ISSN 1948-9358 (online)

World Journal of *Diabetes*

World J Diabetes 2023 December 15; 14(12): 1717-1884

Published by Baishideng Publishing Group Inc

World Journal of Diabetes

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Monthly Volume 14 Number 12 December 15, 2023

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The *WJD* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2023 Edition of Journal Citation Reports® cites the 2022 impact factor (IF) for *WJD* as 4.2; IF without journal self cites: 4.1; 5-year IF: 4.5; Journal Citation Indicator: 0.69; Ranking: 51 among 145 journals in endocrinology and metabolism; and Quartile category: Q2.

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NAME OF JOURNAL
World Journal of Diabetes

ISSN
ISSN 1948-9358 (online)

LAUNCH DATE
June 15, 2010

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Lu Cai, Md. Shahidul Islam, Michael Horowitz

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-9358/editorialboard.htm

PUBLICATION DATE
December 15, 2023

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World J Diabetes 2023 December 15; 14(12): 1849-1861

DOI: 10.4239/wjd.v14.i12.1849

ISSN 1948-9358 (online)

ORIGINAL ARTICLE

Basic Study

Atorvastatin ameliorated myocardial fibrosis in db/db mice by inhibiting oxidative stress and modulating macrophage polarization

Xian-Min Song, Meng-Nan Zhao, Gui-Zhi Li, Na Li, Ting Wang, Hong Zhou

Specialty type: Endocrinology and metabolism

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Mostafavinia A, Iran; Pappachan JM, United Kingdom

Received: September 15, 2023
Peer-review started: September 15, 2023
First decision: September 20, 2023
Revised: September 29, 2023
Accepted: October 23, 2023
Article in press: October 23, 2023
Published online: December 15, 2023

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Abstract

BACKGROUND

People with diabetes mellitus (DM) suffer from multiple chronic complications due to sustained hyperglycemia, especially diabetic cardiomyopathy (DCM). Oxidative stress and inflammatory cells play crucial roles in the occurrence and progression of myocardial remodeling. Macrophages polarize to two distinct phenotypes: M1 and M2, and such plasticity in phenotypes provide macrophages various biological functions.

AIM

To investigate the effect of atorvastatin on cardiac function of DCM in db/db mice and its underlying mechanisms.

METHODS

DCM mouse models were established and randomly divided into DM, atorvastatin, and metformin groups. C57BL/6 mice were used as the control. Cardiac function was evaluated by echocardiography. Hematoxylin and eosin and Masson staining was used to examine the morphology and collagen fibers in myocardial tissues. The expression of transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β),M1 macrophages ($iNOS^+$), and M2 macrophages ($CD206^+$) were demonstrated by immunohistochemistry and immunofluorescence staining. The levels of TGF-β1, IL-1β, and TNF-α were detected by ELISA and real-time quantitative polymerase chain reaction. Malondialdehyde (MDA) concentrations and superoxide dismutase (SOD) activities were also measured.

RESULTS

Treatment with atorvastatin alleviated cardiac dysfunction and decreased db/db mice. The broken myocardial fibers and deposition of collagen in the myocardial interstitium were relieved especially by atorvastatin treatment. Atorvastatin also reduced the levels of serum lactate dehydrogenase, creatine kinase isoenzyme, and troponin; lowered the levels of TGF-β1, TNF-α and IL-1β in serum and myocardium; decreased the concentration of MDA and increased SOD activity in myocardium of db/db mice; inhibited M1 macrophages; and promoted M2 macrophages.

CONCLUSION

Administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization.

Key Words: Atorvastatin; Diabetic cardiomyopathy; Myocardial fibrosis; Macrophage polarization; Inflammation; Oxidative stress

Core Tip: The occurrence and development of diabetic cardiomyopathy are accompanied by a few pathological mechanisms. The present study showed that atorvastatin had antioxidant properties on diabetic hearts. Cardiac tissues include many resident macrophages. In high glucose conditions, macrophages can upregulate glucose uptake and utilization and enhance the production of inflammatory cytokines. Dysregulation of macrophages between M1 and M2 phenotypes causes excessive inflammation and cardiac injury. Our study suggests that administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization.

Citation: Song XM, Zhao MN, Li GZ, Li N, Wang T, Zhou H. Atorvastatin ameliorated myocardial fibrosis in db/db mice by inhibiting oxidative stress and modulating macrophage polarization. *World J Diabetes* 2023; 14(12): 1849-1861
URL: https://www.wjgnet.com/1948-9358/full/v14/i12/1849.htm
DOI: https://dx.doi.org/10.4239/wjd.v14.i12.1849

INTRODUCTION

The prevalence of diabetes mellitus (DM) is rising around the world, and is becoming a significant concern for global health. People with DM suffer from multiple chronic complications due to sustained hyperglycemia, especially diabetic cardiomyopathy (DCM). DCM is defined as myocardial structural abnormality and cardiac dysfunction, characterized by early diastolic dysfunction and further obstacle of systolic function, which ultimately leads to refractory heart failure (HF), independent of hypertension, coronary artery disease, or heart valvular disease[1]. Autophagy dysregulation, abnormal mitochondrial energetics, oxidative stress, inflammation, impaired calcium homeostasis, and activation of the renin–angiotensin–aldosterone system are all involved in the pathogenesis of DCM[2]. The pathological changes of DCM mainly contain cardiomyocyte hypertrophy, apoptosis, and myocardial fibrosis. Cardiac fibrosis is a major contributor to cardiac dysfunction, which ultimately increases the incidence of hospitalization due to HF and the mortality in patients with DM. However, there is currently no specific treatment for DCM at the early stage.

