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Original Article

Mangiferin ameliorates cardiac fibrosis in D-galactose-induced aging rats by inhibiting TGF-β/p38/MK2 signaling pathway

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ABSTRACT Aging is the process spontaneously occurred in living organisms. Cardiac fibrosis is a pathophysiological process of cardiac aging. Mangiferin is a well-known C-glucoside xanthone in mango leaves with lots of beneficial properties. In this study, rat model of cardiac fibrosis was induced by injected with 150 mg/kg/d D-galactose for 8 weeks. The age-related cardiac decline was estimated by detecting the relative weight of heart, the serum levels of cardiac injury indicators and the expression of hypertrophic biomakers. Cardiac oxidative stress and local inflammation were measured by detecting the levels of malondialdehyde, enzymatic antioxidant status and proinflammatory cytokines. Cardiac fibrosis was evaluated by observing collagen deposition *via* masson and sirius red staining, as well as by examining the expression of extracellular matrix proteins *via* Western blot analysis. The cardiac activity of profibrotic TGF-β1/p38/MK2 signaling pathway was assessed by measuring the expression of TGF-β1 and the phosphorylation levels of p38 and MK2. It was observed that mangiferin ameliorated D-galactose-induced cardiac aging, attenuated cardiac oxidative stress, inflammation and fibrosis, as well as inhibited the activation of TGF-β1/p38/MK2 signaling pathway. These results showed that mangiferin could ameliorate cardiac fibrosis in D-galactose-induced aging rats possibly *via* inhibiting TGF-β/p38/MK2 signaling pathway.

INTRODUCTION

Population aging is a global public health problem [1]. Aging is the deteriorative process spontaneously occurred in living organisms and characterized by progressive decline in multiple organ functions and systemic immunity [2]. As a result, aging is suggested to be a major risk factor for varieties of diseases. It is reported that cardiovascular diseases (CVDs) (including hypertension, cardiac hypertrophy and heart failure) contribute to about 31% of all deaths worldwide [3]. In fact, CVDs have already been the major health problems in both developed countries and developing countries [4]. Aging affects the heart at functional, structural and molecular levels [5,6]. These aging-related CVDs have become the

leading cause of death in elderly people [1]. CVDs, especially cardiac hypertrophy, are the common pathological changes in aging process [7]. Cardiac hypertrophy is a compensatory change of the heart in order to adapt to hypertension, vascular heart diseases and so on [7]. Cardiac hypertrophy and its accompanied myocardial fibrosis are usually responsible for heart failure [4]. Extracellular matrix (ECM) remodeling and collagen deposition are the characteristics of organ fibrosis. Aging promotes cardiac fibrosis [8]. However, there is no effective treatment for attenuating the aging-associated cardiac hypertrophy and fibrosis [9].

Mangiferin is a well-known C-glucoside xanthone and widespread in herbal medicines and fruits including mango leaves (*Mangifera indica* Linn) [10]. Mangiferin is known to has varies

of beneficial properties including anti-apoptotic, anti-oxidative and anti-inflammatory [11]. Reports showed that mangiferin administration ameliorated isoproterenol-induced apoptosis [12] and hydrogen peroxide-induced cell injury [10] in cardiomyocytes. Mangiferin was also believed to can protect against myocardial ischemia-reperfusion injury [11]. Furthermore, mangiferin has the functions of attenuating transverse aortic constriction-induced cardiac fibrosis [13]. Therefore, the potential protective effects of mangiferin on heart attract a great deal of attentions. This study was aimed to investigate the potential ameliorative effects of mangiferin against cardiac fibrosis in D-galactose (D-gal)-induced aging rats and its underlying molecular mechanisms.

METHODS

Chemicals and reagents

Mangiferin (purity > 95%) and D-gal (purity > 99%) were purchased from Aladdin Co, Ltd. (Shanghai, China). MMI-0100 (sequence: YARAAARQARAKALARQLGVAA) was synthesized by Genscript Biotechnology, Co. (Jiangsu, China). The commercial kits for the analysis of creatine kinase (CK), lactate dehydrogenase (LDH), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The commercial kits for the analysis of interleukin (IL)-1β IL-6 and tumor necrosis factor (TNF)-α were purchased from ELISA Lab, Co. Ltd. (Hubei, China). The antibodies of phosphorylation-mitogen activated protein kinase activated protein kinase II (p-MK2) and β-myosin heavy chain (β-MHC) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The antibodies of p-p38, α-smooth muscle actin (α-SMA), collagen (Col)-I, Col-III, natriuretic peptides A (ANP) and BNP were purchased from from Abcam, Co. (Cambridge, UK). The antibody of transforming growth factor-β1 (TGF-β1) was purchased from Proteintech Group, Inc. (Chicago, IL, USA).

