Salvianolic Acid B (Sal B) ameliorates myocardial fibrosis in diabetic cardiomyopathy through deubiquitinating Smad7

Hong Luo (luohong1011@163.com)
Guizhou Medical University School of Basic Medicine

Lingyun Fu
Guizhou Medical University School of Pharmaceutical Sciences

Xueting Wang
Guizhou Medical University School of Pharmaceutical Sciences

Yini Xu
Guizhou Medical University School of Pharmaceutical Sciences

Ling Tao
Guizhou Medical University School of Pharmaceutical Sciences

Xiangchun Shen
Guizhou Medical University School of Pharmaceutical Sciences

Research Article

Keywords: Salvianolic acid B, Diabetic cardiomyopathy, Myocardial fibrosis, Deubiquitinating Smad7

Posted Date: October 6th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3377735/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Salvianolic Acid B (Sal B) ameliorates myocardial fibrosis in diabetic cardiomyopathy through deubiquitinating smad7

Hong Luo1,2, Lingyun Fu2, Xueting Wang2, Yini Xu1,2, Ling Tao3, Xiangchun Shen1,2*

1 The State Key Laboratory of Functions and Applications of Medicinal Plants, School of Basic Medical Sciences, Guizhou Medical University; 6 Ankang Road, Guian New District, Guizhou 561113, China, 2 The Key Laboratory of Optimal Utilization of Natural Medicine Resources, School of Pharmaceutical Sciences, Guizhou Medical University, 6 Ankang Road, Guin New District, Guizhou 561113, China, 3 The Experimental Animal Center of Guizhou Medical University, Guizhou Medical University, 6 Ankang Road, Guian New District, 561113, Guizhou, China.

Correspondence:
Xiangchun Shen
shenxiangchun@126.com

1 The State Key Laboratory of Functions and Applications of Medicinal Plants, School of Basic Medical Sciences, Guizhou Medical University, 6 Ankang Road, Guian New District, Guiyang, Guizhou 561113, China

Abstract

Background Salvianolic acid B (Sal B), a water-soluble phenolic compound derived from Salvia Miltiorrhiza Bunge, is commonly used in Chinese Traditional Medicine for the treatment of cardiovascular diseases. Sal B has shown protecting effects against myocardial fibrosis induced by diabetic cardiomyopathy (DCM) in our previous experiment. This study aimed to investigate the ameliorative effects and potential mechanisms of Sal B in mitigating myocardial fibrosis induced by DCM.

Methods In this study, a variety of methods were utilized to investigate the effects of Sal B on improving myocardial fibrosis induced by DCM in vivo and in vitro. These
methods included weight measurement, blood glucose analysis, echocardiography, HE staining, Masson's trichrome staining, Sirius red staining, cell proliferation assessment, determination of hydroxyproline levels, immunohistochemical staining, evaluation of fibrosis-associated protein expression (Collagen I, Collagen III, TGF-β1, p-Smad3, Smad3, Smad7, and α-smooth muscle actin), analysis of Smad7 gene expression, and analysis of Smad7 ubiquitin modification.

**Results** The animal test results indicated Sal B could significantly improve cardiac function, inhibit collagen deposition and phenotypic transformation, ameliorate myocardial fibrosis in DCM by up-regulating Smad7, thereby inhibiting TGF-β1 signaling pathway. Additionally, cell experiments demonstrated Sal B could significantly inhibit the proliferation, migration, phenotypic transformation and collagen secretion of cardiac fibroblasts (CFs) induced by high glucose (HG). Sal B could significantly decrease the ubiquitization modifications of Smad7, stabilize the protein expression of Smad7, therefore increase the protein expression of Smad7 in the CFs, inhibit TGF-β1 signaling pathway, that maybe a potential mechanism of Sal B in mitigating myocardial fibrosis induced by DCM.

**Conclusion** In summary, this study revealed that Salvianolic acid B can improve myocardial fibrosis in DCM by deubiquitinating Smad7, stabilizing the protein expression of Smad7, blocking the TGF-β1 signaling pathway.