DM is a mild, chronic inflammatory condition characterized by the excessive secretion of proinflammatory cytokines, which can lead to cardiovascular complications[3]. Oxidative stress and inflammatory cells play crucial roles in the occurrence and progression of myocardium remodeling. High-glucose-induced oxidative stress can induce cardiac fibroblasts switching to a profibrotic phenotype that leads to cardiac fibrosis[4-6]. Cardiac fibrosis is mediated by a number of inflammatory cytokines, such as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). As a profibrotic regulator, TGF-β1 regulates fibroblast to myofibroblast transformation[7]. In our previous study, we observed an increase in Smad2/3 phosphorylation in the hearts of diabetic rats, coupled with elevated mRNA and protein levels of TGF-β1. JNK and Smad2/3 may serve as downstream signaling molecules within the RhoA/ROCK pathway and play a role in the development of myocardial fibrosis in type 2 DM (T2DM) rats[8]. Che *et al*[9] also discovered that inhibiting the TGF-β1/Smads signaling pathway significantly ameliorated cardiac dysfunction and reduced collagen production in DM mice. Inhibition of TNF-α has been shown to reduce cardiac fibrosis and improve cardiac function, contributing to the amelioration of DCM[10,11]. Hsuan *et al*[2] demonstrated that inhibiting the p38 mitogen-activated protein kinase stress pathway decreased inflammatory cytokines such as TNF-α and IL-1β in diabetic hearts, thus improving left ventricular dysfunction in DCM. Similarly, IL-1β plays a significant role in the pathophysiology of DCM, and targeting the NACHT, LRR, and PYD domains-containing protein 3 (NLRP3)/IL-1β pathway may prove effective in alleviating this disease burden. Incorporating IL-1β inhibition alongside statin therapy may offer added cardiovascular protection benefits[3,12]. Inflammatory cells and macrophages belong to the family of mononuclear phagocytes and play vital roles in immune responses, homeostasis, tissue damage, and restoration[13]. Macrophages

polarize to two distinct phenotypes: M1 and M2, and such plasticity in phenotypes provide macrophages various biological functions. M1 and M2 macrophages are closely associated with inflammatory responses; M1 macrophages are mainly involved in proinflammatory responses through secreting various proinflammatory mediators, leading to tissue injury. For example, a hyperglycemic state triggers aggregation of M1 macrophages, while proinflammatory cytokines such as TNF-α, IL-1β and TGF-β1 are elevated, stimulating myocardial fibrosis[14,15]. M2 macrophages exert anti-inflammatory effects, contributing to tissue repair[16]. It has been shown that the imbalance of the M1/M2 ratio accelerates the development of DCM, and the regulation on macrophage polarization can improve cardiac dysfunction in DCM mice[17,18].

Metformin is a first-line glycemic control drug that can decrease glycogen output and increase peripheral tissue uptake of glucose, thus ameliorating insulin resistance[19]. It is reported that metformin protects against DCM through attenuating cardiac apoptosis and fibrosis[20,21]. Therefore, metformin has been commonly used as the positive drug control for T2DM[22]. Statins are lipid-lowering drugs, possessing anti-inflammatory and antioxidative effects. This study aimed to investigate the effect of atorvastatin on cardiac function of DCM in db/db mice and its underlying mechanisms.

MATERIALS AND METHODS

Animals and treatment

Six-week-old male db/db mice and C57BL/6 mice were purchased from the HFK Bio-Technology Co. Ltd. (Beijing, China) (Approval No. SCXK 2020-0004). All procedures were approved by the Animal Experimental Ethics Committee of the Second Hospital of Hebei Medical University and the Animal Health Care Guidelines to minimize animal suffering. All mice were housed in standard cages (5 mice/cage) and were fed in a room with moderate temperature (22 ± 2°C), appropriate humidity (55% ± 5%), a 12/12 h light/dark cycle, with chow diet and water *ad libitum*. At 8 wk of age, blood glucose levels were measured from the tail vein using a portable glucometer (Accu-Chek Active; Roche Diagnostics, Mannheim, Germany). The db/db mice were considered to have T2DM if their blood glucose level was ≥ 16.7 mmol/L [23]. The diabetic mice were randomly divided into three groups: DM: db/db mice received daily oral gavage of sterilized water; atorvastatin group (DM + ATO): db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; metformin group (DM + MET): db/db mice received daily oral gavage of 200 mg/kg metformin. The C57BL/6 mice were used as the control group (CON). Each group contained five mice. Metformin hydrochloride tablets were purchased from Sino-American Shanghai Squibb Pharmaceutical Co. Ltd. (Shanghai, China), and atorvastatin calcium was purchased from Pfizer (New York, USA). Both drugs were dissolved in sterilized water and administrated through gastric gavage once per day for 16 wk.

At 24 wk of age, following a 12-h fast, blood samples were collected from the ophthalmic vein of mice under anesthesia. Systolic arterial blood pressure (SABP) was measured by tail-cuff micro-photoelectric plethysmography. Fasting blood was collected from the mice. Fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), total cholesterol (TC), triglyceride (TG), lactate dehydrogenase (LDH), creatine kinase isoenzyme (CK-MB), troponin (cTnI), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using the automatic biochemical instrument of the Second Hospital of Hebei Medical University. Cardiac function was evaluated by echocardiography. The mice were killed by cervical dislocation after 4% chloral hydrate anesthesia (0.20 mL/20 g; i.p.), and the cardiac muscle tissues were collected during chest surgery for analysis.

Cardiac echocardiography

Mice were anesthetized using 1.5% maintenance of isoflurane, and their heart rates were maintained between 400 and 500 beats/min. VEVO 3100 imaging system (Visual Sonics Inc., Toronto, ON, Canada) was used to perform echocardiography under M-mode. The left ventricle, the left ventricular ejection fraction (LVEF), the left ventricular fractional shortening (LVFS), the left ventricular internal dimension in systole (LVIDs), and the left ventricular end-diastolic diameter (LVIDd) were recorded.

Hematoxylin and eosin and Masson staining

The heart tissues were fixed in 4% paraformaldehyde and then embedded in paraffin. Tissue sections from the paraffin-embedded samples were stained following hematoxylin and eosin (HE) and Masson's trichrome staining. The myocardial staining was visualized and recorded under an optical microscope. HE and Masson staining was used to examine the changes of morphology and collagen fibers in myocardial tissues. Three sections and fields were investigated in histological evaluations in each group.

Immunohistochemistry and immunofluorescence staining

Specimens of myocardium were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were cut into 5-μm thick sections and heated for 10 min in 0.01 mol/L sodium citrate buffer with a microwave oven for antigen retrieval. Subsequently, 3% hydrogen peroxide was added to quench endogenous peroxidase activity. The sections were then blocked with 10% nonimmune goat serum to reduce nonspecific binding and incubated with 1:200 diluted anti-TGF-β1, 1:500 diluted anti-TNF-α, and 1:500 diluted anti-IL-1β antibodies (Abways, China) overnight at 4°C. After washing in phosphate-buffered saline (PBS), horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated with diaminobenzidine tetrahydrochloride. The sections were mounted on slides, stained with hematoxylin, and dehydrated in graded alcohol.

The paraffin blocks were cut into 5-μm thick sections, blocked with 10% nonimmune goat serum to reduce nonspecific binding, incubated with 1:100 diluted CD206 (Santa Cruz Biotechnology, Dallas, TX, USA) and 1:100 diluted inducible nitric oxide synthase (iNOS) (eBioscience, San Diego, CA, USA) antibodies overnight at 4C. After washing in PBS, HRP-conjugated secondary antibody was added and incubated for an additional 30 min at 37C. 4,6-diamidino-2-phenylindole (DAPI) was used to detect the nucleated cells. Images were visualized under a fluorescence microscope (Olympus, Tokyo, Japan). Three sections and fields were investigated for histological evaluations in each group.

