Am J Transl Res 2026;18(2):1719-1731
www.ajtr.org /ISSN:1943-8141/AJTR0169911

Original Article

Shuangshen Ningxin capsules ameliorate diabetic cardiomyopathy in mice by inhibiting ferroptosis via the NRF2/HO-1 signaling pathway

Yitong Cheng[1], Lanlan Li[2], Shujuan Xu[2], Xiaoqing Zhang[2], Chikun Li[3], Jianhua Fu[2]

[1]Beijing University of Chinese Medicine, Beijing 100105, China; [2]Institute of Basic Medical Sciences, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing 100091, China; [3]The Research Center of The Affiliated Hospital to Changchun University of Chinese Medicine, Changchun 130021, Jilin, China

Received November 10, 2025; Accepted January 21, 2026; Epub February 15, 2026; Published February 28, 2026

Abstract: Objective: To investigate the effects of Shuangshen Ningxin Capsules (SSNX) on myocardial function in patients with diabetic cardiomyopathy (DCM) and its underlying mechanism. Methods: A streptozotocin (STZ)-induced DCM model was established in C57BL/6J mice. The mice were administered low or high doses of SSNX (90 mg/kg/d or 180 mg/kg/d). Cardiac function was evaluated by echocardiographic parameters, H&E staining, Masson staining, and TUNEL assays. Transcriptomics analysis and Western blotting analysis were performed to explore potential molecular mechanisms. An *in vitro* high glucose (35 mmol/L)-induced DCM cell model was also established. Cells were treated with SSNX (40 μg/mL or 80 μg/mL) alone or in combination with the ferroptosis activator erastin (10 μM) or the NRF2 inhibitor ML385 (20 μM). Biochemical assays, EdU staining, and Western blotting were performed to investigate the effects of SSNX on cell proliferation, ferroptosis, and the NRF2/HO-1 pathway in DCM cells. Results: SSNX significantly improved cardiac dysfunction and attenuated cardiomyocyte hypertrophy, myocardial fibrosis, and apoptosis in DCM mice. Transcriptomics analysis revealed that after SSNX intervention, 16 originally upregulated genes were downregulated, while 33 originally downregulated genes were upregulated. These differentially expressed genes (DEGs) were associated with ferroptosis-related pathways. *In vitro* experiments showed that SSNX inhibited ferroptosis in the myocardium of DCM mice through activating the NRF2/HO-1 signaling pathway. Moreover, SSNX significantly reversed high glucose-induced suppression of the proliferation of cardiomyocytes, inhibition of the NRF2/HO-1 signaling pathway, and induction of ferroptosis. Conclusion: SSNX alleviates myocardial injury in DCM mice, and the mechanism underlying the effect of SSNX may involve activation of the NRF2/HO-1 signaling pathway to inhibit ferroptosis.

Keywords: Shuangshen Ningxin, diabetic cardiomyopathy, NRF2/HO-1 signaling pathway, ferroptosis

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia. The incidence of DM is on the rise globally. DM is associated with serious complications, such as diabetic cardiomyopathy (DCM), which is a major contributor to cardiovascular events and mortality in DM patients. The progression of DCM is insidious and chronic. In the early stage, only mild pathological changes, such as interstitial hyperplasia and cardiomyocyte degeneration, may be observed. In the later stages, myocardial fibrosis and ventricular hypertrophy may gradually develop as the disease aggravates, ultimately leading to systolic and diastolic dysfunction [1, 2]. Currently, the management of DCM mainly relies on conventional cardiovascular drugs, including glycemic control, agents that improve myocardial metabolism, angiotensin-converting enzyme inhibitors, and β-blockers. Although these approaches can slow disease progression, their long-term clinical benefits are limited and fail to reverse the pathological process of DCM [3, 4]. Therefore, searching for new and effective treatment regimens is of substantial clinical significance.

Shuangshen Ningxin (SSNX) is a traditional Chinese compound formulation primarily com-

https://doi.org/10.62347/DLRZ8628

posed of *Panax ginseng* C.A. Meyer (total ginsenosides), *Salvia miltiorrhiza* Bunge (total salvianolic acids), and *Corydalis yanhusuo* W.T. Wang (total alkaloids). It is traditionally used to replenish qi and promote blood circulation, eliminate stasis, and alleviate pain, and has been shown to effectively ameliorate myocardial ischemia-reperfusion injury [5, 6]. Its active constituents, including ginsenosides, salvianolic acid, and *Corydalis yanhusuo* alkaloids, exert multiple pharmacological effects, including anti-inflammatory, antioxidant, and cardioprotective properties. For example, ginsenoside Rg3 and salvianolic acid A can improve mitochondrial respiratory function in cardiomyocytes of DCM rats, reduce myocardial fibrosis and hypertrophy, and decrease lipid accumulation, inflammatory responses, and cardiomyocyte apoptosis [7, 8]. Additionally, the alkaloid dehydrocorydaline from *Corydalis yanhusuo* ameliorated atherosclerosis by suppressing inflammation in apolipoprotein E (ApoE)-deficient mice [9]. However, whether SSNX exerts specific therapeutic effects against DCM remains insufficiently investigated, and its potential clinical application in DCM treatment needs to be further assessed.

The pathogenesis of DCM involves multiple complex mechanisms, including hyperglycemia, oxidative stress, and the apoptosis of cardiomyocytes, among which hyperglycemia serves as a key initiating factor [10]. Sustained hyperglycemia induces the production of large amounts of reactive oxygen species (ROS) in cardiomyocytes, triggering mitochondrial dysfunction, thereby activating programmed cell death pathways and ultimately leading to cardiac dysfunction [11]. Ferroptosis, a form of regulated cell death, has attracted considerable attention due to its close association with DCM. Ferroptosis is closely associated with the dysregulation of intracellular iron homeostasis and excess accumulation of ROS. When aberrantly activated in cardiomyocytes, it accelerates myocardial injury and exacerbates the progression of DCM [12, 13]. However, whether SSNX can inhibit ferroptosis in individuals suffering from DCM remains unclear.

The NRF2/HO-1 signaling pathway has been reported to play an essential role in alleviating oxidative stress, suppressing ferroptosis, and maintaining cellular homeostasis [14]. Consequently, targeting the NRF2/HO-1 pathway emerges as an effective way to ameliorate DCM. Evidence has demonstrated that activation of this pathway mitigates myocardial injury and enhances cardiac activity by suppressing ferroptosis, demonstrating significant therapeutic potential across various cardiovascular disease models [15, 16]. However, whether SSNX ameliorates DCM by suppressing ferroptosis through the NRF2/HO-1 pathway remains uncertain. Accordingly, this study comprehensively evaluated the effects of SSNX on DCM in order to elucidate the underlying mechanisms, aiming to provide a novel candidate drug for preventing and treating DCM.

Materials and methods

Preparation of SSNX

The detailed protocol for preparing SSNX capsules and the ultra-high performance liquid chromatography analysis of SSNX extract have been described previously [5, 17]. The SSNX capsules (Lot No. 20240220) used in this study were provided by Xiyuan Hospital, China Academy of Chinese Medical Sciences.

