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Original Article

Effect and mechanism of sacubitril/valsartan on ventricular remodeling in diabetic rats with cardiomyopathy

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Abstract: Background: Diabetic cardiomyopathy (DCM) has a high incidence of heart failure and poor prognosis, so its prevention and treatment need attention. In this study, we investigate the effect and mechanism of sacubitril/valsartan (Sac/Val) on ventricular remodeling in rats with DCM, in order to discuss the clinical prevention of DCM. Methods: Twenty 8-week-old male Wistar rats were randomly divided into four groups: a normal rat group (NOR), a DCM group, a DCM + Sac/Val group and a DCM + Perindopril (PER) group, with 5 rats in each group. The DCM rat model was established by intraperitoneal injection of 1% streptozotocin (STZ). The rast in the NOR and DCM groups were gavaged with normal saline (1 ml/100 g/d) for 8 weeks, while the rats in the Sac/Val and PER groups were gavaged with Sac/Val (60 mg/kg/d) and PER (2 mg/kg/d) for 8 weeks. H&E and Masson staining were used to evaluate the pathological changes of myocardial tissue, the left ventricular/body mass index was calculated to evaluate ventricular remodeling, the levels of IL-6 and TNF-α in the myocardial tissue were detected by ELISA, and the protein expression of p-38 and phos-p38 was detected. Results: Compared with the NOR group, the arrangement of cardiac myocytes in the DCM group was disordered and the content of collagen fibers increased. In the DCM group, the left ventricular/body mass index increased, the levels of IL-6 and TNF-α increased, and the phos-p38 protein expression increased ($P<0.01$). Compared with DCM group, fewer collagen fibers were found and the degree of fibrosis was reduced in the Sac/Val group and the PER group. The levels of left ventricular/body mass index, IL-6, TNF-α and phos-p38 protein expression in the Sac/Val group were all lower than those in the DCM group, and the differences were statistically significant ($P<0.01$). Conclusion: Sac/Val can improve ventricular remodeling in DCM modeled rats, and its mechanism may be related to its anti-inflammatory effect and inhibition of p38 signaling pathway in myocardial tissue.

Keywords: Sacubitril valsartan, diabetic cardiomyopathy, ventricular remodeling, p38

Introduction

Diabetic cardiomyopathy (DCM) is a kind of cardiomyopathy, which is caused by diabetes and leads to a series of malignant heart events such as heart failure and arrhythmia. Studies have shown that the changes of the myocardial structure and function in DCM patients are not related to coronary heart disease, hypertensive heart disease or other cardiovascular diseases [1]. The pathophysiological process of DCM is complex [2]. In the early stage of DCM, myocardial fibrosis worsens, left ventricular weight (LVW) increases, and eventually leads to cardiac enlargement, severe systolic and diastolic dysfunction, as well as microvascular and macrovascular lesions [3]. The incidence of heart failure in type 2 diabetic patients is 2.5 times higher than that in non-diabetic patients, and the prognosis of patients with heart failure due to diabetic cardiomyopathy is poor, with a 5-year survival rate of only about 50%, which seriously threatens people's health [4]. Therefore, further study on the pathogenesis of DCM and search for appropriate treatment can help to reduce the morbidity and mortality of DCM.

Sacubitril/valsartan (Sac/Val) is an angiotensin-receptor enkephalin inhibitor consisting of an enkephalin inhibitor (Sacubitril) and an angiotensin II receptor antagonist (Valsartan), which was approved to be marketed in China in 2017. According to the PARADIGM study, compared with enalapril, Sac/Val reduced the risk of major composite endpoint events by 20%, all-cause mortality by 16%, sudden cardiac death by 20%, and heart failure death by 21% [5]. A total of 4822 heart failure patients with ejection fraction retention were included in the 57-month PARAGON-HF study. The results showed that compared with valsartan alone, Sac/Val reduced NT-proBNP levels at week 12 and improved NYHA cardiac grade at week 36 [6]. At causal analysis of the PARADIGM HF test, Sac/Val reduces the risk of cardiovascular death and hospitalization from heart failure, regardless of blood sugar status, compared with enalapril [7]. However, there is a lack of basic research on the effect of Sac/Val on ventricular remodeling in DCM patients. In this study, we observed the effects of Sac/Val on ventricular structural changes, pathological changes and inflammatory changes in the hearts of DCM modeled rats, to explore the primary mechanism of Sac/Val's improvement on ventricular remodeling in DCM rats, and to provide a new idea for clinical prevention and treatment of DCM.