**Keywords** Salvianolic acid B, Diabetic cardiomyopathy, Myocardial fibrosis, Deubiquitinating Smad7
Background

Since the 1950s, the incidence of diabetes mellitus (DM) has increased dramatically worldwide, becoming a major public health problem. In China there were 140.9 million diabetes patients in 2021, that meant one in five people is diabetic patient[1]. Diabetic cardiomyopathy (DCM) which caused by DM, is one of the most common reason of morbidity and mortality in patients globally, contributing to over 50 % diabetic death[2], while myocardial fibrosis is the main pathological features of DCM[3,4]. The pathological features of myocardial fibrosis involves an excess deposition of extracellular matrix (ECM)[5], which can cause cardiac dysfunction, arrhythmias, and heart attacks[6,7]. Cardiac fibroblasts (CFs) are known as one of the main effectors in fibrotic scar formation in the heart due to their role in ECM turnover[8]. When myocardial fibrosis occurs, cardiac fibroblasts (CFs) proliferation, migration ability, phenotypic transformation, and collagen secretion significantly increase[9,10]. The differentiation of CFs into myofibroblasts is a crucial process in myocardial fibrosis, α–smooth muscle actin (α-SMA) considered a marker of myofibroblast differentiation[11]. Myofibroblasts play a key role in collagen deposition during fibrogenesis[12]. Inhibiting the proliferation, migration, phenotypic transformation, and collagen secretion of CFs is a dominant strategy for attenuating myocardial fibrosis[13,14]. Additionally, the TGF-β1 signaling pathway is the most important target in DCM fibrosis pathogenesis[15]. The reduction of TGF-β1 signaling pathway plays a role in decreasing myocardial fibrosis in DCM.[16]. Mothers Against decapentaplegic homolog 7 (Smad7) is a crucial negative feedback factor in TGF-β1 signaling pathway[17,18]. Smad7 can block TGF-β1 signaling pathway through inhibiting the phosphorylation of Receptor Regulated Smads, such as Smad3[19]. Large amounts of evidence have shown that down-regulating the expression of Smad7, myocardial fibrosis could significantly deteriorate[20]. However, myocardial fibrosis induced by DCM cannot be effectively treated as of now. Salvinolic acid B (Sal B) is a phenolic compound found in Salvia Miltiorrhiza Bunge[21], which is commonly used for treating cardiovascular diseases in Chinese Traditional Medicine. The chemical formula consists of three tanshinol molecules and
one caffeic acid molecule[22]. It has antioxidant properties because of the structure contains multiple phenolic hydroxyls[23]. Our previous experimental results showed that Sal B exhibited potential protective effect on myocardial fibrosis induced by DCM. This study investigates the ameliorative effects and potential mechanism of Sal B on myocardial fibrosis of DCM in vivo and in vitro.

Materials and Methods

Chemicals and reagents

Salvianolic acid B (purity 98%) was purchased from Chengdu Purufa Technology Co., Ltd. (Chengdu, China). High fat and high sucrose diet (60% k cal fat, D12492i) was obtained from Research Diets Co., Ltd. (New Jersey, USA). Streptozotocin was purchased from Solaibao Biological Technology Co., Ltd (Beijing, China). Metformin-HCl was purchased from Shanghai Yisheng Biotechnology Co. Ltd (Shanghai, China). The Hyp assay kit was obtained from Nanjing Jiancheng Biochemical Co., Ltd. (Nanjing, China). E.Z.N.A.® Total RNA Kit I was purchased from OMEGA Biochemical Co., Ltd. (GA, USA). PrimeScript™ RT reagent kit and SYBR® Premix Ex Taq™ II was obtained from Takara Biotechnology Co. Ltd (Dalian, China). The proteasome inhibitor MG132 was purchased from Biyuntian Biotechnology Institute (Shanghai, China). Cycloheximide (CHX) was obtained from Master of Bioactive Molecules Biotechnology Co. Ltd (New Jersey, USA). Vimentin, GAPDH, TGF-β1, Col-I, Col-III, α-SMA antibody was obtained from Wuhan Boster Biological Technology Co., Ltd (Wuhan, China). Ubiquitin antibody, Coralite594-conjugated goat anti-Rabbit IgG was obtained from Proteintech Co. Ltd (Wuhan, China).