Materials

Streptozotocin (STZ; Cat. No. HY-13753), Erastin (Cat. No. HY-15763), and ML385 (Cat. No. HY-100523) were purchased from MedChemExpress (NJ, USA). Metformin hydrochloride (Cat No. 317240) was purchased from Sigma (St Louis, USA). Creatine kinase (CK; Cat No. S03024) and aspartate aminotransferase (AST; Cat No. S03040) ELISA kits were purchased from Rayto (Shenzhen, China). Cardiac troponin I ELISA kit (cTnI; Cat. No. LJS-E-02103), hematoxylin (Cat. No. G1140), and eosin (Cat. No. E8090) were purchased from Lingjiesi (Wuhan, China). The Masson kit was purchased from Baso (Zhuhai, China). The TUNEL detection kit (Cat. No. A113-03) was purchased from Vazyme (Nanjing, China). Claycomb medium (Cat. No. iCell-0021) was purchased from Cellverse (Shanghai, China). Fetal bovine serum (Cat. No. FB15015) was purchased from CLARK (Virginia, USA). CCK-8 kit (Cat. No. C0037) and EdU kit (Cat. No. C0071S) were purchased from Beyotime (Shanghai, China). The cellular iron content assay kit (Cat. No. BC5310) was purchased from Solarbio (Beijing, China). Detection kits for malondialdehyde (MDA, Cat. No. A003-1-2), total superoxide dismutase (T-SOD, Cat. No. A001-3-2), and glutathione (GSH, Cat. No. A006-2-1) were purchased from Jiancheng (Nanjing, China). GPX4 antibody (Cat. No. YN3047), FTH1

antibody (Cat. No. YT1692), NRF2 antibody (Cat. No. YT1692), NRF2 antibody (Cat. No. YM4294), and HO-1 antibody (Cat. No. YM330-779) were purchased from Immunoway (Beijing, China). ACSL4 antibody (Cat. No. LJS-D-12141) was purchased from Lingjiesi (Wuhan, China).

Cells and animals

Mouse cardiomyocytes (HL-1, Cat. No.: iCell-m077) were purchased from Cellverse (Shanghai, China). Male C57BL/6J mice (6-8 weeks old, 18-22 g) were obtained from Sibeifu (Beijing, China; SCXK [Jing] 2024-0001), and housed under specific pathogen free (SPF) environments at 22-26°C with 50-60% relative humidity and a 12-h/12-h light/dark cycle. Mice acclimated for seven days upon arrival before the initiation of experiments. All animal experiments were approved by the Animal Ethics Committee of Changchun University of Chinese Medicine (Approval No. 2024854).

DCM model establishment and grouping

For DCM model establishment, mice were administered STZ via intraperitoneal injection at a dose of 50 mg/kg for five consecutive days, while mice in the control group were administered the same amount of normal saline. Two weeks after the last STZ injection, animals were fasted for 12 h and the tail vein blood was collected to determine fasting blood glucose (FBG) levels. Mice with FBG levels ≥16.7 mmol/L, accompanied by polydipsia, polyphagia, polyuria, and weight loss, were considered to have developed DM. After an additional three weeks of feeding with a high-fat, high-sugar diet, echocardiographic assessment revealed significant cardiac dysfunction in the DM mice, indicating successful construction of the DCM model [18]. All DCM mice were randomized into the Model, SSNX low-dose (SSNX-L, 90 mg/kg/d), SSNX high-dose (SSNX-H, 180 mg/kg/d), positive control metformin (350 mg/kg/d) groups [19], with an additional Control group. Each group consisted of six mice. The SSNX-L, SSNX-H, and metformin groups received the corresponding drugs by oral gavage once daily for four consecutive weeks, while mice in the Model and Control groups received an equal volume of saline.

At the end of the experiment, all animals underwent echocardiography. Subsequently, blood samples were collected from the orbital sinus under anesthesia (2.5% isoflurane inhalation), followed by euthanasia via cervical dislocation. All invasive procedures were performed carefully to minimize suffering. Blood specimens were centrifuged at 1,000×*g* for 10 min at 4°C to obtain serum. Cardiac tissue samples were collected, with one portion fixed in 4% paraformaldehyde and the remaining portion preserved at -80°C for subsequent use.

Echocardiography for cardiac function assessment

Mice were anesthetized with 2.5% isoflurane, and transthoracic echocardiography was performed using a Mylab X5 Vet echocardiography system (Esaote, Shenzhen, China). Left ventricular ejection fraction (EF) and left ventricular fractional shortening (LVFS) were measured to evaluate cardiac systolic function. Additionally, serum levels of myocardial enzyme profile markers, including creatine kinase (CK), aspartate aminotransferase (AST), and cardiac troponin I (cTnI), were detected using a Chemray 800 fully automated biochemistry analyzer (Rayto, Shenzhen, China) and corresponding ELISA kits.

Histological analysis

Myocardial tissues were fixed, dehydrated, and embedded in paraffin, followed by sectioning into 4-μm slices. Hematoxylin-eosin (H&E) staining was performed to evaluate myocardial hypertrophy, while Masson staining was performed to assess myocardial fibrosis.

TUNEL staining

Cell apoptosis was evaluated using the TUNEL kit following the manufacturer's instructions. Paraffin sections were fixed with an immunofluorescence fixative and incubated with terminal deoxynucleotidyl transferase (TdT), followed by labeling with fluorescein-dUTP. A Nikon Fi3 laser confocal microscope (Nikon, Japan) was used to acquire images after nuclear staining with DAPI.

Transcriptomics analysis

Myocardial tissue samples from six mice per group in the Control group, Model group, and SSNX-H group were homogenized in TRIzol reagent to extract total RNA. After treatment with oligo(dT) magnetic beads, the mRNA was

fragmented to the target size range. The fragments were then purified, end-repaired, adaptor-ligated, and amplified by PCR to construct sequencing libraries. High-throughput sequencing was subsequently performed. Sequencing data quality was assessed using fastQC software, ensuring that the data were suitable for downstream analyses. Differentially expressed genes (DEGs) were identified using the DESeq method. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed.

Cell culture and treatment

HL-1 cells were cultivated in Claycomb medium containing 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin, and 0.1 mM norepinephrine, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. To establish the *in vitro* DCM model [20], HL-1 cells were incubated in normal-glucose medium (5.5 mmol/L) for 16 h and then exposed to high-glucose medium (35 mmol/L) for 24 h. Cells were divided into the Control group, Model group, SSNX-L (40 μg/mL) group, SSNX-H (80 μg/mL) group, SSNX-L+Erastin (10 μM) [21] group, SSNX-H+Erastin group, SSNX-L+ML385 (20 μM) [22] group, and SSNX-H+ML385 group. Cells were pretreated with SSNX, erastin, and/or ML385 in normal-glucose medium for 16 h, after which high-glucose medium was added to all groups except the Control group for an additional 24 h incubation.

CCK-8 assay

To determine the optimal concentration of SSNX for HL-1 cells, the cells were seeded at a density of 3×10^3 cells/well in 96-well plates. Cells were treated with increasing concentrations of SSNX (0, 20, 40, 80, 160, and 320 μg/mL) in normal-glucose medium for 16 h, followed by incubation in high-glucose median for 24 h, with an additional Control group. Next, the CCK-8 reagent (10 μL/well) was added for incubation in the dark for 2 h. An HBS-1096A microplate reader (DeTie, Nanjing, China) was used to determine absorbance at 450 nm, and proliferation activity was calculated.

EdU assay

HL-1 cells were inoculated in 12-well plates. After drug administration, an equal volume of pre-warmed 2 × EdU working solution (20 μM) was introduced into each well and incubated at 37°C for 2 h. Supernatants were removed, followed by cell fixation, permeabilization, and Click reaction. Nuclei were counterstained with DAPI for 10 min, and images were captured using an XD202 fluorescence microscope (Yongxin, Jiangnan, China).