Materials and methods

Materials

A total of twenty 8-week-old male Wistar rats were selected and provided by the Animal Model Center of Nanjing University, with the animal qualification number SCXK (Su) 2015-0001. They were raised in an environment of 22 ± 2°C, freely feeding and drinking water, and were adaptively fed for 1 week before the experiment. Streptozotocin (STZ) (Sigma, St. Louis, MO, USA), Sacubitril valsartan tablets (Novartis, Switzerland), H&E kits, and Masson kits were provided by Jiangsu KeyGEN Biotech, Nanjing, China. Rat IL-6 ELISA Kits (ab100772), Rat TNF-ELISA Kits (ab100785), Anti-p38 antibodies (ab31828), and Anti-phos-p38 antibodies (Thr180/Tyr182) (ab4822) were purchased from Abcam, Cambridge, UK. The study was approved by the Ethics review Committee of the Affiliated Hospital of North Sichuan Medical College, and all authors and technical operators complied with the ARRIVE guidelines.

Model establishment

At a dose of 70 mg/kg, 1% STZ (pH = 4.5, compound sodium citrate buffer 4°C, made and immediately used) was injected into the abdominal cavity of rats to destroy the function of the islet cells, and venous blood from the tail of rats was extracted 3 days and 1 week after injection of STZ to detect fasting blood glucose (fasting for 8 h). This was considered the diabetic cardiomyopathy model, which was established, when rats were polydipsic, polyphagous, polyuric and blood glucose was ≥16.7 mmol/L.

Group setting

Rats were divided into the following four groups. Firstly, normal rats (NOR) were given normal saline (1 ml/100 g/d) by gavage for 8 weeks without modeling. Secondly, in the DCM model group, after modeling, normal saline (1 ml/100 g/d) was given by gavage for 8 weeks. Thirdly, in the DCM + Sac/Val group (Sac/Val), after modeling, Sac/Val (60 mg/kg/d) was given orally for 8 weeks. Fourthly, in the DCM + Perindopril group (PER), PER (2 mg/kg/d) was given orally for 8 weeks after modeling.

Oral glucose tolerance test (OGTT)

After the last administration, the rats were forbidden to eat for 12 hours (drinking water was allowed), and tail vein blood was collected and fasting blood glucose was measured and recorded the next morning. Then, we gavaged the rats with 20% glucose solution (2 G/kg) and started the timing. At 30, 60, 90 and 120 minutes after intragastric administration, blood samples were collected from the tail vein to measure and record the blood glucose value. The OGTT curve was drawn and the area under curve (AUC) was calculated. At the end of the experiment, the rats in each group were weighed, and then pentobarbital sodium (30 mg/kg) was injected into the tail vein to kill the rats. The heart was removed, the left ventricle was separated and weighed.

Body mass index

After measuring body weight (BW), the rats were killed immediately, and then the heart was taken out immediately and washed repeatedly in 0.9% sodium chloride. After removing the residual blood, the atrial, great vessels

and epicardial adipose tissue were removed, and the left ventricle was dissociated and dried by filter paper. Finally, we use an electronic balance to weigh the left ventricular weight (LVW). LVW/BW (mg/g) = left ventricular weight (mg)/body weight (g).