ELISA

The levels of TGF-β1, IL-1β, and TNF-α in the serum samples were detected using ELISA kits (Multi Sciences, Hangzhou, China). Fasting insulin (FINS) was measured using another ELISA kit (Senberga, Nanjing, China).

Measurement of malondialdehyde and superoxide dismutase

Cardiac muscle tissues were homogenized, and the supernatants were harvested. Malondialdehyde (MDA) concentrations and superoxide dismutase (SOD) activities in myocardium were examined using commercial assay kits (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China).

Real-time quantitative polymerase chain reaction

Total RNA was extracted using RNA-easyTM Isolation Reagent (Vazyme, Nanjing, China), and real-time quantitative polymerase chain reaction (RT-qPCR) was performed using GoTaq qPCR Master Mix (Promega, Madison, WI, USA). The primers were provided by Sangon Biotechnology (Shanghai, China). The primers are shown in Table 1 The relative expression of the target mRNA was calculated by the $2^{\Delta\Delta CT}$ method.

Statistical analysis

Data were analyzed by GraphPad Prism.9.0 software (GraphPad, La Jolla, CA, USA) and expressed as mean ± SD. The differences among multiple groups were analyzed using one-way analysis of variance, followed by the Tukey test if F was significant. $P < 0.05$ was considered a statistical difference.

RESULTS

Effects of atorvastatin on biochemical parameters and SABP in db/db mice

FBG, FINS, HbA1c, TG and TC of the db/db mice were higher than those of the control mice (Table 2). Atorvastatin treatment of db/db mice markedly decreased their TC, but had no effects on FBG, FINS, HbA1c or TG. The db/db mice treated with metformin exhibited lower levels of FBG, FINS and HbA1c than those in the atorvastatin treatment group. There were no significant differences in SABP among the four groups.

Effects of atorvastatin on cardiac function and structure in db/db mice

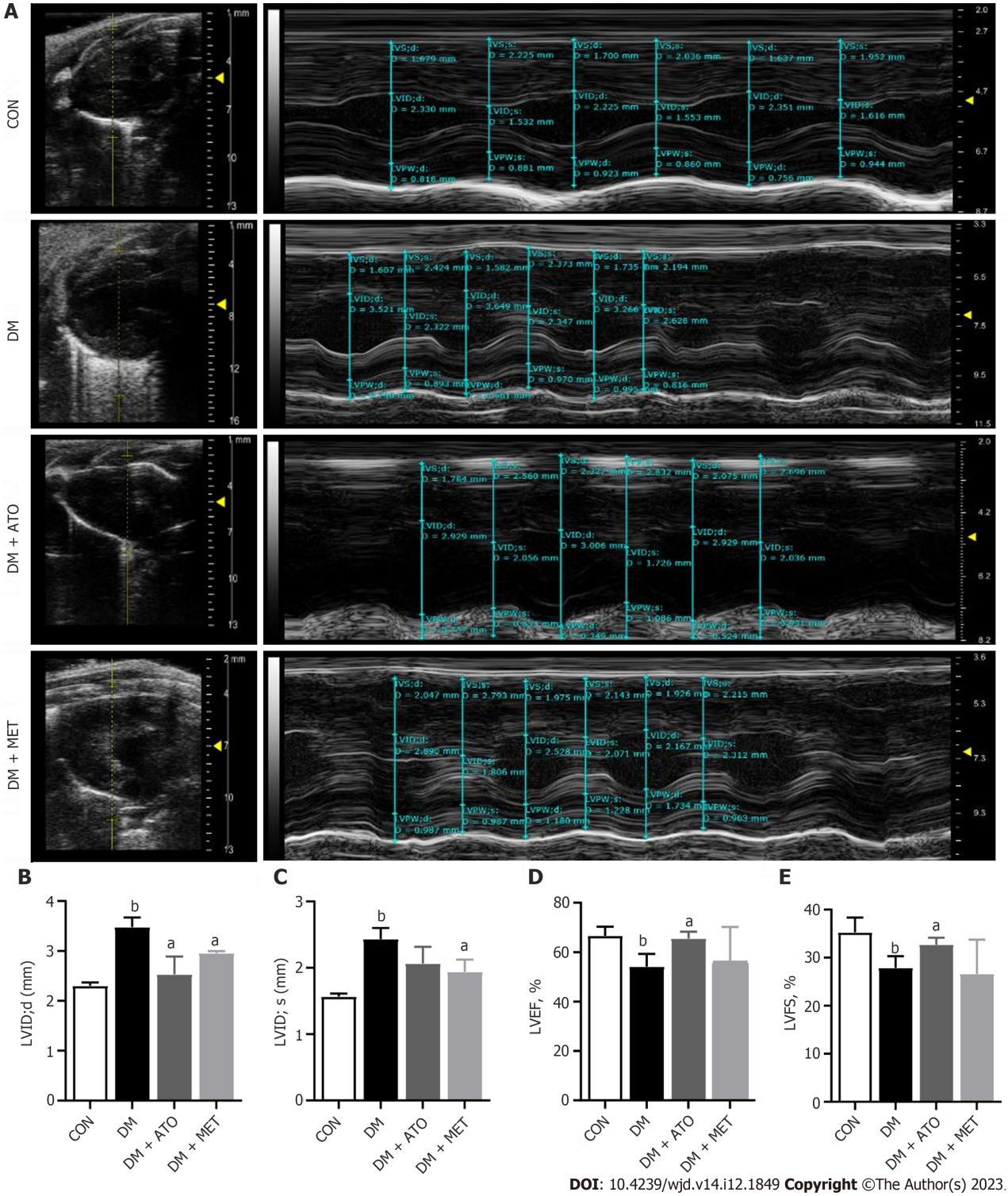
db/db mice had significant increased LVIDd and LVIDs, accompanied by a significant reduction in LVFS and LVEF, while the atorvastatin treatment significantly decreased their LVIDd level and augmented their LVFS and LVFF levels (Figure 1). Metformin treatment of db/db mice decreased LVIDd and LVIDs, but had no effects on LVFS and LVEF. The heart weight/body weight (HW/BW) of the db/db mice was significantly higher than that of the control mice, and treatment with atorvastatin or metformin significantly lowered HW/BW (Table 2). To evaluate the histological changes in the myocardium, HE and Masson staining was performed. db/db mice displayed disorganized and broken myocardial fibers and irregular nucleus and deposition of collagen in the myocardial interstitium, which were relieved by either atorvastatin or metformin treatment, especially atorvastatin (Figure 2). Compared with the control mice, the db/db mice had elevated serum levels of CK-MB, LDH and cTnI, indicating myocardial injury, while atorvastatin or metformin treatment decreased the serum levels of LDH, CK-MB and cTnI in db/db mice (Figure 3).

