involved in the degradation of misfolded proteins, pathogenic proteins, and ECM.[41] Kim *et al*.[42] reported that mice lacking autophagic protein Beclin1 exhibited a profibrotic phenotype, leading to an elevated collagen-I level. On the other hand, knockdown of Beclin1 promoted the activation of myofibroblasts via inducing TGF-β in myofibroblasts.[43] As a classic tissue-promoting factor, TGF-β1 not only induces the generation of collagen but also represses the degradation of collagen by MMP-2 and MMP-9. MMP-2 and MMP-9, which can degrade the collagen, are two well-studied members of the MMPs family in the heart of diabetic rats.[44] Our experimental findings showed that the levels of Beclin1, MMP-2, and MMP-9 were decreased, whereas the levels of TGF-β1, collagen-I, and collagen-III were increased in the DCM group, indicating that the repression of autophagy not only increased myocardial collagen contents but also further accelerated myocardial fibrosis by regulating TGF-β1/MMPs signaling pathway in type 2 diabetic rats. Following ingestion with different doses of EGCG, the expression of autophagic markers increased, myocardial collagen contents decreased, and the TGF-β1 signaling pathway was inhibited in the LEG and HEG groups, which indicated that EGCG could attenuate myocardial fibrosis through activating autophagy.

In conclusion, EGCG attenuated cardiac fibrosis in type 2 diabetic rats, and the results suggested that its underlying mechanism may be related to the activation of autophagy via modulation of the AMPK/mTOR pathway and then repression of the TGF-β/MMPs pathway, thereby decreasing the excess deposition of collagen in the diabetic hearts. The findings suggest that EGCG might be an important therapeutic strategy for patients with DCM.

AUTHORS' CONTRIBUTIONS

QJ and RY designed the experiments. QJ, RY, and YL performed the experiments. QJ, SM, and YL collected and analyzed the data. RY and SM wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID IDS

Qiang Jia https://orcid.org/0000-0001-6689-5255
Rui Yang https://orcid.org/0000-0003-3862-9069

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