Animals and groups

Eight weeks adult male Wistar rats (180–220 g) were obtained from the Centers of Disease Control and Prevention of Hubei Province, China. Rats were maintained under standard environment (temperature of 22°C ± 3°C, humidity of 50% ± 10% and 12 h light:12 h dark cycle). After acclimatization to the laboratory conditions for one week, the rats were randomly assigned into 5 groups (n = 8): the vehicle control group (Veh), the D-gal-induced aging group (aging), the aging-MMI-0100 group (MMI-0100), the aging-100 mg/kg mangiferin group (100 mangiferin) and aging-50 mg/kg mangiferin group (50 mangiferin). The rats from aging, MMI-0100, 100 mangiferin and 50 mangiferin groups were injected with 150 mg/kg/d D-gal for 8 weeks [14]. Animals

from 100 mangiferin and 50 mangiferin groups were daily intragastric administration of 100 and 50 mg/kg mangiferin for 8 weeks, respectively. The low dose of mangiferin was set according to previous report [13]. The high dose of mangiferin was set as 2 times of low dose. The rats from MMI-0100 were daily intraperitoneal injected with 50 μg/kg MMI-0100 [15]. At the end of the 8 weeks experimental period, the animals were anesthetized with pentobarbital Na (i.p. 100 mg/kg). Heart tissue samples were collected, weighted and excised. One part fresh tissue was used for subsequent Western blot analysis. One part tissue was fixed in 4% paraformaldehyde, embedded in paraffin and cut into thin sections. Then, the sections were stained by masson and sirius red. One part tissue was made to the 10% tissue homogenate using ice cold 0.9% sodium chloride for the subsequent assay of cardiac oxidative stress and inflammation. All the experiments were performed in accordance with the Chinese legislation and the ethical rules of NIH Guidelines for the Care and Use of Laboratory Animal, and were approved by the ethics committee of affiliated Puai Hospital of Tongji Medical College, Huazhong University of Science and Technology (approval No. PAH2018-12).

Cardiac aging and fibrosis evaluation

The age-related cardiac decline was estimated by detecting the relative weight of heart, the levels of serum cardiac markers (CK and LDH) and the expression of hypertrophic makers (ANP, BNP, and β-MHC) [16]. The relative weight of heart was showed as heart index (HI). HI (mg/g) = the weight of heart (mg) / the weight of body (g). The levels of serum CK and LDH were detected using commercial kits according to instructions. The expression of cardiac ANP, BNP, and β-MHC was detected by Western blot analysis. Rat heart tissue was ground in liquid nitrogen. The protein samples were extracted and separated by 10% SDS-polyacrylamide gel electrophoresis. Then, the samples were transferred to a PVDF membrane *via* electrophoretic transferring and blocked for 1 h using 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBST). Subsequently, the membranes were incubated with the primary antibody (ANP, BNP, and β-MHC) overnight at 4°C, washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST with 3% nonfat milk for 0.5 h. The chemiluminescence reaction was developed and the quantification of bands was determined by integrated optical density analysis using Alpha Innotech software. The data were normalized using GAPDH as an internal control.

Cardiac fibrosis was evaluated by observing collagen deposition *via* masson and sirius red staining, as well as by examining the expression of ECM proteins (Col-I, Col-III, and α-SMA) *via* Western blot analysis. The proportion of masson- and sirius red-positive area in sections were measured using Image-Pro Plus 6.0. The mean percent area of positive stained was calculated for 10 randomly selected fields of each section.

Cardiac oxidative stress and inflammation evaluation

Rat cardiac oxidative stress was assessed by detecting the activities of enzymatic antioxidants SOD and CAT, as well as the level of lipid peroxidation marker MDA. Cardiac inflammation was evaluated by detecting the levels of IL-1β, IL-6, and TNF-α. The performance was carried out in accordance with the instructions of commercial kits.