Animal protocol

Health male C57BL/6J mice (body weight 18–20g) were obtained from Sibeifu Biotechnology Co., Ltd. (Beijing, China). In these experiments, mice were housed at a controlled temperature (25°C) and photoperiod (12 h:12 h light-dark cycle). Animal protocols were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996). Mice were divided into control (20) and DCM (100) group. During the 3 months period of the study, DCM
mice were fed a high fat and high sucrose diet, while control mice were fed a normal chow diet. Glucose tolerance tests (GTTs) and ELISA test which detected the blood glucose level and serum fasting insulin level were performed to confirm the mice had developed insulin resistance. Type 2 diabetes animal model in mice was induced by streptozotocin (30 mg/kg/time, intraperitoneal injection times every other day). The mice blood glucose levels were measured after the last induction for one month when they blood glucose levels are stably maintained. Type 2 diabetes was confirmed by the glucose level of fasting glucose above 11.1 mmol/L[24]. The mice of type 2 diabetes were randomly divided into 4 groups: diabetic cardiomyopathy model group (DCM, normal saline 10 mL/kg/day, ig), Sal B low-dose group (Sal B.L, 1.5 mg/kg/day, ig), Sal B high-dose group (Sal B.H, 3 mg/kg/day, ig), Metformin group (Met, 200 mg/kg/day, ig), the mice which fed normal chow diet as the control group (Control, normal saline 10 mL/kg/day, ig). All mice were administered by gavage 6 days every week. The ejection fraction (EF) of control and DCM group were assessed monthly, DCM model was successfully established when the EF of DCM group was significantly less than the control group[25].

Echocardiographic recordings

Echocardiographic measurement was performed at the end of the study. The mode of 2D and M-Mode echocardiography equipped with 30MHz linear transducer system: VINNO echocardiography system (shuzhou, China) was used to evaluate the cardiac morphology and function[26]. The following structural variables were evaluated: left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), fractional shortening (FS), ejection fraction (EF).

Histological analysis

The heart tissues were fixed in a 4% paraformaldehyde solution in a 0.1 M phosphate buffer for a duration of 48 hours. Following fixation, the tissues were dehydrated and embedded in paraffin. Sections of the tissues, with a thickness of 4 μm, were then prepared and mounted on glass slides. To evaluate the extent of fibrosis in the heart tissues, histological staining techniques such as HE staining, Masson's trichrome staining, and Sirius red staining were employed. The
quantification of fibrotic regions and the total tissue area was performed using Image Pro Plus (version 6.0). The degree of fibrosis was determined by calculating the ratio between the area exhibiting fibrosis and the total tissue area, expressed as a percentage: [% fibrosis = (area exhibiting fibrosis/total area) *100].

**Isolation and culture of neonatal rat CFs**

Neonatal rat CFs were obtained using the trypic digestion method[27]. Hearts from neonatal Sprague Dawley rats aged 1 to 3 days old were digested with 0.125% trypsin at 4 °C for 6 hours, followed by a 5 minute digestion at 37 °C. This process was repeated 4-6 times. The isolated CFs were then cultured in DMEM supplemented with 15% fetal bovine serum at 37 °C in a 5% CO2 humidified atmosphere. To separate CFs from cardiomyocytes, a differential adhesion technique was employed for 1.5 hours, as CFs and cardiomyocytes adhere to surfaces at different rates[28]. The resulting primary neonatal rat CFs were subsequently used for subculture. The second or third passage of CFs was utilized for further experiments.

**Immunofluorescence analysis**

Immunofluorescence staining of CFs with anti-vimentin and anti-Smad7 antibody (1:500 dilutions) was performed to identify cell and observe the effect of Sal B on Smad7 expression in CFs. Images of cells were captured by using a fluorescence microscope (Leica DMi 8, Germany). Quantification of fluorescence intensity was performed using Image Pro Plus(version 6.0).

**Cell proliferation evaluation**

This study utilizes the xCELLigence Real-Time Cell Analyzer system, which employs sensor impedance technology to assess the cell status through a unit-less parameter known as the cell index. As a result of measuring the relative changes in electrical impedance in the presence and absence of cells in the wells, the cell index reflects the cell status[29,30]. xCELLigence Real-Time Cell Analyzer was used to optimize the concentration of high glucose(HG) induced CFs proliferation and evaluate the inhibitory effect of Sal B on the proliferation of CFs induced by HG. Briefly, CFs were seeded into E-plate for 24 h at 37°C in 100 µl media. By adding
different concentrations glucose and Sal B medium incubated for 120h, detected the cell index of CFs every 15 min.