Biochemical detection

Following the manufacturers' protocols, biochemical assay kits were used to evaluate the levels of Fe^{2+}, malondialdehyde (MDA), glutathione (GSH), and SOD in HL-1 cells and mouse myocardial tissues.

Western blotting analysis

Using RIPA lysis buffer, total proteins were isolated from mouse myocardium tissues or HL-1 cells, with protein content measured using the BCA assay. Equal amounts of protein (30 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with skim milk for 1 h at ambient temperature, membranes were incubated overnight 4°C with primary antibodies against GPX4 (1:1,000), FTH1 (1:1,000), ACSL4 (1:1,000), NRF2 (1:1,000), HO-1 (1:1,000), and GAPDH (1:1,000). Then, membranes were incubated with horseradish peroxidase-conjugated rabbit IgG secondary antibody (1:10,000) at room temperature for 2 h. Next, an ECL developing solution was added for visualizing protein bands, which were imaged using a 4300 chemiluminescence instrument (CLINX, Shanghai, China). The ImageJ software was used to quantify protein levels, with the levels of the GAPDH protein serving as the internal reference.

Statistical analysis

All data were analyzed using the SPSS software (version 26.0). Experimental data were presented as the mean ± standard deviation ($\overline{x}$ ±s). The differences among multiple groups were determined by conducting one-way analysis of variance (ANOVA), whereas those between the two groups were determined by conducting the LSD *t*-test. A P value < 0.05 was considered statistically significant.

Results

SSNX ameliorated cardiac dysfunction and myocardial injury in DCM mice

DCM mice in the Model group exhibited decreased EF and LVFS along with elevated CK,

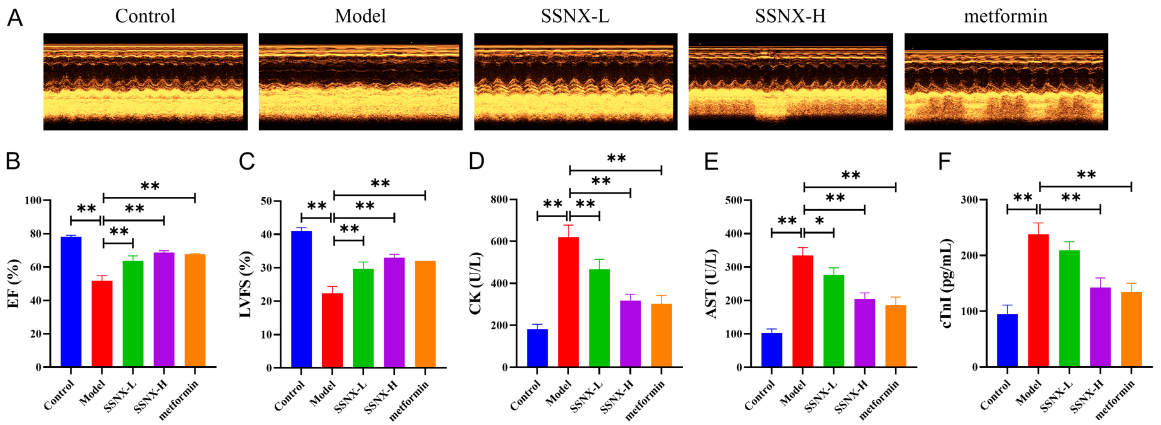

Figure 1. SSNX ameliorated cardiac dysfunction and myocardial injury in DCM mice. A. Representative echocardiograms; B. Left ventricular ejection fraction (EF); C. Left ventricular fractional shortening (LVFS); D-F. Serum levels of creatine kinase (CK), aspartate aminotransferase (AST), and cardiac troponin I (cTnI). Notes: DCM, diabetic cardiomyopathy; EF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; CK, creatine kinase; AST, aspartate aminotransferase; cTnI cardiac troponin I. $^{*}P < 0.05$ and $^{**}P < 0.01$.

AST, and cTnI levels, indicating myocardial injury and impaired systolic function. After treatment with SSNX or metformin, EF and LVFS were significantly increased, whereas CK, AST, and cTnI levels were markedly decreased in DCM mice (**Figure 1A-F**). These findings indicate that SSNX effectively mitigated cardiac dysfunction and myocardial injury in DCM mice.

SSNX alleviated cardiomyocyte hypertrophy, fibrosis, and apoptosis in DCM mice

Cardiomyocyte hypertrophy, apoptosis, and fibrosis are hallmark pathological features of DCM. H&E, Masson's trichrome, and TUNEL staining results showed that, compared with the Control group, mice in the Model group showed cardiomyocyte hypertrophy, collagen deposition in the myocardial interstitium, and an increase in cardiomyocyte apoptosis. After intervention with SSNX or metformin, cardiomyocyte hypertrophy was alleviated compared to that in the Model group, with concomitant reductions in collagen deposition and apoptotic cells (**Figure 2A-E**).

Cardioprotective effects of SSNX at the transcriptional level in DCM mice

Transcriptomics analysis was further performed to elucidate the potential molecular mechanisms underlying the cardioprotective mechanisms of SSNX in DCM. Compared with the Control group, 357 DEGs were identified in the Model group, comprising 203 upregulated genes and 154 downregulated genes. Compared with the Model group, 348 DEGs were screened in the SSNX-H group, encompassing 176 upregulated genes and 172 downregulated genes (**Figure 3A**, **3B**). Further intersection analysis of the two comparisons (Control vs. DCM and DCM vs. SSNX-H) revealed that after SSNX-H intervention, 16 originally upregulated genes in the myocardium of DCM mice were downregulated, while 33 originally downregulated genes were upregulated (**Figure 3C**, **3D**).

The results of the KEGG enrichment analysis showed that these DEGs were significantly enriched in ferroptosis-related pathways, supporting a potential regulatory effect of SSNX on the ferroptosis signaling pathway (**Figure 3E**). GO annotation results revealed that these DEGs were mainly associated with the integral component of membranes (cellular component), structural molecule activity (molecular function), and transmembrane transporter activity (biological process) (**Figure 3F**).

SSNX inhibited oxidative stress and ferroptosis in STZ-induced DCM mice

Compared to the Control group, ferrous ion (Fe^{2+}) contents were significantly elevated in both serum and myocardial tissues of mice in the Model group (**Figure 4A**, **4B**). Consistently, biochemical assays showed that serum MDA contents were notably elevated, while SOD and GSH contents were markedly decreased in the Model group compared to the Control group