Cardiac TGF-β/p38/MK2 signaling pathway evaluation

The cardiac activity of profibrotic TGF-β1/p38/MK2 signaling pathway was assessed by measuring the expression of TGF-β1 and the phosphorylation levels of p38 and MK2 *via* Western blot analysis. The data were normalized using GAPDH as an internal control.

Statistical analysis

The values were presented as mean ± standard deviation. The bar graphs were made using Origin 6.0. Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison using SPSS 17.0.1 (SPSS Inc., Chicago, IL, USA). Differences were considered as significant at $p < 0.05$.

RESULTS

Mangiferin ameliorated D-gal-induced cardiac aging and fibrosis in rats

It can be seen from Table 1 that D-gal injection significantly enhanced the heart weight (wt) and HI compared to the Veh. When compared to the aging group, MMI-0100 and mangiferin (50 and 100 mg/kg/d) significantly reduced the heart wt and HI. As showed in Fig. 1, when compared to the Veh, the serum levels (Fig. 1A) of CK and LDH, as well as the cardiac expression (Fig. 1B) of hypertrophic makers ANP, BNP, and β-MHC were significantly increased in the rats from Aging group. When compared to the Aging group, MMI-0100 and mangiferin (50 and 100 mg/kg/d) significantly decreased the levels of CK and LDH, and the expression of ANP and BNP. MMI-0100 and 100 mg/kg/d man-

Table 1. Effects of mangiferin on body weight (wt), heart wt and heart index (HI) in D-gal-induced aging rats

	Body wt (g)	Heart wt (g)	HI (mg/g)
Veh	449.1 ± 12.5	1.35 ± 0.07**	3.00 ± 0.07**
Aging	454.4 ± 15.6	1.64 ± 0.07##	3.61 ± 0.04##
MMI-0100	452.1 ± 12.0	1.40 ± 0.06**	3.09 ± 0.06**,##
50 mangiferin	451.8 ± 13.8	1.54 ± 0.07*,##	3.40 ± 0.07**,##
100 mangiferin	449.9 ± 13.1	1.47 ± 0.08**,#	3.27 ± 0.08**,##

D-gal, D-galactose; Veh group, vehicle control group; Aging group, D-gal-induced aging group. Compared to aging group, *p < 0.05, **p < 0.01. Compared to Veh group, #p < 0.05, ##p < 0.01.
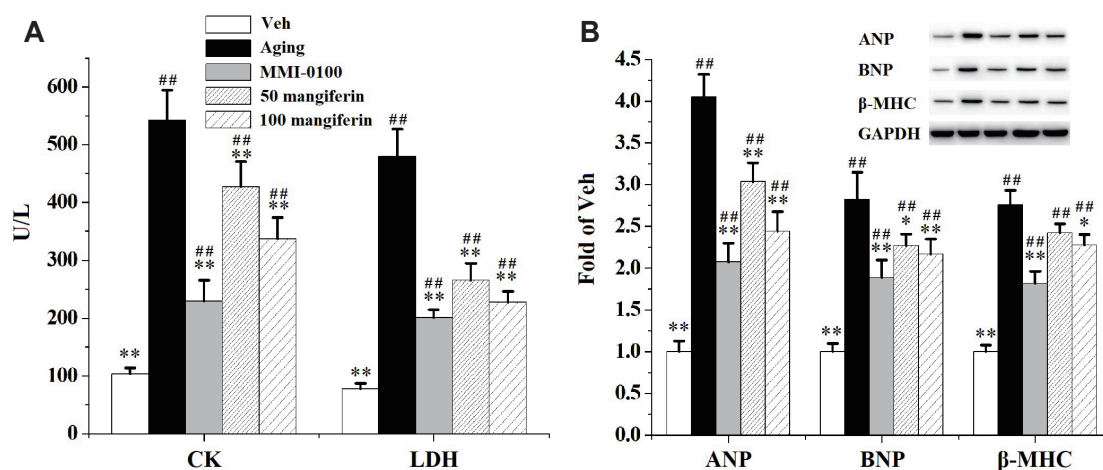