**Cell Scratch Assay**

CFs migration was assessed by Cell Scratch Assay as previously described[31]. CFs were cultured in 6 well plates for 24 h, made the cell scratch area using a 100μl pipette tip, added 40 mM glucose and different concentrations of Sal B medium incubated for 24 h. The cell scratch are was imaged by microscopy at 0 h and 24 h. From five averaged regions, the width of the cell scratch area was measured using Image Pro Plus (version 6.0). Migration distances after 24 h were subtracted from baseline distances. The relative migrating distance of cells was measured as the distance of cell migration and the wound distance at 0 h, expressed as a percentage[

\[
\text{% migration}=\left(\frac{\text{wound distance at T0 h} - \text{wound distance at T24 h}}{\text{wound distance at 0 h}}\right) \times 100\%
\]

**Myocardial hydroxyproline concentration**

The hydroxyproline (Hyp) content of cell supernatants was quantified by using a Hyp assay kit according to the manufacturer’s protocols. Microplate readers ELX800 were used to measure the OD values of the samples at 550 nm. The results were expressed as ug/ml total protein.

**Western blotting analysis**

Protein samples were extracted from mice heart tissues and CFs as previously described [32]. Briefly, Myocardial tissue was lysed in an ice-cold 200 uL RIPA buffer containing 1mM PMSF (Solarbio, China). CFs was lysed by the same lysate. BCA Protein Assay Kits (Solarbio, China) were used to determine the total protein concentration. Proteins were separated by SDS-PAGE using 12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), then transferred from the gel to PVDF membranes. An overnight incubation of primary antibodies(Col-I, Col- III, α-SMA , TGF- β1, Smad3, Smad7, 1:1000) was performed after blocking the membrane with 5% bovine serum albumin. The membranes were incubated for 1 hour at room temperature with the appropriate secondary antibody (1:10000). The membranes were then incubated for 1 hour at room
temperature (about 20 °C ± 5) with the appropriate secondary antibody (1:10000). ECL Kit was used to detect bands. GAPDH (1:10000) was used as a reference of total cell protein. To evaluate protein expression, the protein bands were scanned and analyzed using Image Pro Plus (version 6.0) for gray values. 

[Relative protein expression = gray value of the target proteins/gray value of the GAPDH protein bands]

**RNA extraction and quantitative real-time polymerase chain reaction**

The qRT-PCR method was employed to investigate the mechanisms by which Sal B enhances the expression of Smad7[33]. In accordance with the standard protocol, total cellular RNA was extracted from CFs using the E.Z.N.A.® Total RNA Kit I (R6834-01, OMEGA). RNA was reverse-transcribed into complementary DNA (cDNA) using PrimeScriptTM RT reagent kit (AK6003, Takara Bio, Inc.) and SimpliAmp Thermal Cycler (Applied Biosystems, Life Technologies). CFX Manager 3.0 Real-Time PCR System (Bio-Rad) was used to perform quantitative real-time PCR using SYBR® Premix Ex TaqTM II (Takara Bio, Inc.). GAPDH was used as an internal control for mRNA expression. The primers that we designed were as follows:

rat Smad7 forward, 5’-GTGGCATACTGGGAGGAGAA-3’;
reverse, 5’-AGCTGACTCTTGTTGTCCGA-3’;
rat GAPDH, forward, 5’-GACATGCCGCCTGGAGAAAC-3’,
reverse, 5’-AGCCCAGGATGCCCTTTTAGT-3’.

**Immunoprecipitation analysis of Smad7**

The inhibitory effect of Sal B on the ubiquitination modification of Smad7 was investigated by immunoprecipitation[34,35]. CFs divided into the Ctrl (5.5 mM glucose), HG (40 mM glucose), HG (40 mM glucose)+Sal B (25 μM), IgG. the cells were incubated for 24 hours. The proteasome inhibitor MG132 were used at final concentrations of 2 μM. Cells were lysed in 500 μl of lysis buffer. The total protein from the cultured cells was extracted using a previously described method. The extracted protein was then combined with Smad7 antibodies. As a negative control, the rabbit IgG was added to form antibody-antigen complexes. The mixture was
incubated overnight at 4 °C with gentle shaking to allow for the formation of these complexes. To obtain the antigen-antibody-magnetic bead mixture, the pretreated magnetic beads were added to the antigen-antibody mixture and incubated overnight at 4 °C. Finally, the antigen-antibody complex was eluted from the mixture, and the beads were boiled for 5 minutes. The supernatant was collected for Westernblotting. Smad7 was detected by using an anti-ubiquitin antibody (1:1000).