Effects of atorvastatin on inflammation and oxidative stress in db/db mice

Compared with the control mice, the serum levels and mRNA expression of TGF-β1, TNF-α and IL-1β in the myocardium were markedly elevated, while atorvastatin treatment markedly lowered these indicators in the serum and myocardium of db/db mice. Metformin treatment of db/db mice had no effects on the mRNA expression of IL-1β in the myocardium. The results of immunohistochemical staining of TGF-β1, TNF-α and IL-1β in the myocardium were consistent with those in the serum (Figures 3 and 4). Compared with the control mice, MDA concentration in cardiac muscle tissues of db/db mice was significantly increased, while SOD activity was significantly decreased. Compared with the db/db mice, atorvastatin or metformin treatment reduced MDA concentration and enhanced SOD activity in the myocardium (Figure 5).

Effects of atorvastatin on myocardial macrophage phenotypes in db/db mice

Immunofluorescence staining of macrophages showed increased M1 proinflammatory macrophages ($INOS^+$) and decreased M2 anti-inflammatory macrophages ($CD206^+$) in the myocardium of db/db mice. Atorvastatin treatment reduced the expression of M1 macrophages and promoted expression of M2 macrophages. However, metformin treatment increased expression of M2 macrophages and had no effects on the expression of M1 macrophages in the myocardium of db/db mice (Figure 5).



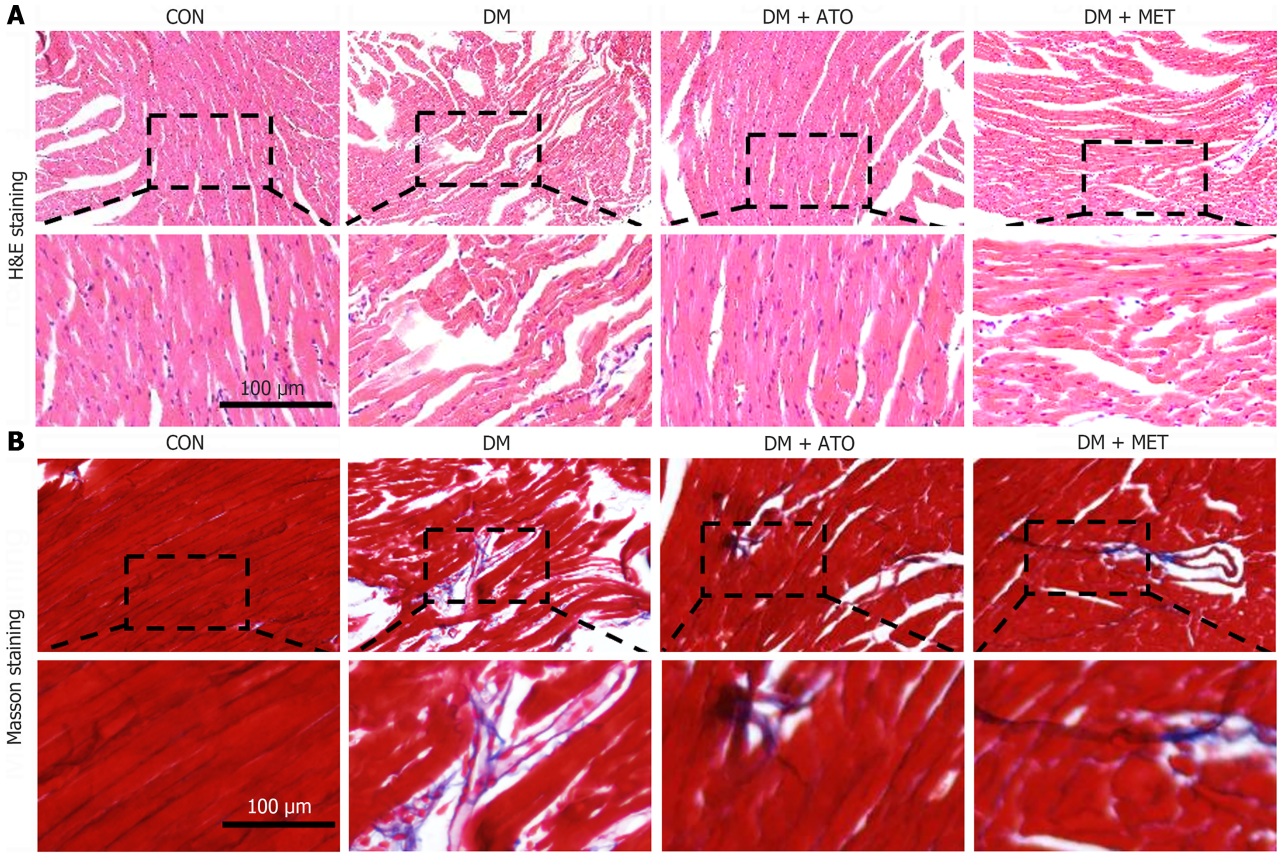
DOI: 10.4239/wjd.v14.i12.1849

Figure 1 Comparison of echocardiographic indices of cardiac systolic and diastolic function between each group. A: Representative pictures of cardiac echocardiography in each group; B–E: db/db mice received daily oral gavage of sterilized water (DM group) showed a significant increase in left ventricular end-diastolic diameter (LVIDd) and left ventricular internal dimension in systole (LVIDs), accompanied by a significant reduction in left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF), while db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin group showed decreased levels of LVIDd, and augmented levels of LVFS and LVEF. The DM+MET group had decreased levels of LVIDd and LVIDs, but no effects on LVFS and LVEF. Data represent the means ± SD (n = 5). ^{a}P < 0.05 compared with db/db mice received daily oral gavage of sterilized water group. ^{b}P < 0.05 compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening; LVIDs: Left ventricular internal dimension in systole; LVIDd: Left ventricular end-diastolic diameter.

