Exogenous spermidine alleviates diabetic cardiomyopathy via suppressing Pannexin-1-mediated ferroptosis in db/db mice

Jian Sun
Mudanjiang Medical University

Jiyu Xu
Mudanjiang Medical University

Yong Liu
Mudanjiang Medical University

Yitong Lin
Mudanjiang Medical University

Fengge Wang
The Fifth Affiliated Hospital of Southern Medical University

Yue Han
Mudanjiang Medical University

Shumin Zhang
Mudanjiang Medical University

Xiaoyan Gao
Mudanjiang Medical University

Changqing Xu
Harbin Medical University

Hui Yuan (✉ yuanhui@mdjmu.edu.cn)
Mudanjiang Medical University

Research Article

Keywords: Diabetic cardiomyopathy, Spermidine, Ferroptosis, Pannexin-1, P2X7

Posted Date: October 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2140318/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Diabetic cardiomyopathy (DCM) is a serious complication and death cause of diabetes mellitus (DM). Recent cardiology studies suggest that spermidine has cardioprotective effects. Here, the hypothesis was tested the spermidine effects of DCM. Therefore, db/db mice and primary neonatal mouse cardiomyocytes were used to observe the effects of spermidine. Immunoblotting showed that ornithine decarboxylase (ODC) and SPD/spermine N1-acetyltransferase (SSAT) were downregulated and upregulated in the myocardium of db/db mice, respectively. We found that diabetic mice showed cardiac dysfunction in 12 weeks. Conversely, exogenous spermidine could improve cardiac functions and reduce the deposition of collagens, myocardial damage, ROS levels and endoplasmic reticulum stress in diabetic mouse hearts. Our results also demonstrated that cardiomyocytes appeared ferroptosis and then activated Pannexin-1 expression, which resulted in increasing the extracellular ATP. Subsequently, increased ATP as a paracrine molecule combined to P2X7 receptors to activate ERK1/2 signaling pathway in cardiomyocytes, and activated NCOA4-mediated ferroptinophagy to promote lipid peroxidation and ferroptosis. Interestingly, spermidine could reverse these molecular processes. Our findings indicate an important new mechanism for DCM, and suggest spermidine have potential applicability to protect against deterioration of cardiac function with DCM.

Introduction

Diabetes mellitus (DM) is a metabolic disease, divided into type 1 and type 2. The former is usually induced by congenital pancreatic islet dysfunction, and the latter with the higher incidence is mostly caused by insulin resistance[1]. DM can cause persistent hyperglycemia and metabolic disorders, and then can lead to the impairment of tissues and organs, particularly damage the cardiovascular system[2, 3]. DCM is an impairment of ventricular diastolic and systolic function induced by no coronary artery disease, which is distinct from cardiomyopathy caused by coronary heart disease and hypertension[4]. The pathogenesis of DCM is complex and inconclusive, and the underlying mechanisms are all related to the interaction of multiple damage factors of hyperglycemia, which affect the heart structural function. It may be related to mitochondrial dysfunction in cardiomyocytes, abnormal activation of cardiac fibroblasts, imbalance of redox system and extracellular matrix (ECM) [5, 6]. However, the exact mechanism of DCM remains unknown. Therefore, finding accurate and effective therapeutic target drugs is the priority.

Polyamines (PAs) is small branched chain cationic molecule derived from linear amino acid, including spermine (SP), spermidine(SPD) and putrescine (PU), are widely distributed in all types of mammalian cells[7]. Intracellular levels of SPD are maintained and tightly controlled by enzymes that catalyze rate-limiting steps of their biosynthesis by ornithine decarboxylase (ODC) and catabolism by spermidine/spermine-N1-acetyltransferase (SSAT)[8, 9]. SPD participates in various biological processes of organism, including regulation of DNA synthesis, cell cycle, cell proliferation and differentiation, aging, endoplasmic reticulum stress, oxidative stress and ion channel switching, and also has anti-apoptosis[10], anti-inflammatory[11], antioxidant and induction of autophagy[12]. Some studies highlight
the effects of SPD for aging via improving mitochondrial biogenesis and function[7, 13, 14], and recent researches demonstrate that SPD can prevent heart injury by inhibiting oxidative stress and endoplasmic reticulum stress[8]. However, it is unclear whether administration of exogenous SPD to diabetic heart can reduce myocardial damage.

Our team, and others, recently corroborated that spermidine and spermine levels were decreased in diabetic rats[15, 16], and treatment of exogenous spermine can protect cardiomyocytes to attenuate rat diabetic cardiomyopathy[17, 18]. However, whether SPD can mitigate DCM has not yet been elucidated. Hence, in this study, we examined whether SPD exerted beneficial effects on DCM. The therapeutic implication of spermidine in DCM development was evaluated in db/db mouse models, the mechanism of SPD was determined using cardiomyocytes undergoing high glucose model.