Fig. 1. Effects of mangiferin on serum levels (A) of CK and LDH, as well as on cardiac expression (B) of ANP, BNP, and β-MHC in D-gal-induced aging rats. The levels of serum CK and LDH were detected using commercial kits according to instructions (n = 8). The expression of cardiac ANP, BNP, and β-MHC was detected by Western blot analysis (n = 3). Values were presented as mean ± standard deviation. CK, creatine kinase; LDH, lactate dehydrogenase; ANP, natriuretic peptides A; β-MHC, β-myosin heavy chain; D-gal, D-galactose; Veh group, vehicle control group; Aging group, D-gal-induced aging group. *p < 0.05, **p < 0.01 compared to the Aging group. ##p < 0.01 compared to the Veh group.

giferin significantly decreased the expression of β-MHC.

Fig. 2 describes the cardiac collagen deposition. When compared to the Veh, D-gal injection significantly enhanced both the proportion of masson-positive and sirius red-positive area. When compared to the Aging group, MMI-0100 and mangiferin (50 and 100 mg/kg/d) significantly reduced the proportion of masson-positive and sirius red-positive area.

Mangiferin attenuated D-gal-induced cardiac oxidative stress and inflammation in rats

Fig. 3 describes that D-gal injection stimulates cardiac oxidative stress (Fig. 3A) and inflammation (Fig. 3B). When compared to the Veh, the cardiac activities of SOD and CAT in rats from Aging group were significantly decreased. Meanwhile, the levels of cardiac MDA, IL-1β, IL-6, and TNF-α were significantly increased in rats from Aging group compared to the Veh. When compared to the Aging group, MMI-0100 and mangiferin (50
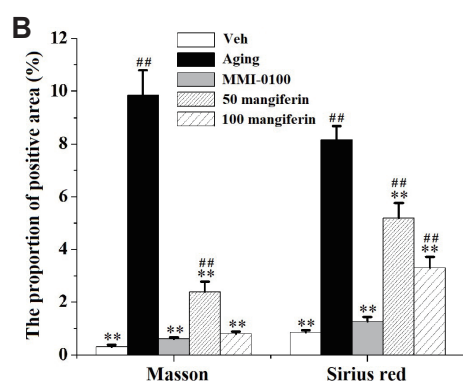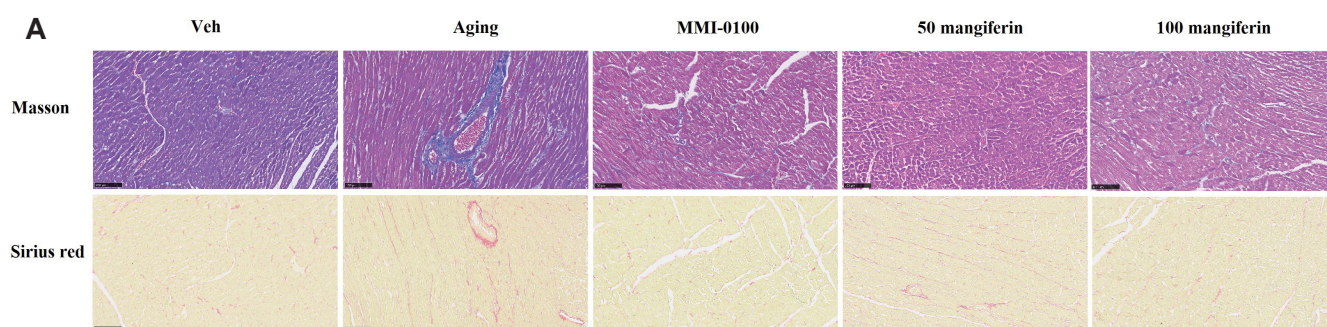