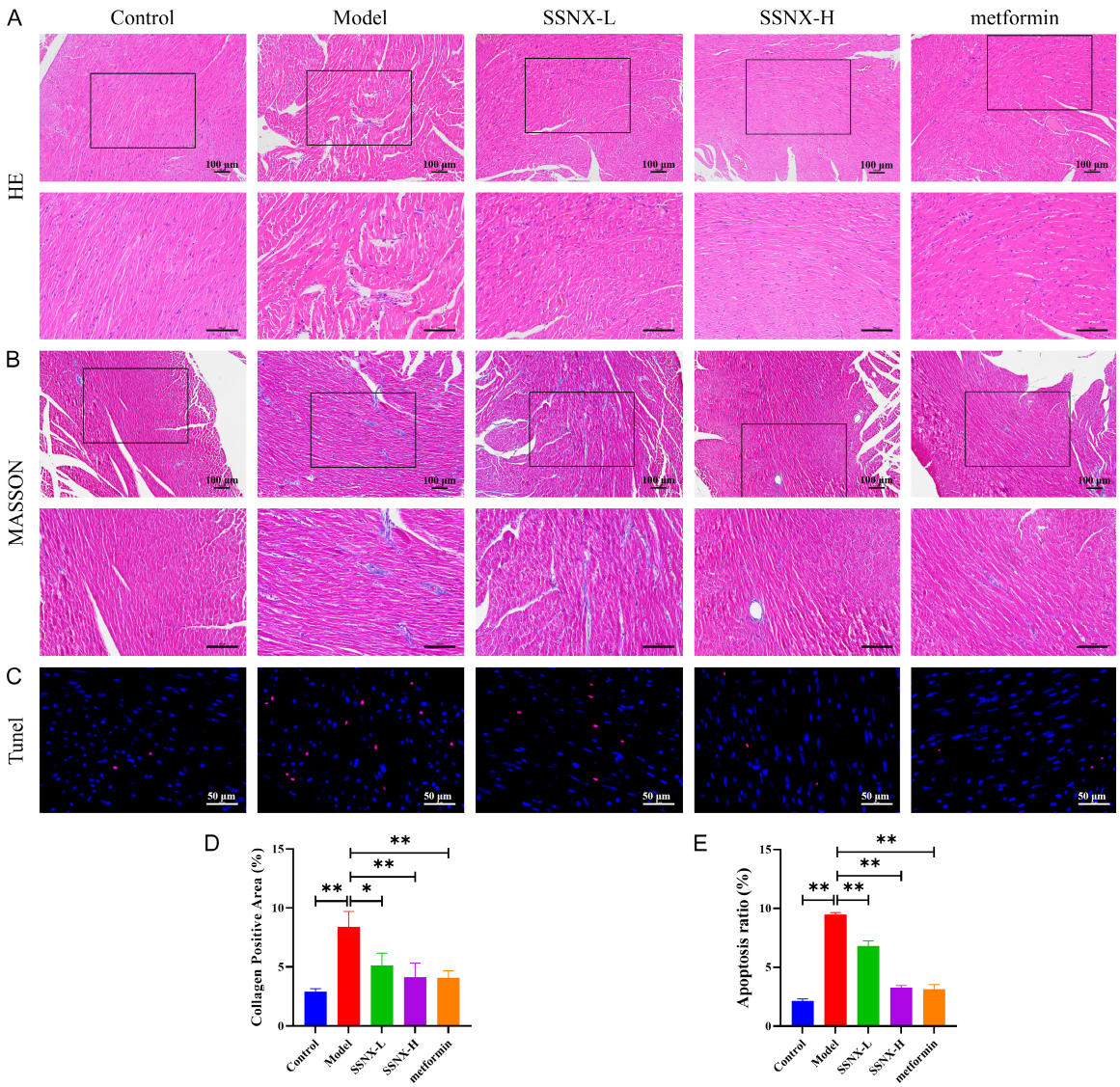

Figure 2. SSNX ameliorated cardiomyocyte hypertrophy, fibrosis, and apoptosis in DCM mice. A. H&E staining showing myocardial histopathological changes; B. Masson staining for assessment of myocardial fibrosis; C. TUNEL staining for detecting cardiomyocyte apoptosis; D. Quantification of collagen fiber-positive areas; E. Quantification of apoptotic cells. Notes: DCM, diabetic cardiomyopathy. $^{*}P < 0.05$ and $^{**}P < 0.01$.

(**Figure 4C-E**). These results demonstrate enhanced ferroptosis and oxidative stress in the myocardium of DCM mice.

Compared to the Model group, intervention with SSNX or metformin significantly reduced Fe^{2+} accumulation and alleviated oxidative stress in DCM mice. Concurrently, Western blotting analysis revealed that the protein levels of FTH1 and GPX4 were significantly decreased, while ACSL4 protein levels were considerably increased in the myocardium of the mice in the Model group compared with those in the Control group. However, SSNX treatment significantly reversed the alterations in the expression of these ferroptosis-associated proteins (**Figure 4F-I**).

SSNX reversed suppression of the NRF2/HO-1 pathway in DCM mice

A schematic diagram of ferroptosis-related pathways shows that the NRF2/HO-1 pathway plays a key role in regulating ferroptosis (**Figure 5A**). To investigate the involvement of this pathway, we first analyzed gene expression patterns. Heatmap analysis of DEGs revealed that SSNX treatment significantly altered the expres-