Myocardial histology

We prepared paraffin sections of heart tissue for routine xylene dewaxing, followed by gradient ethanol hydration, distilled water washing, and then stained with H&E to evaluate the myocardial arrangement. The paraffin sections of heart tissue were placed in xylene I and xylene II for 10 min. After dewaxing, they were washed with water, stained with hematoxylin for 10 min, and then washed with water until they turned blue. Then they were stained with Ponceau and fuchsin for 5 min, 1% molybdic acid solution for 10 min, aniline blue solution for 15 min, 0.2% glacial acetic acid solution for 5 min, and 0.2% glacial acetic acid solution for 5 min. Finally, the myocardial histological changes were evaluated by Masson's trichrome staining. Myocardial collagen volume fraction (CVF): Masson staining sections were taken and CVF of myocardial tissue was calculated by Image Pro Plus 6.0 image analysis system to evaluate myocardial fibrosis. The higher the proportion of CVF, the more severe the fibrosis. Six cross sections of intramural arterioles were taken from each specimen for measurement, and the average value was taken. CVF was used to quantitatively evaluate the degree of myocardial fibrosis in rats.

ELISA

We quickly extracted the hearts of the rats immediately after they were killed and we then removed about 100 mg of myocardial tissue, and rinsed them with PBS buffer. The tissue homogenate was centrifuged at 12,000 g for 10 minutes, and the contents of IL-6 and TNF-α in the supernatant were determined with ELISA kits.

Western blot

We isolated cardiac tissue and placed about 100 mg tissue into a precooled EP tube. Then we added 400 μl RIPA lysate into a homogenizer for homogenization of the tissue, we crushed the tissue as much as possible, put it on ice for 30 min, then transferred the lysate to 1.5 ml Eppendorf tube with a pipette, centrifuged for 5 min, and part of the supernatant was put into 200 μl Eppendorf tube, and the total protein concentration was determined according to the instructions of BCA kit. The SDS-PAGE gel was prepared. The gel was transferred to PVDF film and activated in methanol for 1 min, and then immersed in the membrane buffer. The filter paper was placed in the transfer film buffer to soak for 15 min. According to the principle of PVDF membrane ≥ gel ≥ filter paper, we made the transfer sandwich to ensure constant pressure transfer after bubble removal. We added TBST diluted to the appropriate concentration of a single antibody, close the bag, rested it overnight at 4°C, added the appropriate amount of secondary antibody, closed the bag, and incubated it for 1 h at room temperature. Tanon 6600 luminous imaging workstation was used for image acquisition. Finally, we detected the expression levels of p38 and phos-p38 in myocardium tissue of rats in each group by Western blot.

Statistical analysis

We used single factor analysis of variance (ANOVA) to compare the mean of multiple independent samples. Kruskal-wallis test was used for analysis if there were other conditions such as uneven variance or non-normal distribution. The significance level was bilateral $\alpha = 0.05$. All data were analyzed using SPSS 20.0.

Results

The OGTT-AUC after the establishment of DCM rat model

Compared to the NOR group, the OGTT-AUC in the DCM group increased significantly (1497.60 ± 70.11 mmol/l*min vs. 3596.60 ± 104.29 mmol/l*min, $P<0.01$). After treatment with Sac/Val or PER, the OGTT-AUC of the Sac/Val group (3288.00 ± 240.09 mmol/l*min vs. 3596.60 ± 104.29 mmol/l*min, $P = 0.009$) and the PER group (3098.20 ± 187.48 mmol/l*min vs. 3596.60 ± 104.29 mmol/l*min, $P<0.01$) decreased in the comparison of DCM group (**Figure 1**).