Table 1 Primers used in this study

Gene	Primer	Tm (°C)	Product length	Accession
TGF-β1	Forward: 5′-CTCCCGTGGCTTCTAGTGC-3′	60.15	133	NM_011577.2
	Reverse: 5′-GCCTTAGTTTGGACAGGATCTG-3′	58.73		
TNF-α	Forward: 5′-CCCTCACACTCAGATCATCTTCT-3′	59.29	61	NM_013693.3
	Reverse: 5′-GCTACGACGTGGGCTACAG-3′	60.23		
IL-1β	Forward: 5′-GCAACTGTTCCTGAACTCAACT-3′	59.05	89	NM_008361.4
	Reverse: 5′-ATCTTTTGGGGTCCGTCAACT-3′	59.58		
18S rRNA	Forward: 5′-AGGGGAGAGCGGGTAAGAGA-3′	61.58	241	AH002077.2
	Reverse: 5′-GGACAGGACTAGGCGGAACA-3′	61.26		

Tm: Melting temperature; TGF: Transforming growth factor; TNF: Tumor necrosis factor; IL: Interleukin.



DOI: 10.4239/wjd.v14.i12.1849

Figure 2 Histological changes in the myocardium. A: Hematoxylin and eosin staining was performed for each group. db/db mice that received daily oral gavage of sterilized water (DM group) had disorganized and broken myocardial fibers and irregular nuclei, which were relieved in db/db mice by daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or in db/db mice by daily oral gavage of 200 mg/kg metformin (DM + MET) group, especially in the DM + ATO group; B: Masson staining was performed for each group. DM group displayed deposition of collagen in the myocardial interstitium, which was relieved in the DM + ATO or DM + MET group, especially the DM + ATO group. Scale bar, 100 μm. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; H&E: Hematoxylin and eosin staining.

DISCUSSION

The incidence of HF has increased in patients with DM, which is closely related to DCM. The development of DCM initiates from subtle myocardial changes to myocardial fibrosis and diastolic dysfunction and eventually to stubborn HF. Cardiac fibrosis is one of the primary characteristics of DCM that contributes to the development of adverse cardiac remodeling and myocardial stiffness[24]. Hyperglycemia, hyperlipidemia, hyperinsulinemia, and impaired insulin

Table 2 Biochemical parameters, systolic arterial blood pressure and ratio of heart weight/body weight in mice at 24-wk age

Parameters	CON, *n* = 5	DM, *n* = 5	DM + ATO, *n* = 5	DM + MET, *n* = 5
FBG (mmol/L)	5.12 ± 0.11	31.81 ± 3.27[b]	33.19 ± 3.13[b]	10.16 ± 2.92[a,c,d]
FINS (mU/L)	62.82 ± 4.98	281.24 ± 15.47[b]	290.10 ± 18.39[b]	104.13 ± 8.99[a,c,d]
HbA1c (%)	5.51 ± 0.60	9.57 ± 0.58[b]	9.42 ± 0.47[b]	7.15 ± 0.84[a,c,d]
ALT (IU/L)	58.95 ± 9.88	67.75 ± 11.30	69.03 ± 14.29	73.49 ± 15.68
AST (IU/L)	131.3 ± 27.12	157.3 ± 30.36	139.7 ± 28.49	143.9 ± 32.19
TG (mmol/L)	1.10 ± 0.17	2.48 ± 0.46[b]	2.29 ± 0.33[b]	2.17 ± 0.25[b]
TC (mmol/L)	2.22 ± 0.25	3.77 ± 0.39[a]	2.15 ± 0.38[c]	3.29 ± 0.32[a,d]
SABP (mmHg)	123.9 ± 3.09	133.9 ± 5.96	129.6 ± 4.88	136.4 ± 5.71
HW/BW (× 10^{-3})	4.37 ± 0.55	6.25 ± 0.38[a]	4.89 ± 0.10[c]	5.01 ± 0.46[c]

[a]$P < 0.05$ *vs* control group.
[b]$P < 0.01$ *vs* control group.
[c]$P < 0.05$ *vs* db/db mice group.
[d]$P < 0.05$ *vs* db/db mice + atorvastatin.
CON: Control group; DM: db/db mice group; DM+ATO: db/db mice + atorvastatin; DM+MET: db/db mice + metformin; FBG: Fasting blood glucose; FINS: Fasting insulin; HbA1c: Hemoglobin A1c; ALT: Alanine transaminase; AST: Aspartate aminotransferase; TG: Triglyceride; TC: Total cholesterol; HW/BW: Heart weight/body weight; SABP: Systolic arterial blood pressure.