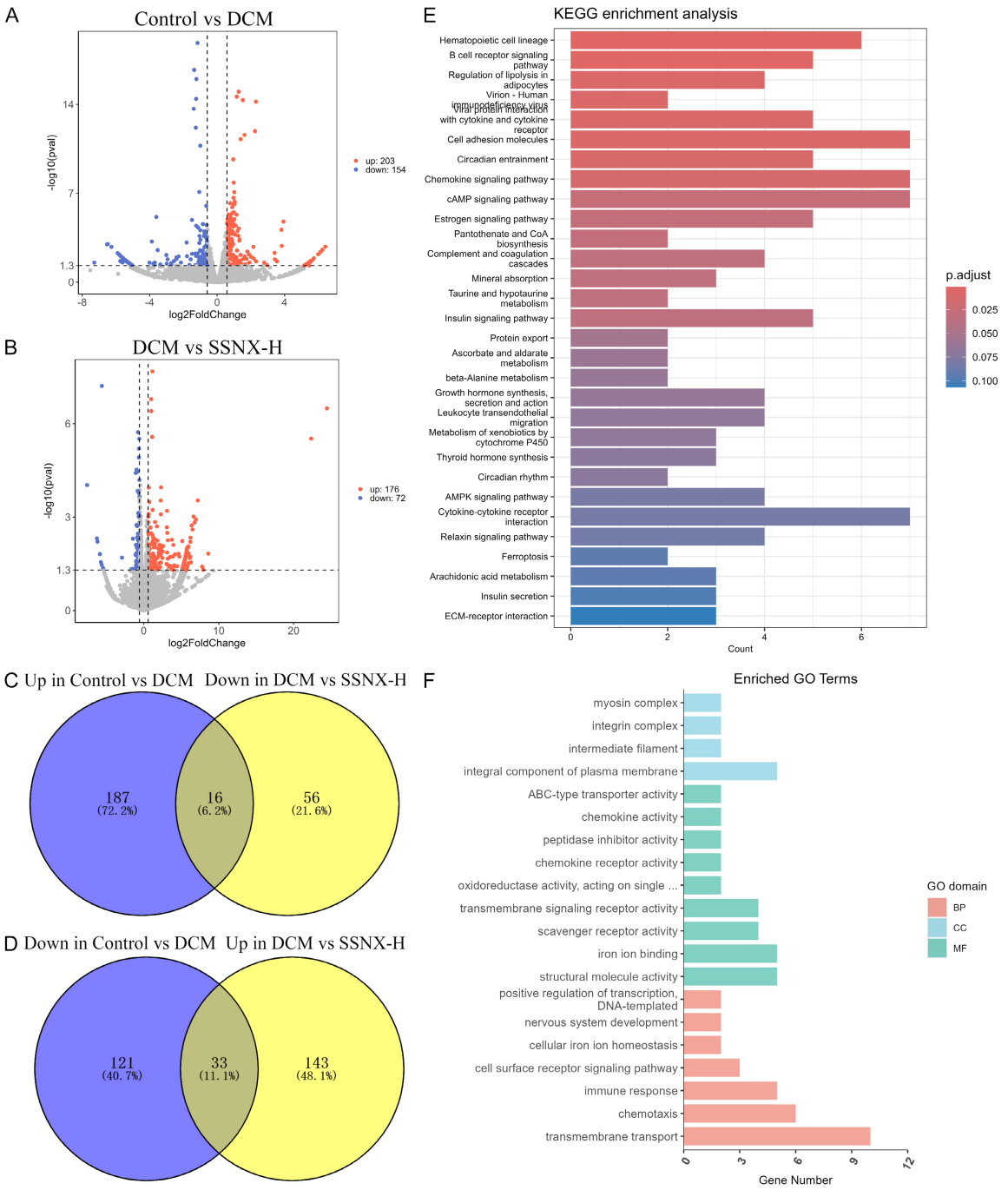

Figure 3. Cardioprotective effect of SSNX on DCM mice at the transcriptional level. A, B. Volcano plots of differentially expressed genes, with red indicating upregulated genes and blue indicating downregulated genes; C. Venn diagram showing 16 upregulated genes in the DCM group that downregulated after SSNX treatment; D. Venn diagram showing 33 downregulated genes in the DCM group that upregulated after SSNX treatment; E. KEGG pathway enrichment analysis; F. GO functional annotation analysis. Notes: DCM, diabetic cardiomyopathy; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

sion of ferroptosis-related genes (FTH1, GPX4, ACSL4) in the myocardium of DCM mice (**Figure 5B**). Western blotting analysis demonstrated that the protein expression levels of NRF2 and HO-1 were significantly reduced in the Model group compared with the Control group, indicating suppression of the NRF2/HO-1 pathway in DCM. In contrast, SSNX or metformin inter-

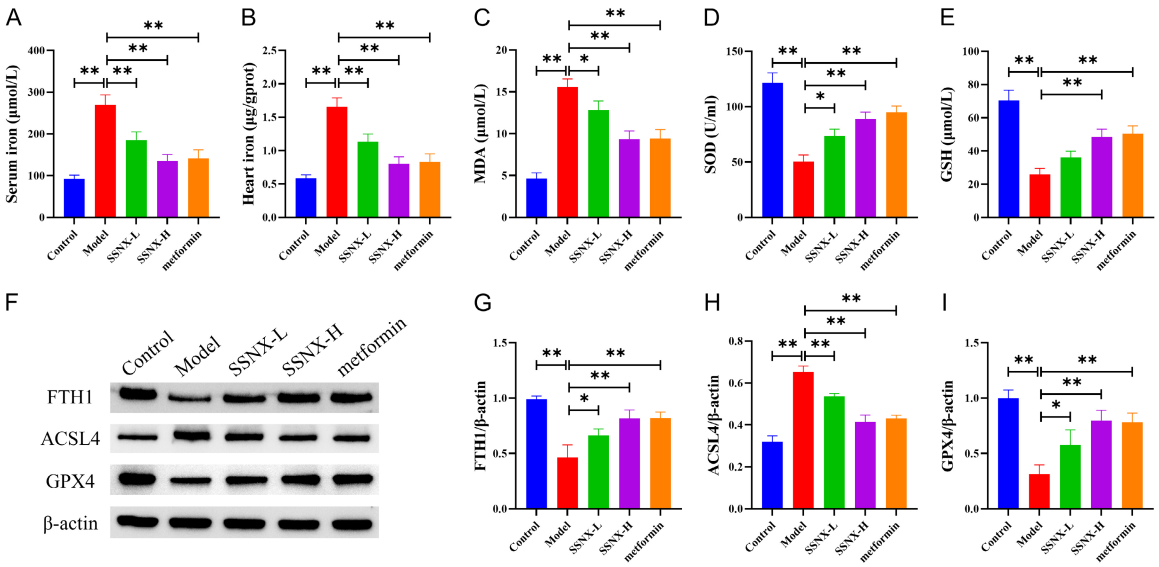

Figure 4. SSNX inhibited oxidative stress and ferroptosis in STZ-induced mice. A. Ferrous ion levels in mouse serum; B. Ferrous ion levels in mouse myocardium; C-E. Levels of MDA, SOD, and GSH in mouse myocardium; F-I. Protein levels of ferroptosis-related markers FTH1, ACSL4, and GPX4 in mouse myocardium. Notes: DCM, diabetic cardiomyopathy; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione. $^{*}P < 0.05$ and $^{**}P < 0.01$.

vention significantly increased the protein levels of NRF2 and HO-1 compared with the Model group (**Figure 5C-E**).

SSNX inhibited high-glucose-induced ferroptosis in HL-1 cells by activating the NRF2HO-1 signaling pathway

The optimal concentration of SSNX was first determined using a CCK-8 assay. Compared with the Control group, exposure to high glucose (35 mmol/L) significantly decreased the viability of HL-1 cells. Treatment with 20, 40, 80, and 160 μg/mL SSNX significantly attenuated this decrease in a dose-dependent manner, with the maximal protective effect observed at 80 μg/mL (**Figure 6A**). Therefore, 80 μg/mL was selected as the high dose (SSNX-H) for subsequent experiments, while 40 μg/mL was selected as the low dose (SSNX-L).

To evaluate ferroptosis, intracellular levels of Fe^{2+} and oxidative stress markers were examined. High glucose stimulation significantly increased intracellular Fe^{2+} levels (**Figure 6B**) and MDA levels (**Figure 6D**), along with a decrease in SOD (**Figure 6C**) and GSH (**Figure 6E**) levels compared to the Control group, indicating the induction of ferroptosis. Co-treatment with SSNX-L or SSNX-H significantly reversed these changes. However, co-treatment with the ferroptosis inducer erastin or the NRF2 inhibitor ML385 abolished the protective effects of SSNX on these indicators (**Figure 6B-E**).

Moreover, EdU assay revealed that the proliferative capacity of HL-1 cells was significantly impaired under high glucose conditions, which was ameliorated by treatment with SSNX. Similarly, this pro-proliferative effect of SSNX was blocked by co-incubation with erastin or the NRF2 inhibitor ML385 (**Figure 6F**, **6G**).

At the molecular level, Western blotting analysis confirmed the involvement of NRF2/HO-1 pathway in SSNX-mediated protection against ferroptosis. High glucose exposure suppressed the protein expression of NRF2 and HO-1, downregulated the ferroptosis-suppressive proteins FTH1 and GPX4, and upregulated the ferroptosis-promoting protein ACSL4. However, SSNX treatment significantly counteracted these high glucose-induced alterations. Notably, the regulatory effects of SSNX on the expression of these key proteins were largely abolished by co-treatment with either erastin or ML385 (**Figure 6H-M**).