Fig. 2. Effect of mangiferin on cardiac collagen deposition. (A) Histological analysis for the effects of mangiferin on D-gal-induced collagen deposition in rat heart sections stained by masson and sirius red. The proportion of masson- and sirius red-positive area in sections were measured using Image-Pro Plus 6.0. Images were captured at 200× magnification. Scale bar of 100 μm was marked. (B) The proportion of masson-positive area and sirius red in heart sections. Values (n = 8) were presented as mean ± standard deviation. D-gal, D-galactose; Veh group, vehicle control group; Aging group, D-gal-induced aging group. **p < 0.01 compared to the Aging group. ##p < 0.01 compared to the Veh group.
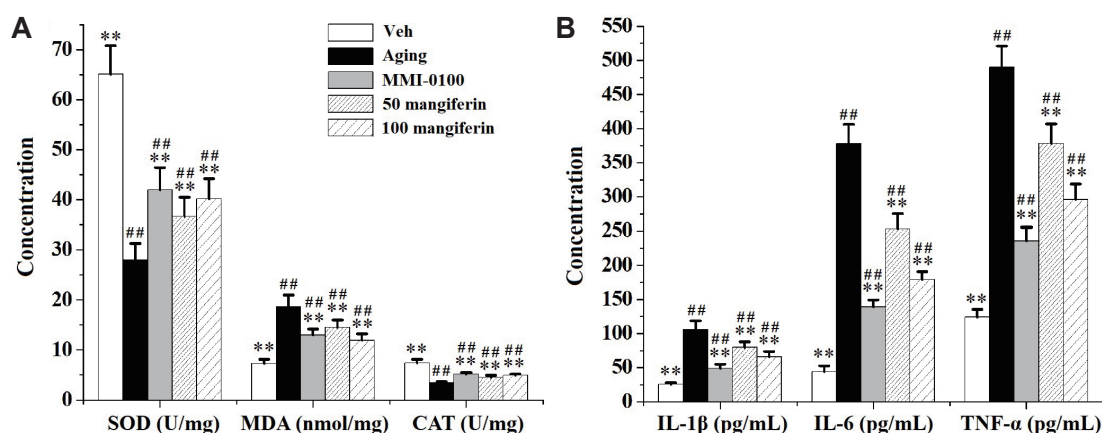
Fig. 3. Effects of mangiferin on cardiac oxidative stress (A) and inflammation (B) in D-gal-induced aging rats. The activities of enzymatic antioxidants SOD and CAT, as well as the level of lipid peroxidation marker MDA and proinflammatory cytokines IL-1β, IL-6 and TNF-α were measured in accordance with the instructions of commercial kits. Values (n = 8) were presented as mean ± standard deviation. D-gal, D-galactose; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; IL, interleukin; TNF, tumor necrosis factor; Veh group, vehicle control group; Aging group, D-gal-induced aging group. **p < 0.01 compared to the Aging group. ##p < 0.01 compared to the Veh group.
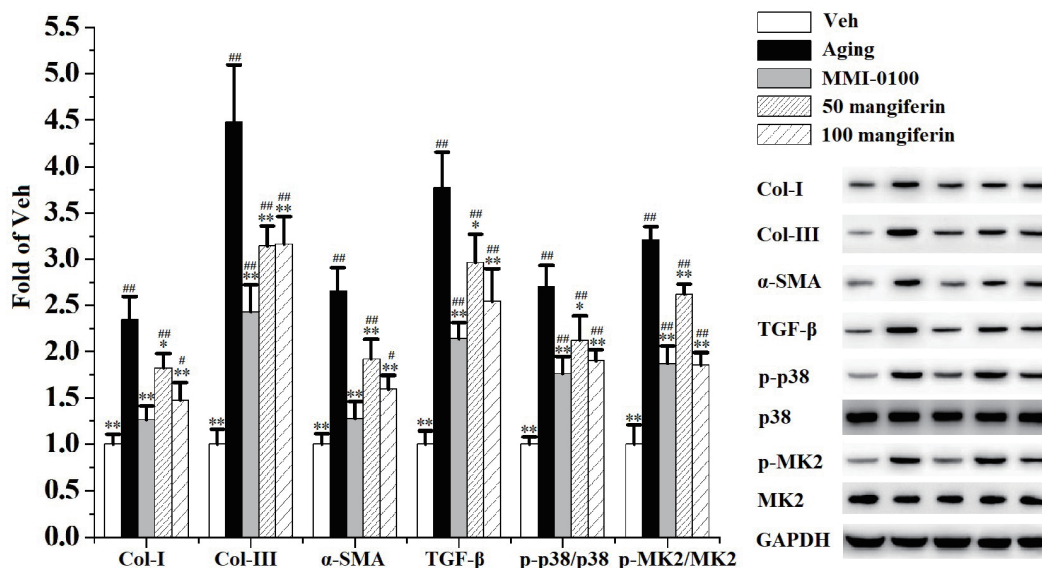
Fig. 4. Analysis for cardiac expression of pro-fibrogenic proteins (TGF-β1, p-p38, p-MK2, p38, and MK2) and ECM proteins (Col-I, Col-III, and α-SMA) in D-gal-induced aging rats. Values (n = 3) were presented as mean ± standard deviation. D-gal, D-galactose; Veh group, vehicle control group; Aging group, D-gal-induced aging group. *$p < 0.05$, **$p < 0.01$ compared to the Aging group. #$p < 0.05$, ##$p < 0.01$ compared to the Veh group.