**Statistical analysis**

The statistical analysis involved the utilization of GraphPad Prism statistical software (version 5.0) to analyze all the data. The data were then presented as the mean and standard deviation (SD). To assess statistical differences among the groups, an analysis of variance (ANOVA) with a Tukey post hoc test was conducted. Statistical significance was determined by considering $p$ values less than 0.05.

**Results**

**Sal B improved cardiac function in DCM mice.**

The type 2 diabetes model were prepared using a high fat and high sucrose diet combined with low dose STZ. Compared to the control group, mice were fed with high fat and high sucrose diet showed significantly impaired glucose tolerance and insulin resistance(Fig.1A, B, C), after induced by STZ, the levels of blood glucoses were remarkably increased (Fig.1D), type 2 diabetes was confirmed by the presence of blood glucose concentrations above 11.1mmol/L. The type 2 diabetes model was considered successfully established. Compared with the control group, the EF decreased after eight weeks of placement, the DCM mice model was reproduced successfully(Fig.1K). Left ventricular hypertrophy and dilated left ventricle was observed in DCM mice hearts(Fig.1E, G, H). The heart viscera index of DCM mice was significantly increased in DCM group(Fig.1F), after Sal B and Met treatment, the heart hypertrophy and left ventricular dilation were significantly improved, the heart viscera index of Sal B and Met mice was significantly lower than in DCM group(Fig.1F). Cardiac ultrasound results showed in DCM mice hearts the LVESV and LVEDV were significantly increased, EF and FS were significantly decreased, which was improved after Sal B and Met treatment.(Fig.1 I, J, K, L). Take
together, these results indicated that Sal B could significantly improve cardiac function in DCM mice.

Fig.1 Effects of Sal B on Cardiac dysfunction in DCM mice. (\(\bar{x} \pm s, \ n=8\))

(A) Intraperitoneal glucose tolerance test (IPGTT). (B) Area under curve (AUC) of the blood glucose. (C) Insulin resistance was observed in rats after HFS diet feeding for 3 months (HOMA-IR). (D) The blood glucose level of type 2 diabetes model. (E) Anatomical image of DCM
mice. (F) The heart visceraindex of DCM mice. (G) Representative images of 2D echocardiography. (H) Representative images of M-mode echocardiography. (I-L) Quantitative results of left ventricular end-systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV), ejection fraction (EF), fractional shortening (FS) of mice heart. \#P<0.05 , \##P<0.01 vs. the control group, *P<0.05 , **P<0.01 vs. the DCM group.

**Sal B ameliorated inflammatory cell infiltration, collagen deposition and phenotypic transformation in DCM mice.**

Histological examination revealed significant myocardial hypertrophy and inflammatory cell infiltration in DCM mice (Fig. 2 A). However, after treatment with Sal B and Met, there was a notable improvement in myocardial hypertrophy and inflammatory cell infiltration (Fig. 2 A). Myocardial fibrosis, characterized by excessive deposition of fibrotic extracellular matrix proteins, particularly collagen I and collagen III, was assessed using Masson and Sirius red staining. These staining techniques demonstrated a substantial amount of collagen deposition in the myocardium of DCM with obvious hypertrophy (Fig. 2 A), after Sal B and Met treatment, the hypertrophy or inflammatory cells infiltration in the myocardium improved significantly. (Fig. 2 A). Myocardial fibrosis is characterized by excessive deposition of fibrotic extracellular matrix proteins, especially collagen I and collagen III[36]. Masson and Sirius red staining showed a large amount of collagen deposition in the myocardial tissue of DCM (Fig. 2 B, C, D). Sal B and Met treatment could decreased the collagen deposition significantly (Fig. 2 B, C, D). Western blotting results always showed that the expressions of Col-I and Col-III in myocardial tissue of DCM mice were significantly up-regulated. After Sal B and Met treatment, The expressions of Col-I and Col-III in mice myocardial tissue were significantly down-regulated (Fig. 2 E, F), indicated Sal B can significantly decrease collagen secretion. Myofibroblasts produce more collagen during fibrogenesis, which were responsible for collagen deposition[37]. The differentiation of fibroblasts to myofibroblasts was assessed by performing Western blot analysis for α-SMA. Western blotting results always showed that the expressions of α-SMA myocardial tissue of
DCM mice were significantly up-regulated. After Sal B and Met treatment, the expressions of α-SMA in mice myocardial tissue were significantly down-regulated (Fig. 2 E, F), indicated Sal B can significantly decrease phenotypic transformation in DCM mice. Western blotting results showed that the expressions of TGF-β1, p-Smad3, Smad3 in myocardial tissue of DCM mice were significantly up-regulated, Smad7 were significantly down-regulated (Fig. 2 E, G). After Sal B and Met treatment, the expressions of TGF-β1, p-Smad3, Smad3 in mice myocardial tissue were significantly down-regulated, Smad7 were significantly up-regulated (Fig. 2 E, G), indicated Sal B can significantly improve myocardial fibrosis in DCM by up-regulating Smad7 to inhibit TGF-β1 signaling pathway.
Fig. 2 Effects of Sal B on inflammatory cell infiltration and collagen deposition in DCM mice. (x±s, n=8)