Discussion

Diabetes mellitus can induce a series of pathological changes primarily driven by insulin deficiency, which is the fundamental pathogenic basis of DCM. DCM is characterized by a high

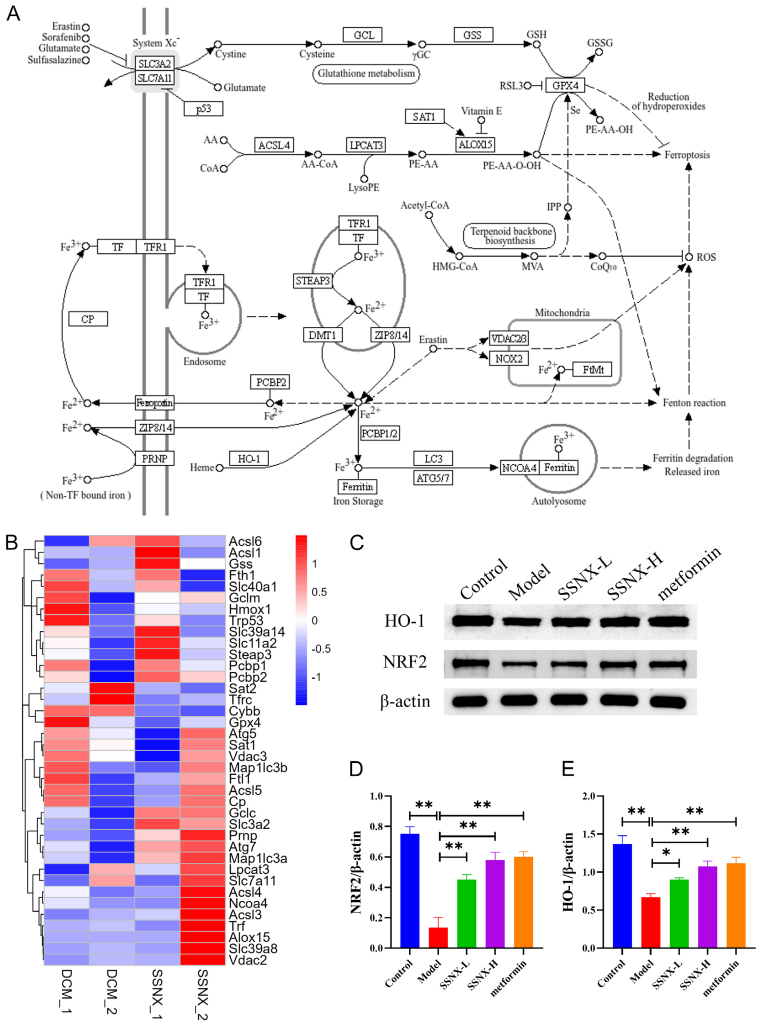

Figure 5. SSNX reversed inhibition of the NRF2/HO-1 pathway in DCM mice. A. Schematic diagram of ferroptosis-related pathways; B. A heatmap showing ferroptosis-related gene expression; C. Protein expression levels of the NRF2/HO-1 pathway-related molecules assessed using Western blot; D and E. Protein expression levels of NRF2 and HO-1 in mouse myocardium. Notes: $^{*}P < 0.05$ and $^{**}P < 0.01$.

clinical incidence and poor prognosis. Currently, unified diagnostic criteria are lacking, and effective treatment methods remain limited.

Previous experimental and clinical studies have indicated that the effective components in SSNX, *ginsenoside* and *salvianolic acid*, have significant effects in improving myocardial injury and related pathological alterations, with their acting mechanism largely associated with the inhibition of ferroptosis. *Ginsenoside Rg3* can suppress ferroptosis, improve cardiac function, and reduce infarct size in mice with myocardial ischemia/reperfusion injury [23]. *Ginsenoside Rb1* similarly ameliorates doxorubicin-induced myocardial hypertrophy and fibrosis by inhibiting mitochondrial damage in cardiomyocytes [24]. Moreover, *salvianolic acid* A inhibits iron deposition and lipid peroxidation, decreases mitochondrial dysfunction, and therefore ameliorates retinal injury [25]. *Salvianolic acid B* can activate the NRF2 signaling pathway, decrease Fe^{2+} and lipid peroxide levels, and downregulate ferroptosis-related protein expression in myocardial infarction rats, which ultimately improves the myocardial injury [26]. However, whether SSNX has specific therapeutic effects against DCM remains insufficiently investigated.

In this study, SSNX effectively alleviated DCM-related phenotypes *in vivo*, extending the findings from its individual components to the compound formula. These results suggest a potential synergistic or additive cardioprotective effects through the coordinated actions of multiple bioactive constituents in SSNX, which needs to be further investigated. We observed that SSNX markedly mitigated STZ-related cardiac dysfunction, myocardial apoptosis, myocardial hypertrophy, and cardiac fibrosis in DCM mice, indicating a protective effect of SSNX on the myocardium in DCM. However, the underlying mechanisms need to be further elucidated.

To further identify potential therapeutic targets of SSNX for ameliorating DCM and its specific mechanisms, high-throughput transcriptomic sequencing was performed on murine myocardial tissues. The results of the DEG analysis confirmed that in the myocardium of DCM mice, 16 originally upregulated genes exhibited a downward trend after intervention with high-dose SSNX, while 33 originally downregulated genes showed an upward trend. GO annotation showed that these DEGs were primarily associ-