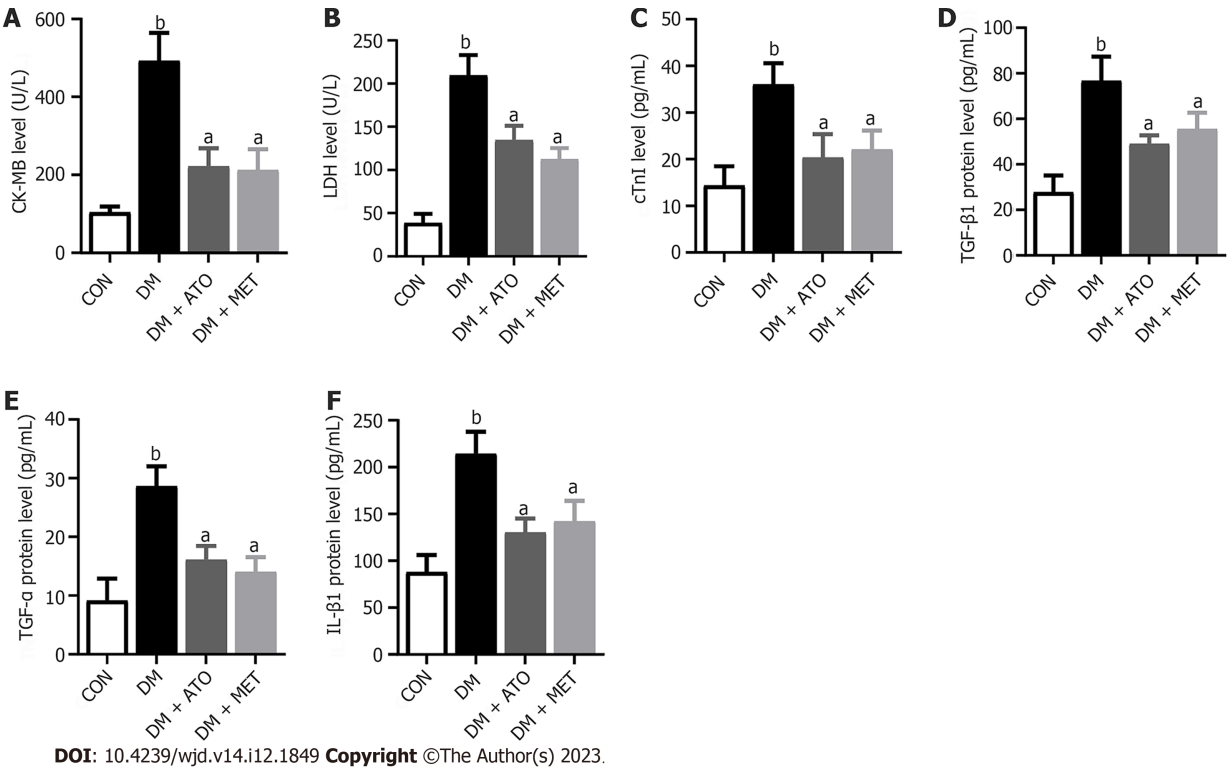


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Figure 3 Expression of markers of cardiac injury and indicators of inflammation in the serum of each group. A–C: Serum creatine kinase isoenzyme (CK-MB), lactate dehydrogenase (LDH) and troponin (cTnI) were measured using the automatic biochemical instrument. Compared with C57BL/6 mice (CON group), db/db mice that received daily oral gavage of sterilized water (DM group) had elevated serum CK-MB, LDH and cTnI. db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or db/db mice that received daily oral gavage of 200 mg/kg metformin (DM + MET) group had decreased serum CK-MB, LDH and cTnI; D–F: The levels of transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β in the serum samples were detected by ELISA. Compared with CON group, the serum levels of TGF-β1, TNF-α, and IL-1β were markedly elevated. In the DM + ATO and DM + MET groups, these indicators were markedly lower. Data represent the means ± SD ($n = 5$). [a]$P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. [b]$P < 0.05$ compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin. CON: C57BL/6 mice.

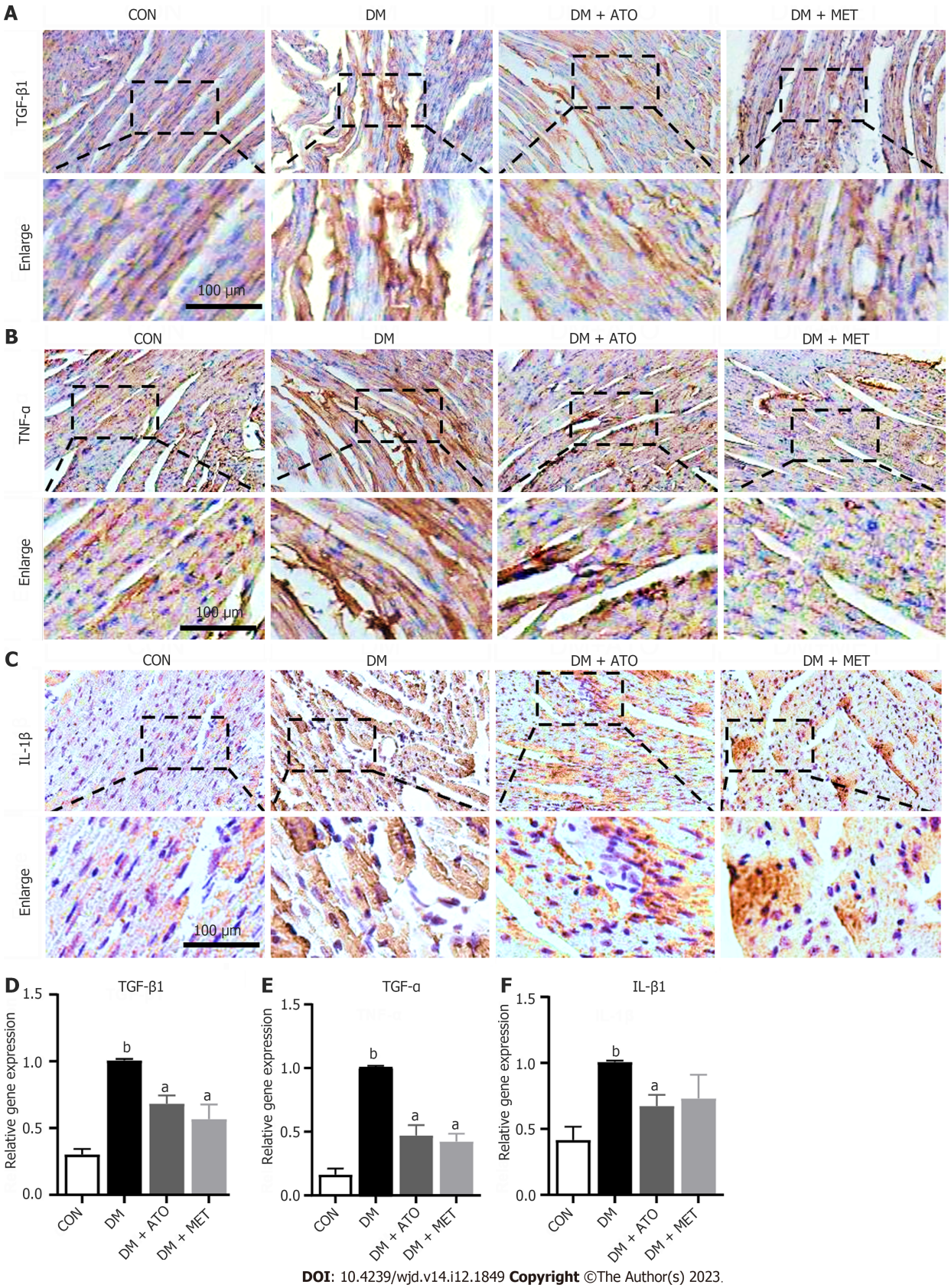

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Figure 4 Immunohistochemical staining and relative gene expression of transforming growth factor-β1, tumor necrosis factor-α, and interleukin-1β in each group. A–C: Immunohistochemistry staining of TGF-β1, TNF-α, and IL-1β in each group; D–F: relative gene expression of TGF-β1, TNF-α, and IL-1β in each group. Compared with C57BL/6 mice (CON group), the expression of TGF-β1, TNF-α and IL-1β in db/db mice that received daily oral gavage of sterilized water (DM) group was markedly elevated, and daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or 200 mg/kg metformin (DM + MET) alleviated these manifestations. The DM + MET group showed no effects on IL-1β mRNA expression in the myocardium. Data represent the means ± SD (n = 5). $^{a}P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. $^{b}P < 0.05$ compared with C57BL/6 mice. DM: db/db mice received daily oral gavage of

sterilized water; DM+ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM+MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice; TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β.