(A-C) HE staining, Masson staining and Sirius red staining of heart tissue. (D) Masson trichromatic quantitative analysis of collagen area. (E) Expression level of myocardial fibrosis related proteins.
Quantitative results of proteins expression. The expression of all proteins was normalized to GAPDH. *$P<0.05$, **$P<0.01$ vs. the control group, *$P<0.05$, **$P<0.01$ vs. the DCM group.

**Morphology and cell identification of primary neonatal rat CFs**

Under the microscope, neonatal rat cardiac fibroblasts (CFs) showed a spindle-shaped morphology with multiple projecting processes (Fig. 3A). Vimentin, a specific marker for CFs, was detected using immunocytochemistry, revealing its filamentous structure[38]. Control cells, treated with PBS instead of the anti-vimentin antibody, showed no red fluorescence and only blue nucleus staining (Fig. 3B). In contrast, primary cells stained with the anti-vimentin antibody exhibited a strong red fluorescence along with blue nucleus staining, confirming the presence of vimentin (Fig. 3C). Quantitative analysis demonstrated that 95% of the cells were positive for vimentin.

![Fig. 3 Morphology and cell identification of primary neonatal rat CFs.](image)

(A) CFs grow after 3 days. (B) Negative control cells. (C) Vimentin positive cells.

**Sal B inhibit the proliferation of CFs induced by HG**

Compared to the control group, the proliferation of CFs is significantly increased by 40 mM and 45 mM glucose. Previous literature suggests that 40 mM glucose is the optimal concentration for inducing CFs proliferation (Fig. 4A)[39]. The results of cell proliferation and HE staining always indicated that Sal B 12.5–50 $\mu$M could significantly inhibit the proliferation of CFs induced by HG(Fig. 4B, C, D).
Fig. 4 Effects of Sal B on the proliferation of CFs induced by HG. (X±s, n=3)

(A) The dose-effect relation of cardiac fibroblasts proliferates induced by HG. (B) Effect of Sal B on HG-induced CFs cell index by xCELLigence Real-Time Cell Analyzer (0-120 h). (C) Effect of Sal B on HG-induced CFs cell index (24 h). (D) HE stains. *P<0.05, **P<0.01 vs. the control group, *P<0.05, **P<0.01 vs. the HG group.

Sal B decreased the migration and hydroxyproline secretion of CFs

Cell scratch assay were used to detect the effects of Sal B on migration of CFs induced by HG. Compared to the control group, HG can significantly increase the migration of CFs (Fig. 5A, B), Sal B can significantly inhibit the migration ability of CFs induced by HG (Fig. 6A, B). Myocardial collagen content was estimated by measuring myocardial hydroxyproline concentrations. Compared to the control group, HG can significantly increase the secretion of hydroxyproline level of CFs induced by HG (Fig. 5C). Sal B can significantly inhibit the secretion of hydroxyproline level from CFs induced by HG (Fig. 5C).
Fig. 5 Effects of Sal B on migration and hydroxyproline secretion of CFs induced by HG.

(A) Effects of Sal B on migration of the primary neonatal rat CFs induced by HG (24h).

(B) Quantitative results of migration. (C) Effect of Sal B on hydroxyproline level secretion from CFs induced by HG. *P<0.05, **P<0.01 vs. the control group, *P<0.05, **P<0.01 vs. the HG group.