Sac/Val reduced LVW/BW ratio in DCM rats

Compared with healthy rats in the NOR group, the left ventricular weight of rats in the DCM

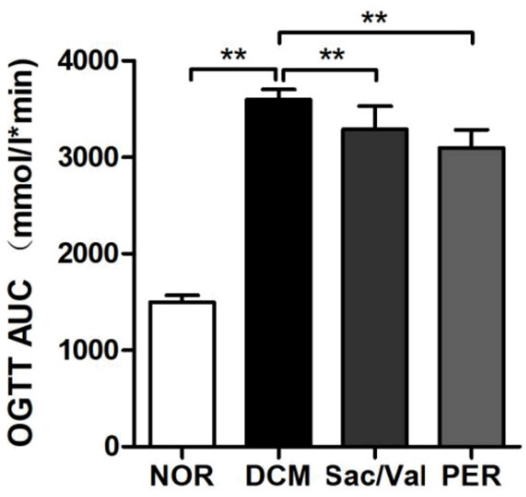

Figure 1. The OGTT-AUC after the establishment of the DCM rat model. *P<0.05; **P<0.01.

group increased (842.34 ± 53.18 mg vs. 536.27 ± 22.97 mg, P<0.01), LVW/BW ratio increased (1.59 ± 0.12 mg/g vs. 3.03 ± 0.24 mg/g, P<0.01), after treatment with Sac/Val or PER, the LVW/BW ratio of the Sac/Val group (3.03 ± 0.24 mg/g vs. 1.98 ± 0.15 mg/g, P<0.01) and the PER group (3.03 ± 0.24 mg/g vs. 1.95 ± 0.37 mg/g, P<0.01) was lower than that of the DCM group (**Figure 2**).

Sac/Val alleviated the degree of myocardial injury and fibrosis in DCM modeled rats

As shown in **Figure 3**, compared with the NOR group, the arrangement of cardiomyocytes in the DCM group was disordered and the content of collagen fibers increased. Compared with the DCM group, the collagen fiber and fibrosis degree were reduced in the Sac/Val and PER groups.

Effect of Sac/Val on CVF in DCM rats

Compared with the NOR group, CVF in the DCM group increased significantly (6.68 ± 1.23% vs. 18.62 ± 3.02%, P<0.01). CVF in the Sac/Val group (12.17 ± 1.35% vs. 18.62 ± 3.02%, P<0.01) and the PER group (12.30 ± 4.78% vs. 18.62 ± 3.02%, P<0.01) were reduced remarkably in the comparison with the DCM group (**Figure 4**).

Sac/Val reduced the levels of inflammatory cytokines IL-6 and TNF-α in DCM modeled rats

Compared with the NOR group, the levels of inflammatory factors IL-6 (125.28 + 10.44 pg/mg vs. 295.49 + 25.31 pg/mg, P<0.01) and TNF-α (80.03 + 9.46 pg/mg vs. 240.61 + 40.53 pg/mg, P<0.01) in the myocardial tissue of the DCM group were significantly increased, and the difference was statistically significant. Compared with the DCM group, the inflammatory cytokines IL-6 (295.49 ± 25.31 pg/mg vs. 167.25 ± 18.74 pg/mg, P<0.01. 295.49 ± 25.31 pg/mg vs. 171.50 ± 13.12 pg/mg, P<0.01) and TNF-α (240.61 ± 40.53 pg/mg vs. 138.10 ± 3.58 pg/mg, P<0.01. 240.61 ± 40.53 pg/mg vs. 137.35 ± 7.36 pg/mg, P<0.01) decreased in the Sac/Val and PER groups after treatment with Sac/Val and PER, and the differences were statistically significant (**Figure 5**).

Sac/Val inhibits the p38 signaling pathway in myocardial tissue of DCM modeled rats

Compared with the NOR group, the expression of phos-p38 protein in myocardial tissue of rats in the DCM group was significantly increased (1.00 ± 0.12 vs. 2.27 ± 0.53, P = 0.001); after treatment with Sac/Val and PER, the expression of phos-p38 protein in the Sac/Val group (2.27 ± 0.53 vs. 1.25 ± 0.23, P = 0.004) and the PER group (2.27 ± 0.53 vs. 1.29 ± 0.16, P = 0.004) was lower than that in the DCM group, and the difference was statistically significant (**Figure 6**).