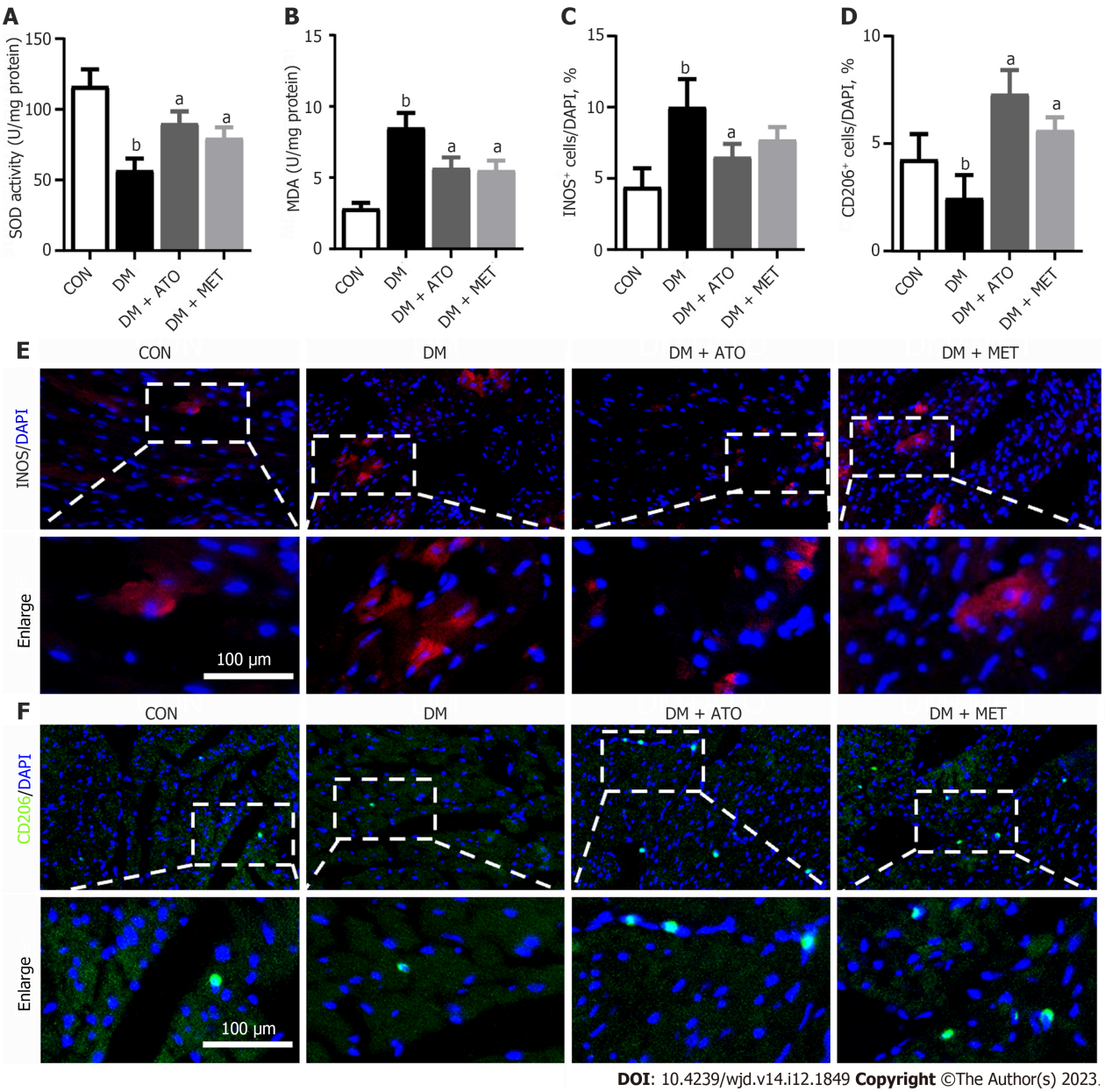

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Figure 5 Levels of superoxide dismutase activity and malondialdehyde content and immunofluorescence staining of macrophages. A and B: SOD activity was decreased and MDA content was significantly increased in db/db mice that received daily oral gavage of sterilized water (DM group). db/db mice that received daily oral gavage of 10 mg/kg/d atorvastatin (DM + ATO) or 200 mg/kg metformin (DM + MET) group had markedly decreased MDA content and enhanced SOD activity; C and E: Compared with C57BL/6 mice (CON group), iNOS$^+$ in the DM group were markedly increased, and the DM + ATO group had reduced the expression of M1 macrophages. The DM + MET group showed no effects on expression of M1 macrophages in the myocardium of db/db mice; D and F: Compared with the CON group, CD206$^+$ in the DM group were markedly decreased, and the DM + ATO and DM + MET groups had increased expression of M2 macrophages in the myocardium. Data represent the means ± SD (n = 5). $^a P < 0.05$ compared with db/db mice received daily oral gavage of sterilized water group. $^b P < 0.05$ compared with C57BL/6 mice. iNOS: Inducible nitric oxide synthase; MDA: Malondialdehyde; SOD: Superoxide dismutase; DM: db/db mice received daily oral gavage of sterilized water; DM + ATO: db/db mice received daily oral gavage of 10 mg/kg/d atorvastatin; DM + MET: db/db mice received daily oral gavage of 200 mg/kg metformin; CON: C57BL/6 mice.

signaling are the main initiators of DCM[25]. db/db mice are often used as a typical animal model of T2DM. In our study, the db/db mice exhibited hyperglycemia, hyperlipidemia and hyperinsulinemia, indicating a feature of T2DM. At 24 wk old, the db/db mice showed a significant increase in LVIDd and LVIDs and a significant reduction in LVFS and LVEF, as shown by echocardiography, suggesting cardiac diastolic and systolic dysfunction. The HW/BW of the db/db mice was significantly higher than that of the control mice; in combination with the results from HE and Masson staining, these findings manifested as myocardial pathological hypertrophy and fibrosis in the db/db mice. The elevated serum levels of

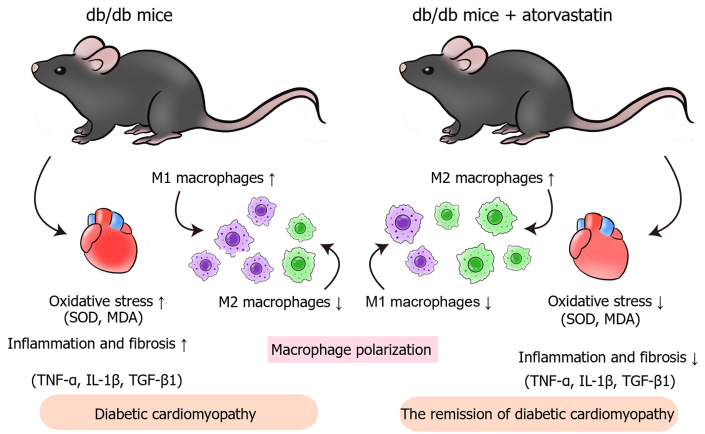

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Figure 6 Graphical abstract. MDA: Malondialdehyde; SOD: Superoxide dismutase; TGF-β1: Transforming growth factor-β1; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β.