Myocardial fibrosis and TGF-β1 signaling pathway related proteins from CFs induced by HG.

Compared to the control group, the the protein levels of Col-I, Col-III, TGF-β1, p-Smad3, Smad3 and α-SMA of CFs induced by HG significantly increased, Sal B can significantly decrease these protein levels (Fig. 6A, B, C). The results indicated Sal B can significantly increase the the protein levels of Smad7, this was consistent with the immunofluorescence staining results (Fig. 6D, E).
Fig. 6  Effects of Sal B on the expression of myocardial fibrosis and TGF-β1 signaling pathway related proteins from CFs induced by HG. (x±s,  n=3)
(A) Expression level of myocardial fibrosis and TGF-β1 signaling pathway related proteins. (B) Quantitative results of myocardial fibrosis proteins expression. The expression of all proteins was normalized to GAPDH. (C) Quantitative results of TGF-β1 signaling pathway related proteins. The expression of all proteins was normalized to GAPDH. (D) Effects of Sal B on the Smad7 expression of CFs induced by high glucose. (E) Quantitative results of Smad7 expression. *P<0.05, #P<0.01 vs. the control group, *P<0.05, **P<0.01 vs. the HG group.

**Sal B can improve myocardial fibrosis in DCM by deubiquitinating Smad7, stabilizing the protein expression of Smad7**

We hypothesized that the effect of Sal B increase the expression of Smad7 was through gene transcription, qRT-PCR was used to explore the effect of Sal B on Smad7 mRNA expression in CFs induced by HG. Compared with the control group, the mRNA expression levels of Smad7 slightly decreased induced by HG, but the difference was not statistically significant. After Sal B treatment, Smad7 mRNA levels increased slightly, but the difference was not statistically significant (Fig. 7A, B, C). The result showed that Sal B didn’t increase the mRNA expression of Smad7. Therefore, we speculated that Sal B maybe inhibet the degradation of Smad7. In order to determine the effect of Sal B in stabilizing Smad7 protein levels, the inhibitor of protein synthesis CHX was used to inhibit new proteins synthesis. The protein expression of Smad7 were detected at 1 h, 2 h, 4 h, 8 h and 12 h after CHX treatment (20 μM), respectively. The result showed that the expression of Smad7 in HG group presented a gradual decline. The expression of Smad7 in the Sal B group was highest at 1 h, and then maintained a high level. There were significant differences in Smad7 expression at 2 h, 4 h, 8 h and 12 h, indicating that Sal B could significantly stabilize Smad7 expression level in CFs (Fig. 7D, E, F). According to the documented reports, myocardial fibrosis promotes ubiquitination modifications of Smad7 and reduce stability of Smad7, which results the expression of Smad7 decreased. To confirm Sal B can prevent ubiquitination modifications of Smad7 to improve myocardial fibrosis in DCM. Immunoprecipitation was performed to observe the inhibitory effect of Sal B on the ubiquitization modifications of Smad7. Compared with the Control group, Sal B reduced the ubiquitization modifications of Smad7 significantly (Fig. 7 G).
results were verified Sal B acts as an antifibrotic agent by inhibiting the ubiquitization modifications of Smad7.

![Graphs and images showing the mechanisms of Sal B on the expression of Smad7.](image)

**Fig. 7** The mechanisms of Sal B on the expression of Smad7. (±s, n=3)

(A) The amplification curve of Smad7 and GAPDH. (B) The melting curve of Smad7 and GAPDH. (C) Quantitative results of Smad7 mRNA levels induced by Sal B. The expression of Smad7 mRNA levels was normalized to GAPDH\( (2^{-\Delta\Delta C_t}) \). (D) Quantitative results of Smad7 proteins expression. The expression of Smad7 proteins was normalized to GAPDH. (E) Expression level of
Smad7 at different time points induced by HG. (F) Expression level of Smad7 at different time points induced by Sal B. (G) Effect of Sal B on Smad7 ubiquitination modifications. *P<0.05, **P<0.01 vs. the control group, *P<0.05, **P<0.01 vs. the HG group.