Discussion

The hospitalization rate and mortality of patients with heart failure caused by diabetic cardiomyopathy are higher than those of patients with heart failure caused by other reasons [8], so its prevention and treatment require investigation. In this study, a rat model of DCM was established by STZ to investigate the effect of Sac/Val on ventricular remodeling in DCM modeled rats. STZ is the most widely used chemical inducer of the DCM model, which has the advantages of less toxicity to other tissues and organs, and a high success rate of modeling [9, 10]. Angiotensin converting enzyme inhibitors (ACEI) is a traditional medicine for heart failure. A large number of studies have proved that ACEI can inhibit myocardial fibrosis and improve ventricular remodeling [11, 12]. So we take perindopril as the positive control group. This study found that Sac/Val could reduce LVW/BW, myocardial inflammatory factors IL-6 and TNF-α levels, phos-p38 protein expression in myocardial tissue of DCM modeled rats, and improve the degree of myo-

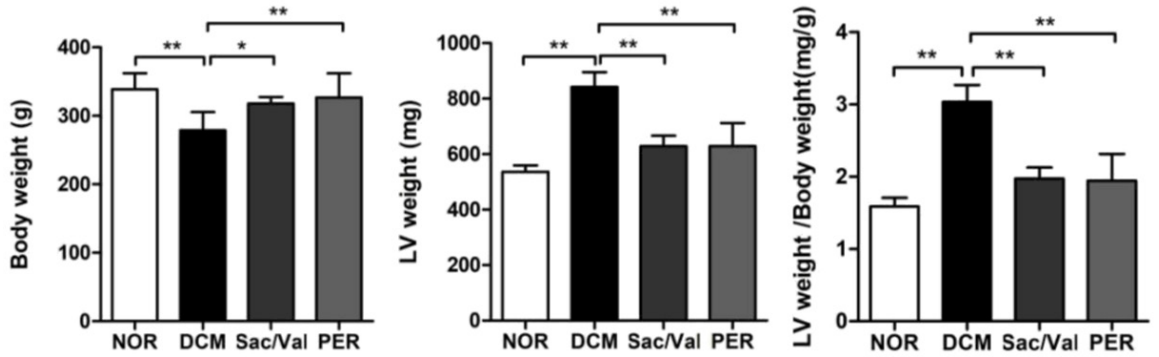

Figure 2. Effect of Sac/Val on LVW/BW ratio in DCM modeled rats. *P<0.05; **P<0.01.

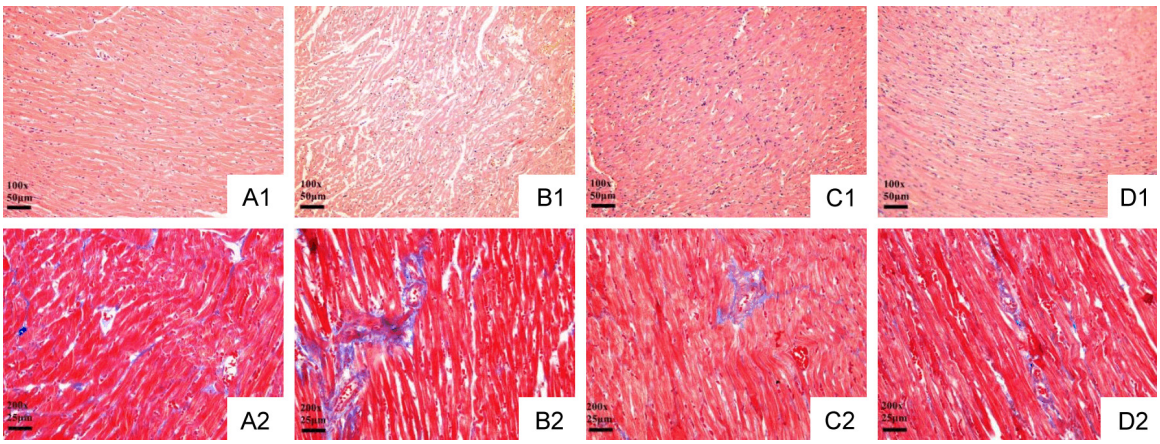

Figure 3. Comparison of H&E staining (magnification × 100) and Masson staining. (magnification × 200) in myocardial tissues of rats in each group. H&E staining: CON group (A1), DCM group (B1), Sac/Val group (C1) and PER group (D1). Masson staining: CON group (A2), DCM group (B2), Sac/Val group (C2) and PER group (D2).