CK-MB, LDH and cTnI also suggested myocardial injury of the db/db mice. Therefore, the db/db mice had developed DCM.

Metformin is a widely used glucose-lowering drug and has been confirmed to exert cardioprotective effects by improving the morphology and structure of the heart in db/db mice[20,26]. Metformin ameliorates DCM by inhibiting myocardial inflammation and oxidative stress, mainly through the activation of mitogen-activated protein kinase and promotion of autophagic flux[20,27,28]. At present, there is ample clinical evidence supporting the notion that metformin can reduce the risk of cardiovascular rehospitalization in diabetic patients with HF, and decrease the high risk of exacerbating DCM in prediabetic patients[29,30]. A recent meta-regression analysis has demonstrated that metformin is associated with reduced mortality in patients with HF with preserved ejection fraction, resulting in an 18% decrease in mortality for all HF patients[31]. Although metformin can be used safely in T2DM patients complicated with HF, it should be noted that currently metformin is drugs and have become the first-line choice for patients suffering from cardiovascular diseases. In addition to lowering blood fat, statins possess multiple pleiotropic effects. Previous studies have shown that statins can prevent DCM by alleviating myocardial fibrosis through antioxidative stress and anti-inflammatory pathways[33,34]. Greig *et al*[35] showed that atorvastatin reduced the levels of oxidative stress and inflammation and restored endothelial dysfunction in patients with HF. Taking a daily dose of 40 mg of simvastatin reduced the risk of major adverse cardiovascular events by ~25% in diabetic patients[36]. Similarly, our study also demonstrated that atorvastatin inhibited myocardial injury and fibrosis, which contribute to DCM attenuation.

The occurrence and development of DCM is accompanied by oxidative stress and chronic inflammation[37,38]. Oxidative stress can prompt the transformation of fibroblasts to myofibroblasts, leading to cardiac fibrosis[39,40]. The present study displayed that atorvastatin had antioxidant properties in diabetic hearts. Cardiac tissues include a plenty of resident macrophages. Under high glucose conditions, macrophages can upregulate glucose uptake and utilization and enhance the production of inflammatory cytokines, such as TGF-β1 and TNF-α, which act on macrophages and promote the activation of inflammatory phenotype M1[41]. Dysregulation of macrophages between M1 and M2 phenotypes causes excessive inflammation and cardiac injury[42]. Macrophages can also promote cardiac fibrosis through either directly producing extracellular matrix proteins or stimulating fibroblasts to secret TGF-β1[25]. Widiapradja *et al*[6] showed that only M2 macrophages were found in normal mouse hearts without inflammation, but there was a predominant increase in M1 macrophages in diabetic hearts, leading to a significant increased M1/M2 ratio. Liu *et al*[43] also had similar findings for the hearts of T2DM mice. We observed the distribution of M1 ($CD86^+$) and M2 ($CD206^+$) macrophages in the hearts of the db/db mice and found that M1 macrophages were increased in diabetic hearts. It has been reported that the imbalance of M1/M2 ratio can accelerate the development of DCM[17], and the regulation of macrophage polarization can improve the cardiac function of DCM[18]. These results demonstrate that inflammatory polarization of macrophages plays an important role in the development of DCM. Our study showed that atorvastatin reduced the expression of M1 macrophages and increased M2 macrophages in the hearts of db/db mice, and concurrently decreased the levels of TGF-β 1, TNF-α and IL-1β in both the myocardium and the serum. These findings are consistent with those from the study by Jia *et al*[28].

CONCLUSION

Our study suggests that administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated with the antioxidative stress and anti-inflammatory effects of atorvastatin on diabetic myocardium through modulating macrophage polarization. The investigation of cardiac macrophage polarization will facilitate DCM treatment by targeting macrophage metabolism in the hearts (Figure 6).

ARTICLE HIGHLIGHTS

Research background

Statins were initially used to lower blood lipids; however, in addition to their lipid-lowering effects, statins are involved in the regulation of the inflammatory response and play an important role in cardiovascular protection. Macrophage polarization is involved in a variety of pathological processes. Macrophage polarization is likewise involved in the development of diabetic cardiomyopathy (DCM).

Research motivation

DCM is one of the serious complications of diabetes mellitus, and we wanted to explore whether atorvastatin could mitigate the effects on DCM by affecting macrophage polarization to reduce oxidative stress, inflammation, and cardiac fibrosis.

Research objectives

We used db/db mice as a type 2 diabetes model and randomly divided into three groups: The db/db mice received daily oral gavage of sterilized water group, atorvastatin group and metformin group. C56BL/6 mice were used as the control group.

Research methods

Cardiac function was evaluated by echocardiography. Histological evaluations are hematoxylin and eosin staining, Masson staining, immunohistochemistry, and immunofluorescence. ELISA and real-time quantitative polymerase chain reaction were also used.

Research results

Treatment with atorvastatin improved cardiac dysfunction in db/db mice. Atorvastatin reduced the levels of serum myocardial injury markers; lowered the levels of Inflammatory cytokines in serum and myocardium; decreased indicators of oxidative stress in myocardium of db/db mice; inhibited M1 macrophages and promoted M2 macrophages.

Research conclusions

Administration of atorvastatin attenuates myocardial fibrosis in db/db mice, which may be associated through modulating macrophage polarization.

Research perspectives

Our study further confirms the protective role of statins in cardiovascular disease and provides a new therapeutic target for DCM.

FOOTNOTES

Author contributions: Zhou H designed the study and revised the manuscript; Song XM performed the experiments and drafted the manuscript; Zhao MN participated in data processing and revised the manuscript; Li GZ and Li N contributed to animal feeding; Wang T performed statistical analyses; and all authors contributed to the article and approved the submission of this manuscript.

Supported by the Health Commission of Hebei Province, No. 20220998.

Institutional animal care and use committee statement: All procedures were approved by the Animal Experimental Ethics Committee of the Second Hospital of Hebei Medical University and the Animal Health Care Guidelines to minimize animal suffering.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Data will be made available on request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

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S-Editor: Wang JJ

L-Editor: Kerr C
P-Editor: Cai YX

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