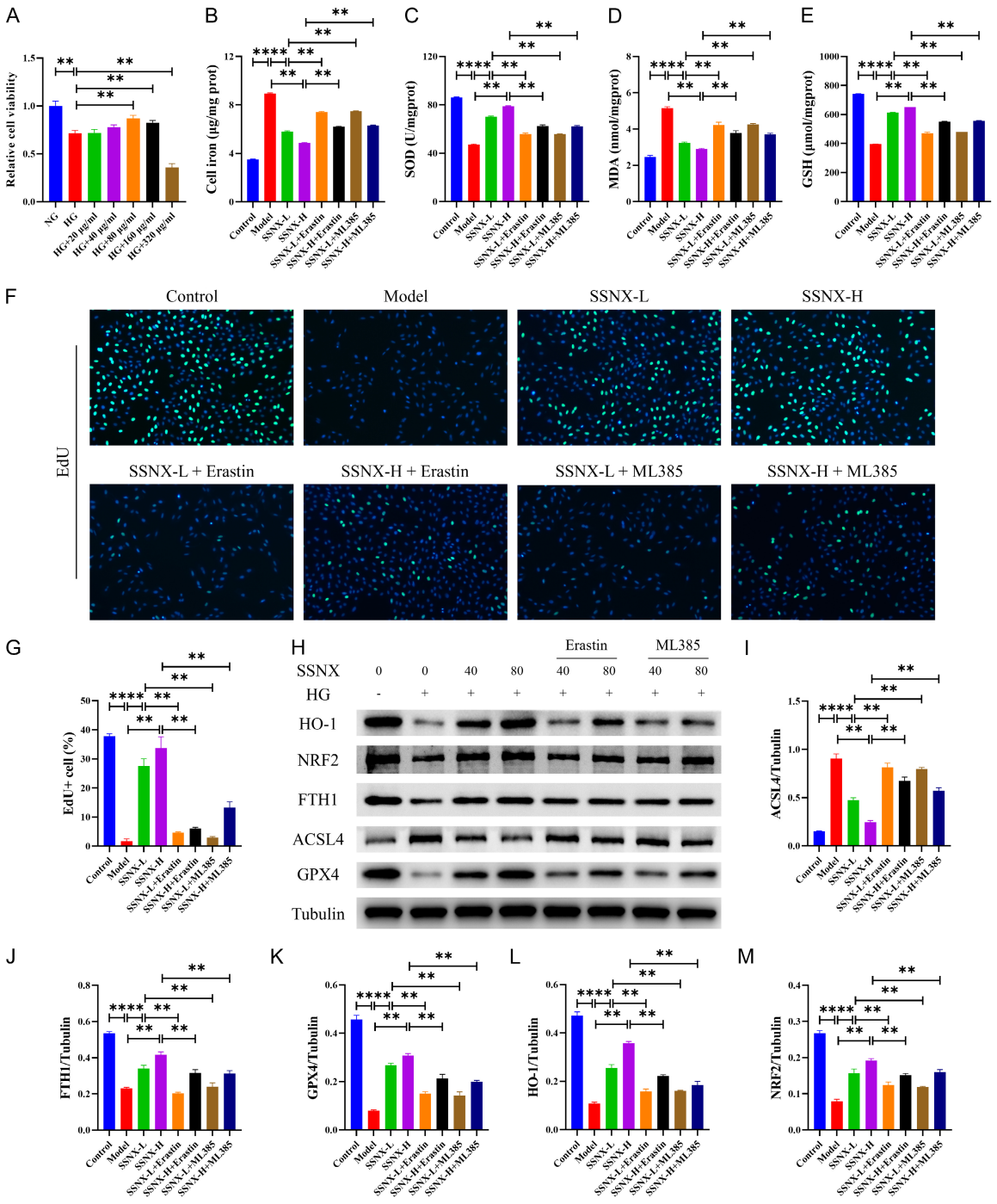

Figure 6. SSNX inhibited high-glucose-induced ferroptosis in HL-1 cells by activating the NRF2/HO-1 signaling pathway. A. Proliferative activity of HL-1 cells; B. Ferrous ion levels in HL-1 cells; C-E. SOD, MDA, and GSH levels in HL-1 cells; F, G. Proliferative capacity of HL-1 cells; H-M. Protein expression levels of HO-1, NRF-2, FTH1, ACSL4, and GPX4 in HL-1 cells. Notes: MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione. $^{*}P < 0.05$ and $^{**}P < 0.01$.

ated with membrane-related cellular components, structural molecular activity, and transmembrane transport-related biological processes. KEGG pathway enrichment analysis demonstrated that SSNX treatment ameliorates DCM through the ferroptosis signaling pathway. These findings are consistent with previous studies by Song et al. and Wang et al. [12, 13],

which reported that ferroptosis plays a critical role in the pathogenesis of DCM. Our study extends these observations by identifying SSNX as a potential intervention targeting ferroptosis in DCM. Collectively, GO functional and KEGG pathway enrichment analyses improved our understanding of gene functions and regulatory networks involved in the cardioprotective effects of SSNX, and provided a ferroptosis-oriented direction for investigating the molecular mechanisms underlying these therapeutic effects at the cellular level.

Iron homeostasis dysregulation and lipid peroxidation represent two hallmarks during ferroptosis, where FTH1, GPX4, and ACSL4 serve as key regulatory nodes in iron and lipid metabolism pathways, respectively [27]. FTH1 facilitates the oxidation of Fe^{2+}, enabling its deposition as ferric hydroxide to prevent ferroptosis triggered by excessive intracellular free iron. GPX4 catalyzes the reduction of glutathione to reduce lipid peroxides into respective alcohols, thus suppressing the lipid peroxidation chain reaction while protecting cells from ferroptotic damage. In contrast, ACSL4 enhances ferroptosis via the catalysis of acyl-CoA generation from polyunsaturated fatty acids while inhibiting the activity of GPX4 [28, 29].

Additionally, the NRF2/HO-1 signaling pathway exerts an important effect on regulating ferroptosis. NRF2 is an important transcription factor for cellular antioxidant response and is translocated to the nucleus upon activation. It can bind to antioxidant response elements (ARE) to promote the expression of HO-1, subsequently modulating lipid and iron metabolism pathways to suppress ferroptosis [30, 31]. Previous studies have demonstrated that ginsenoside Rg3 ameliorates DCM by activating the NRF2/HO-1 pathway, suppressing ferroptosis, reducing inflammation, and counteracting oxidative stress mechanisms [32]. Our *in vivo* and *in vitro* experimental observations suggested that high glucose induces ferroptosis in cardiomyocytes, manifested by an increase in Fe^{2+} levels, oxidative stress levels, and ACSL4 protein expression, along with a decrease in the expression of NRF2, HO-1, FTH1, and GPX4 proteins. Following SSNX intervention, DCM mice and *in vitro* DCM cardiomyocytes exhibited a decrease in Fe^{2+} levels, oxidative stress levels, and ACSL4 protein expression, along with an increase in the expression of NRF2, HO-1, FTH1, and GPX4 proteins, accompanied by an increase in the viability of cardiomyocytes. However, co-treatment with erastin or ML385 reversed the protective effects of SSNX on myocardial injury. These results suggest that SSNX may inhibit ferroptosis and improve DCM by activating the NRF2/HO-1 signaling pathway, in agreement with the findings reported by Wu et al. [32]. Collectively, SSNX may exert protective effects against DCM by activating the NRF2/HO-1 signaling pathways, thus suppressing ferroptosis.

Nevertheless, several limitations of this study should be acknowledged. First, the STZ-induced mouse model does not fully mimic complex pathophysiology of human DCM. Second, as a multi-compound formulation, the specific active components of SSNX responsible for the observed effects remain to be identified. Although pharmacological inhibition with ML385 supports the involvement of NRF2, genetic validation may further strengthen the conclusion. Future studies should isolate the key effective substances of SSNX, validate the mechanism using genetic models, and assess its long-term efficacy and potential for combined therapy.

Conclusion

SSNX ameliorates cardiac dysfunction in DCM mice by suppressing ferroptosis via regulation of the NRF2/HO-1 pathway, while concurrently alleviating myocardial hypertrophy, fibrosis, and injury. These findings indicate that SSNX may represent a promising candidate therapeutic agent for DCM.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (8217-4219), and the High-level TCM key discipline construction project of the State Administration of Traditional Chinese Medicine (zyyzdxk-2023231).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianhua Fu, Institute of Basic Medical Sciences, Xiyuan Hospital, China Academy of Chinese Medical Sciences, National Clinical Research Center for Cardiovascular Diseases of Traditional Chinese Medicine, 1 Xiyuan Playground, Haidian District, Beijing 100091, China. E-mail: 13651043518@139.com

References

[1] Dillmann WH. Diabetic cardiomyopathy. Circ Res 2019; 124: 1160-1162.

[2] Lorenzo-Almorós A, Cepeda-Rodrigo JM and Lorenzo Ó. Diabetic cardiomyopathy. Rev Clin Esp (Barc) 2022; 222: 100-111.

[3] Paolillo S, Marsico F, Prastaro M, Renga F, Esposito L, De Martino F, Di Napoli P, Esposito I, Ambrosio A, Ianniruberto M, Mennella R, Paolillo R and Gargiulo P. Diabetic cardiomyopathy: definition, diagnosis, and therapeutic implications. Heart Fail Clin 2019; 15: 341-347.

[4] Nakamura K, Miyoshi T, Yoshida M, Akagi S, Saito Y, Ejiri K, Matsuo N, Ichikawa K, Iwasaki K, Naito T, Namba Y, Yoshida M, Sugiyama H and Ito H. Pathophysiology and treatment of diabetic cardiomyopathy and heart failure in patients with diabetes mellitus. Int J Mol Sci 2022; 23: 3587.

[5] Liu Z, Han X, You Y, Xin G, Li L, Gao J, Meng H, Cao C, Liu J, Zhang Y, Li L and Fu J. Shuangshen ningxin formula attenuates cardiac microvascular ischemia/reperfusion injury through improving mitochondrial function. J Ethnopharmacol 2024; 323: 117690.

[6] Jia F, Chen Y, Xin G, Li L, Liu Z, Xu S, Gao J, Meng H, Shi Y, Ma Y, Li L and Fu J. Shuangshen Ningxin capsule alleviates myocardial ischemia-reperfusion injury in miniature pigs by modulating mitophagy: network pharmacology and experiments in vivo. Chin Med 2023; 18: 120.