4. Discussion

Myocardial fibrosis is the main pathologic feature in DCM. However, the molecular mechanism has remained elusive. Currently, there are no treatment for DCM fibrosis. Preponderance of evidence proved increasing the expression of Smad7 in CFs has been identified as a critical strategy to improve myocardial fibrosis in DCM. Therefore, Smad7 has emerged as a potential hotspot [40]. This study demonstrated that Sal B can significantly increase the expression of Smad7. However, the mechanism of Sal B on Smad7 expression remains to be elucidated. After a series of experiments, we demonstrate Sal B did not regulate the protein levels of Smad7 at the transcriptional level, instead of inhibiting the degradation of Smad7 protein to increase the stabilization of Smad7 protein expression. The results of immunoprecipitation indicated Sal B acts as an antifibrotic agent by inhibiting the ubiquitination modifications of Smad7. However, due to time limitations, comprehensive investigations regarding the impact of Sal B on the ubiquitination modification of Smad7 has not been conducted. We speculate that Sal B may potentially decrease the ubiquitination modification of Smad7 by suppressing the expression and activity of key enzymes involved in Smad7 ubiquitination, such as Smurf1 and Smurf2 [41,42]. Alternatively, Sal B may enhance the deubiquitination modification of Smad7 by increasing the expression and activity of deubiquitination enzymes like USP2 and OTUD1 [43,44]. In subsequent experiments, we will proceed to confirm the mechanism of Sal B on the ubiquitination modification of Smad7.

Conclusion

In summary, we demonstrated a partial amelioration of Sal B on myocardial fibrosis in DCM mice model by inhibiting collagen formation and deposition through the classical TGF-β/Smad pathway. Furthermore, an 8-week treatment of Sal B improves the structure and function of the left ventricle in diabetic mice. Consequently, Sal B shows promise as a therapeutic agent for preventing and treating
myocardial fibrosis induced by DCM. However, additional clinical data is necessary to confirm the safety and effectiveness of Sal B in treating patients with DCM, and further investigation is required to elucidate its potential mechanism.

Fig. 8 The mechanism of Sal B improving myocardial fibrosis of DCM

Acknowledgements
Not applicable.

Author contributions
Hong Luo contributed to experiment performance and manuscript draft; Lingyun Fu and contributed to animal experiment; Xueling Wang contributed to cell experiment; Yini Xu performed gene expression and analysis of ubiquitin modification.; Ling Tao performed the statistical analysis; Xiangchun Shen contributed to manuscript correcting.

Funding
This study was supported by the National Natural Science Foundation of China (82260827 and U1812403-4-4), the Guizhou Provincial Natural Scientific Foundation ([202
Availability of data and materials
The data in this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All animal handling and experimental conditions were approved by the Laboratory Animal Care and Use Committee of the Guizhou Medical University. C57BL/6J mice (Sibeifu Biotechnology Co., Ltd, production batch: 20160002)

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


[9] Yi Yu, Shuchen Gu, Wenjian Li, Chuang Sun, Fenfang Chen, Mu Xiao, Lei Wang, Dewei Xu, Ye Li, Chen Ding, Zongping Xia, Yi Li, Sheng Ye, Pinglong Xu, Bin Zhao, Jun Qin, Ye-Guang Chen, Xia Lin, Xin-Hua Feng. Smad7 enables STAT3 activation and promotes pluripotency independent of TGF-β signaling. PNAS. 2017; 114 (38) :10113-10118.


[22] Liping Cao, Guojun Yin, Jinliang Du, Rui Jia, Jiancao Gao, Nailin Shao, Quanjie Li, Haojun Zhu, Yao Zheng, Zhijuan Nie, Weidong Ding, Gangchun Xu. Salvianolic Acid B Regulates


[26] Tingting Fang, Congcong Ma, Zhanming Zhang, Luning Sun, Ningning Zheng. Roxadustat, a HIF-PHD inhibitor with exploitable potential on diabetes-related complications. Front Pharmacol. 2023; 14:1088288


[38] Xiaodong Zou, Hongsheng Ouyang, Feng Lin, Huanyu Zhang, Yang Yang, Daxin Pang, Renzhi Han, Xiaochun Tang. MYBPC3 deficiency in cardiac fibroblasts drives their activation and contributes to fibrosis. Cell Death Dis. 2022; 13(11): 948.


[41] Haichao Deng, Xueqiong Yao, Ningning Cui, Shanshan Huang, Yan Yan Ge, Rui Liu, Xuefeng Yang. The protective effect of zinc, selenium, and chromium on myocardial fibrosis in the offspring of rats with gestational diabetes mellitus. Food Funct. 2023; 14(3): 1584-1594.