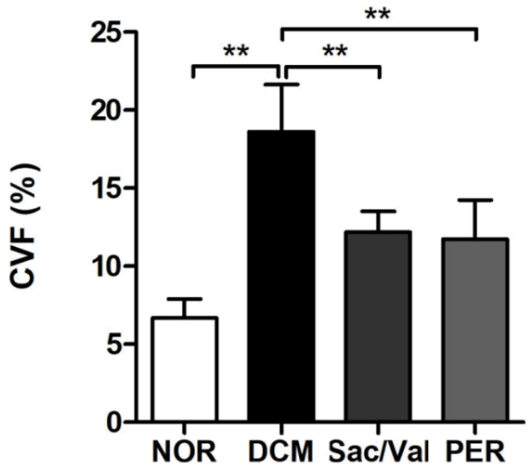

Figure 4. Comparison of CVF of myocardial tissues in each group. *P<0.05; **P<0.01.

cardial injury and fibrosis in DCM modeled rats. Diabetes causes ventricular remodeling, which is independent of factors such as obesity and hypertension [13]. DCM can be seen under cardiac MRI mainly manifested as left ventricular hypertrophy, interstitial and peripheral vascular fibrosis and microvascular abnormalities [14]. This study found that compared with healthy rats, DCM modeled rats showed a significant increase in LVW and a significant increase in the ratio of LVW to body weight, which also proved that the increase of LVW in DCM modeled rats was not related to obesity, which was consistent with previous studies [13]. Sac/Val can improve ventricular remodeling and decrease LVW/BW in rats. This study also found that the arrangement of cardiomyocytes was disordered and the content of collagen fibers increased in the DCM group. Previous animal experiments have demonstrated that the metabolic disorder caused by diabetes mellitus can directly affect cardiomyocytes and fibroblasts, change the function of cardiomyocytes,

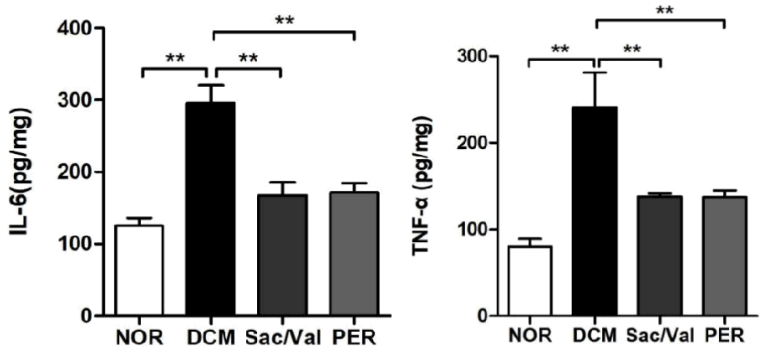

Figure 5. Sac/Val attenuates IL-6 and TNF-α levels in myocardial tissue of DCM modeled rats. *P<0.05; **P<0.01.