[7] Zhang C, Yu H, Ye J, Tong H, Wang M and Sun G. Ginsenoside Rg3 protects against diabetic cardiomyopathy and promotes adiponectin signaling via activation of PPAR-γ. Int J Mol Sci 2023; 24: 16736.

[8] Gong DF, Sun SC, Wang RR, Dawuti A, Kong DW, Liu RQ, Du LD, Wang SB, Lu Y, Yuan TY, Du GH and Fang LH. Salvianolic acid A improve mitochondrial respiration and cardiac function via inhibiting apoptosis pathway through CRYAB in diabetic cardiomyopathy. Biomed Pharmacother 2023; 160: 114382.

[9] Wen B, Dang YY, Wu SH, Huang YM, Ma KY, Xu YM, Zheng XL and Dai XY. Antiatherosclerotic effect of dehydrocorydaline on ApoE(-/-) mice: inhibition of macrophage inflammation. Acta Pharmacol Sin 2022; 43: 1408-1418.

[10] Zhan J, Chen C, Wang DW and Li H. Hyperglycemic memory in diabetic cardiomyopathy. Front Med 2022; 16: 25-38.

[11] El Hayek MS, Ernande L, Benitah JP, Gomez AM and Pereira L. The role of hyperglycaemia in the development of diabetic cardiomyopathy. Arch Cardiovasc Dis 2021; 114: 748-760.

[12] Song Z, Wang J and Zhang L. Ferroptosis: a new mechanism in diabetic cardiomyopathy. Int J Med Sci 2024; 21: 612-622.

[13] Wang R, Zhang X, Ye H, Yang X, Zhao Y, Wu L, Liu H, Wen Y, Wang J, Wang Y, Yu M, Ma C and Wang L. Fibroblast growth factor 21 improves diabetic cardiomyopathy by inhibiting ferroptosis via ferritin pathway. Cardiovasc Diabetol 2024; 23: 394.

[14] Loboda A, Damulewicz M, Pyza E, Jozkowicz A and Dulak J. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell Mol Life Sci 2016; 73: 3221-3247.

[15] Ndisang JF. Synergistic interaction between heme oxygenase (HO) and nuclear-factor E2-related factor-2 (Nrf2) against oxidative stress in cardiovascular related diseases. Curr Pharm Des 2017; 23: 1465-1470.

[16] Li X, Sun YX, Tjahjono AW, Wei Y, Li X, Zheng QH, Qi WC and Liang FR. Acupuncture attenuates myocardial ischemia/reperfusion injury-induced ferroptosis via the Nrf2/HO-1 pathway. Chin Med 2025; 20: 61.

[17] Li L, Li L, Cao C, Guo F, Wang A, Lin L, Liu Z, Meng H, Zhang P, Xin G, Liu J, Ren J and Fu J. Investigation of the active ingredients of Shuangshen Ningxin Fomula and the mechanism underlying their protective effects against myocardial ischemia-reperfusion injury by mass spectrometric imaging. Phytomedicine 2024; 123: 155184.

[18] Tang YJ, Zhang Z, Yan T, Chen K, Xu GF, Xiong SQ, Wu DQ, Chen J, Jose PA, Zeng CY and Fu JJ. Irisin attenuates type 1 diabetic cardiomyopathy by anti-ferroptosis via SIRT1-mediated deacetylation of p53. Cardiovasc Diabetol 2024; 23: 116.

[19] Chen X, Yin J, Zhong Q, Wang K, Zhang X, Liang M, Lin Q, Wang H, Wang W, Wang L, Hu X, Bei W and Guo J. Fufang-zhenzhu-tiaozhi formula protects islet against injury and promotes β cell regeneration in diabetic mice. J Ethnopharmacol 2023; 301: 115791.

[20] Xu Y, Fang H, Xu Q, Xu C, Yang L and Huang C. LncRNA GAS5 inhibits NLRP3 inflammasome activation-mediated pyroptosis in diabetic cardiomyopathy by targeting miR-34b-3p/AHR. Cell Cycle 2020; 19: 3054-3065.

[21] Li W, Li W, Leng Y, Xiong Y and Xia Z. Ferroptosis is involved in diabetes myocardial ischemia/reperfusion injury through endoplasmic reticulum stress. DNA Cell Biol 2020; 39: 210-225.

[22] Li F, Hu Z, Huang Y and Zhan H. Dexmedetomidine ameliorates diabetic cardiomyopathy by inhibiting ferroptosis through the Nrf2/GPX4 pathway. J Cardiothorac Surg 2023; 18: 223.

[23] Zhong G, Chen J, Li Y, Han Y, Wang M, Nie Q, Xu M, Zhu Q, Chang X and Wang L. Ginsenoside Rg3 attenuates myocardial ischemia/reperfusion-induced ferroptosis via the keap1/Nrf2/

GPX4 signaling pathway. BMC Complement Med Ther 2024; 24: 247.
[24] Zhai Y, Bai J, Peng Y, Cao J, Fang G, Dong Y, Wang Z, Lu Y, Wang M, Liu M, Liu Y, Li X, Dong J and Zhao X. Ginsenoside Rb1 attenuates doxorubicin induced cardiotoxicity by suppressing autophagy and ferroptosis. Biochem Biophys Res Commun 2024; 710: 149910.
[25] Shen Y, Shen X, Wang S, Zhang Y, Wang Y, Ding Y, Shen J, Zhao J, Qin H, Xu Y, Zhou Q, Wang X and Shen J. Protective effects of Salvianolic acid B on rat ferroptosis in myocardial infarction through upregulating the Nrf2 signaling pathway. Int Immunopharmacol 2022; 112: 109257.
[26] Zhao Y, Li Q, Jian W, Han X, Zhang Y, Zeng Y, Liu R, Wang Q and Song Q. Protective benefits of salvianic acid A against retinal iron overload by inhibition of ferroptosis. Biomed Pharmacother 2023; 165: 115140.
[27] Zheng J and Conrad M. The metabolic underpinnings of ferroptosis. Cell Metab 2020; 32: 920-937.
[28] Seibt TM, Proneth B and Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. Free Radic Biol Med 2019; 133: 144-152.
[29] Feng S, Tang D, Wang Y, Li X, Bao H, Tang C, Dong X, Li X, Yang Q, Yan Y, Yin Z, Shang T, Zheng K, Huang X, Wei Z, Wang K and Qi S. The mechanism of ferroptosis and its related diseases. Mol Biomed 2023; 4: 33.
[30] Liu Z, Zhou Z, Ai P, Zhang C, Chen J and Wang Y. Astragaloside IV attenuates ferroptosis after subarachnoid hemorrhage via Nrf2/HO-1 signaling pathway. Front Pharmacol 2022; 13: 924826.
[31] Chen Z, Zhu Q, Qi X, Yang LR, Rong YX, Wei Q, Wu SQ, Lu QW, Li L, Jiang MD and Qi H. Dual role of Nrf2/HO-1 pathway in Z-ligustilide-induced ferroptosis against AML cells. Phytomedicine 2024; 124: 155288.
[32] Wu S, Zhu J, Wu G, Hu Z, Ying P, Bao Z, Ding Z and Tan X. 6-gingerol alleviates ferroptosis and inflammation of diabetic cardiomyopathy via the Nrf2/HO-1 pathway. Oxid Med Cell Longev 2022; 2022: 3027514.