and 100 mg/kg/d) significantly increased the cardiac activities of SOD and CAT, decreased the cardiac levels of MDA, IL-1β, IL-6 and TNF-α. It indicated that mangiferin attenuated D-gal-induced cardiac oxidative stress and inflammation in rats.

Mangiferin inhibited D-gal-induced cardiac pro-fibrogenic TGF-β1/p38/MK2 signaling pathway

Fig. 4 represents D-gal injection provokes cardiac pro-fibrogenic TGF-β1/p38/MK2 signaling pathway in rats. When compared to the Veh, the cardiac expression of TGF-β1, the phosphorylation levels of p38 and MK2, as well as the expression of ECM proteins Col-I, Col-III, and α-SMA were significantly enhanced in rats from Aging group. When compared to the Aging group, MMI-0100 and mangiferin (50 and 100 mg/kg/d) significantly reduced the cardiac expression of TGF-β1, p-p38, p-MK2, Col-I, Col-III, and α-SMA. The results showed that mangiferin inhibited D-gal-induced cardiac pro-fibrogenic TGF-β1/p38/MK2 signaling pathway.

DISCUSSION

Aging is the predisposing risk factor for cardiac hypertrophy, which severely harms the public health and brings huge time and financial burden [9]. D-gal has been long used to establish experimental aging models in animals because that it brings an abnormal D-gal accumulation *in vivo* and subsequently leads to harmful effects in the body [1]. The pathological progress of cardiac hypertrophy is accompanied by disorder expressed genes. ANP, BNP, and β-MHC are suggested to be the most consistent mark-

ers for cardiac hypertrophy [3,17]. Moreover, aging promoted-cardiac fibrosis is the characteristic of age-related cardiac decline including cardiac hypertrophy [4,8]. Several studies demonstrated that mangiferin had the abilities of inhibiting pulmonary fibrosis [18], renal interstitial fibrosis [19,20] and cardiac fibrosis [13]. In our present study, mangiferin ameliorated D-gal-induced cardiac hypertrophy and fibrosis by reducing heart index, decreasing the serum levels of cardiac injury indicators and expression of hypertrophic makers, as well as inhibiting cardiac collagen deposition.

Aging is a complex physiological process. The oxidative stress is considered to be one of the possible mechanisms underlying aging-related pathological changes [9]. Evidences indicate that aging causes the decline in mitochondrial function and antioxidant defense systems *in vivo* [5,7]. Accumulated reactive oxygen species (ROS) leads to oxidative damage [21]. These ROS stimulate oxidative burst, and contribute to the damages of cell membrane and DNA [22]. As a major pathophysiological mechanism for heart diseases, oxidative stress participates in the occurrence and development of cardiac hypertrophy [7,21]. Under normal conditions, ROS are scavenged by cellular antioxidant defense mechanisms [2,23]. SOD and CAT are the core members of antioxidant enzymatic defense systems [10]. The activity of SOD is used as an indicator for aging since that SOD protects against ROS-induced cellular injury *via* diminishing toxic oxygen free radicals [2,23]. Similarly, CAT is responsible for catalyzing the harmful hydrogen peroxide into harmless water [2]. MDA is a secondary product of lipid peroxidation, which is triggered by oxygen free radicals [10]. It is found that the level of MDA increases within the mitochondria from failing hearts [23]. So, the content of MDA is used to evaluate the extent of cellular damage and widely used as the biomarker of oxidative stress [2,10]. Our study confirmed that

mangiferin attenuated D-gal-induced cardiac oxidative stress *via* enhancing the activities of SOD and CAT, as well as reducing the level of MDA.