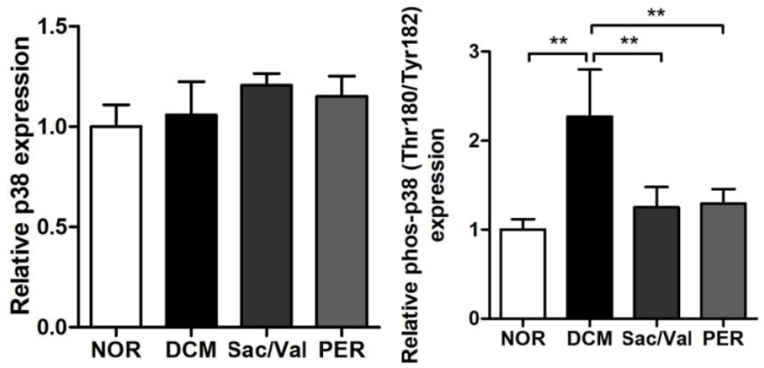

Figure 6. Sac/Val inhibits the p38 signaling pathway in myocardial tissue of DCM modeled rats. *P<0.05; **P<0.01.

make collagen fibers be deposited in the myocardial interstitium, leading to reduced myocardial compliance and diastolic function [15]. Cardiac fibrosis in DCM patients assessed by cardiac MRI was associated with mortality and hospitalization for heart failure [16]. It was found that after treatment with Sac/Val, myocardial collagen fibers were significantly reduced and the degree of fibrosis was significantly reduced in rats, which indicated that Sac/Val could delay the myocardial fibrosis caused by DCM and possibly reduce the mortality and hospitalization rate of heart failure patients with DCM, but this still needs to be confirmed by large-scale multicenter clinical studies. Previous studies have confirmed that the pathophysiological role of inflammatory signals is associated with diabetic complications, and infiltrating immune cell influx has become an important factor in the progression of cardiomyopathy and left ventricular dysfunction [17]. Inflammatory factors and related proteins such as TNF-α, IL-1β, IL-6, TGF-β, IFNγ and NFκB are highly expressed in the myocardial tissue of diabetic modeled rodents. Increased cytokines, chemokines and various white blood cell counts in humans with DCM also prove that inflammation is one of the main pathogenesis of DCM [18]. This study found that compared with the NOR group, the levels of inflammatory factors IL-6 (125.28 + 10.44 pg/mg vs. 295.49 + 25.31 pg/mg, P<0.01) and TNF-α (80.03 + 9.46 pg/mg vs. 240.61 + 40.53 pg/mg, P< 0.01) in myocardial tissue of the DCM group were significantly increased, and the difference was statistically significant. This is consistent with the previous results, and we found that Sac/Val can reduce the levels of IL-6 and TNF-α in the myocardial tissue of DCM modeled rats. The results showed that Sac/Val could inhibit the myocardial inflammatory response induced by diabetes in rats. In addition, the p38 signaling pathway is a classic inflammatory signaling pathway, which mediates a variety of pathophysiological processes. P38 mitogen activated protein kinase (p38 MAPK) is a serine/threonine protein kinase, which can respond to various cellular processes and external stress signals, such as cell differentiation, cell proliferation, and inflammation and cell death. Therefore, the increase of p38 MAPK may lead to cardiac damage in DCM [19]. It has been proven that inhibition of p38 MAPK activity can reduce cardiomyocyte apoptosis and myocardial hypertrophy in DCM modeled rats [20]. The expression of phos-p38 protein was significantly increased in the DCM group, but was decreased after treatment with Sac/val. The results suggest that Sac/val can reduce cardiomyocyte apoptosis and myocardial hypertrophy by inhibiting the p38 signaling pathway in DCM modeled rats.

There are also some shortcomings in this study. Firstly, the establishment of an early dia-

betic animal model by STZ cannot completely simulate human diabetic patients. Secondly, LVW/BW index cannot fully reflect the changes of ventricular remodeling in rats, and there is a lack of cardiac MRI data. Moreover, LVW/BW index cannot fully reflect the changes of ventricular remodeling in rats, and there is a lack of cardiac MRI data. These deficiencies need to be further improved in the follow-up study.

Conclusion

Sac/Val can improve the ventricular remodeling found in DCM, and its mechanism may be related to its anti-inflammatory effect and inhibition of the p38 signaling pathway.

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Disclosure of conflict of interest

None.

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