Chronic inflammation is another pathological mechanism for CVDs besides oxidative stress [24]. The cardiac levels of proinflammatory mediators (like IL-6 and TNF-α) are over-generated under pathological stimuli [25]. D-gal elevates the levels of these inflammatory cytokines in heart [26]. Furthermore, local inflammation promotes myocyte hypertrophy and interstitial fibrosis, and finally induces cardiac hypertrophy and heart failure [25]. We found that mangiferin attenuated D-gal-induced cardiac inflammation *via* decreasing the cardiac levels of IL-1β, IL-6, and TNF-α.

Fibrosis is associated with ECM accumulation and collagen deposition, and subsequently leads to progressive architectural remodeling in nearly all organs [27]. Cardiac fibrosis is an important pathophysiological changes in cardiac aging and observed in several cardiac diseases [8,14]. Oxidative stress can up-regulate cardiac fibrotic signaling pathway [8,17]. For example, ROS can induce cardiac fibrosis *via* enhancing the expression of profibrotic TGF-β1 [14]. The molecular mechanisms of organ fibrosis are not fully elucidated. In the process of cardiac fibrosis, fibroblasts are activated and transformed into myofibroblasts, and subsequently lead to ECM accumulation and collagen deposition [28]. TGF-β1 is found to have the function of inducing cardiac myofibroblasts [29]. Emerging evidence suggests that profibrotic TGF-β1 contains canonical and noncanonical signaling pathways in cardiac fibrosis [15]. On the one hand, TGF-β1/Smad3 signaling pathway is considered to be the canonical fibrosis-regulated signaling pathway [30]. On the other hand, the noncanonical TGF-β1 signaling pathway attracts more and more attentions.

MAPKs family proteins are involved in diverse cellular processes including cell differentiation, proliferation and apoptosis [31]. It is known that oxidative stress enhances the expression of MAPKs proteins [11]. P38 is a member of MAPKs. P38 signaling pathway can be activated by ROS [17]. The phosphorylation activation of p38 promotes the proliferation and differentiation of cardiac fibroblasts, as well as stimulates collagen deposition. Ultimately, cardiac fibrosis occur [32]. MK2 is a MAPK activated protein kinase belonging to serine-threonine kinase family. MK2 can be activated through phosphorylation under inflammation conditions [33]. MK2 is the downstream of p38. The phosphorylation level of MK2 is in direct proportion to the phosphorylation level of p38 [31]. MK2 is involved in regulating the levels of proinflammatory cytokines (such as IL-1β, IL-6, and TNF-α) [33]. Furthermore, MK2 participates in p38-induced heart failure [32]. As a result, MK2 seems to provide a bridge between oxidative stress and inflammation in cardiac fibrosis. Study illustrates that TGF-β is the upstream of MK2 [11]. Blocking MK2 can inhibit TGF-β-induced fibroblast differentiation and ECM protein a-SMA expression [15]. MK2 inhibitor MMI-0100 treatment successfully attenuates cardiac fibrosis and inflammation [32]. It

can be concluded that the TGF-β1/p38/MK2 is an important noncanonical fibrosis-regulated signaling pathway in age-related cardiac decline [30]. Previous study found that mangiferin alleviated cardiac fibrosis *via* activating Nrf2 signaling pathway, and thus promoting reduced glutathione (GSH) synthesis [13]. In our present work, we further identified the abilities of mangiferin on attenuating cardiac oxidative stress, inflammation and fibrosis *via* inhibiting the activation of TGF-β1/p38/MK2 signaling pathway. MMI-0100 is a cell-penetrating peptide inhibitor of MK2 [34]. MMI-0100 had exerted anti-cardiac fibrosis ability [15]. So, MMI-0100 was used as the positive control in this study. MMI-0100 and 100 mg/kg/d mangiferin significantly attenuated age-related cardiac declines and fibrosis.

In conclusion, the whole findings suggested that mangiferin suppressed D-gal-induced cardiac aging, ameliorated cardiac oxidative stress, inflammation and fibrosis possibly *via* inhibiting TGF-β/p38/MK2 signaling pathway. This present study provided new evidences for the protective effects of mangiferin against age-related cardiac hypertrophy and fibrosis.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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