Results

Establishment of type 2 diabetic mouse model

To better observe the effects of SPD, we selected 8-week-old wild type mice and db/db mice as our research objects and intraperitoneally injected with SPD every other day for 12 weeks respectively. Glucose levels, glucose intolerance, insulin levels, triglyceride (TG) and total cholesterol (TC) levels were examined on week 12, which recapitulated the hallmark features of type 2 diabetes. The results showed that compared with WT group and WT+SPD group, the blood glucose levels and glucose intolerance at each time point were higher, the insulin levels were significantly increased, and TG, TC were also increased in the T2D group and T2D+SPD group. Interestingly, compared with T2D group, there were no differences of the above indexes in T2D+SPD group (Figure 1A-F).

Exogenous SPD ameliorates cardiac functions in db/db mice

To determine whether SPD can improve heart function in db/db mice, echocardiography was performed. Compared with the T2D group, we observed that ejection fraction (EF) and left ventricular fractional shortening (FS) were increased, left ventricular internal dimension systole (LVIDs) and left ventricular internal dimension diastole (LVIDd) were decreased in T2D+SPD group, and wild-type mice treated with SPD had no obvious changes of cardiac functions (Figure 2A-E).

To verify that diabetic cardiomyopathy can cause serious cardiac injury, the hearts of mice in each group were excised in week 12, HE staining indicated that the cardiac myocytes were disordered and hypertrophic, TEM results revealed myocardial myofilament lysis and mitochondrial edema, and SPD could mitigate these pathological changes in db/db mice (Figure 2F). Masson and Sirius Red staining results showed large amounts of collagen deposition in T2D group, and SPD could reduce collagen deposition (Figure 2G-H).

We also detected the serum myocardial injury markers (LDH, CK-MB and cTnl) in each group. The results showed that the contents of serum LDH, CK-MB and cTnl in T2D group were significantly higher than
those in WT group. Compared with T2D group, the serum contents of above enzymes in T2D+SPD group decreased significantly. These indicated SPD has protective and therapeutic effects on myocardial injury caused by high glucose (Figure 2I-K). Moreover, body weight (BW) of db/db mice on week 12 was heavier than wild type mice BW, and SPD had no effects on BW. However, we found that SPD can reduce the ratio of heart weight to tibia length (HW/TL) in diabetic mice (Figure 2L-M).

**Comparative proteomic analysis of cardiac tissues from WT mice and db/db mice**

In order to further study the differential variations of protein expression in T2D mice, a liquid chromatography-tandem mass spectrometry (LC-MS) analysis of cardiac tissues in WT and db/db mice group was performed. Using the criteria fold change > 1.5 and \( P \)-value < 0.05, 169 proteins were identified as differentially expressed (DE) between the two groups (115 upregulated proteins and 54 downregulated proteins) (Figure 3A). The major GO terms including the related biological processes were identified, DE proteins were found to be enriched in cellular process, metabolic process, biological regulation and response to stimulus (Figure 3B-C). Subcellular structure results showed that the largest proportion of DE proteins was identified in cytoplasm (35.5 %), followed by mitochondria (21.3 %), nucleus (8.88 %), extracellular (19.53 %), plasma membrane (7.1 %) (Figure 3D). DE proteins between WT and db/db mice group were mapped and enriched in KEGG pathways, the top 10 enriched pathways were shown in the functional KEGG enrichment cluster image (Figure 3F), we found that ferroptosis, MAPK signaling pathway and Gap junction were highly enriched in db/db mice compared to WT mice.

**Exogenous SPD restrains ferroptosis pathway in vivo and vitro**

We detected the levels of pivotal polyamine metabolic enzymes in vivo and vitro, the results showed that the cardiac expression of ODC was downregulated but SSAT was upregulated in db/db mice, and the expression trend is consistent with cell model results (Figure 4A-B). Now that proteomics results have been verified that it is associated with ferroptosis, we then detected the key proteins of ferroptosis, and HG could upregulate ASCL-4 expression and downregulate GPX-4 expression in cardiomyocytes, treatment with SPD could gain the opposite results, which was consistent with the results of animal experiments (Figure 4C-D). Mitochondria is the key organelle of lipid metabolism, and abnormal lipid metabolism is closely related to mitochondrial damage[19]. JC-1 staining is often used as a tool to detect mitochondrial membrane potential (\( \Delta \Psi_m \)). The result revealed that compared with the Control group, the red/green fluorescence intensity significantly decreased in HG group, and supplement of SPD increased the fluorescence intensity apparently (Figure 4E). In addition, we assayed iron, GPX-4 activity, GSH level and GSH/GSSG ratio at 12 weeks in myocardial tissue. Iron, another essential factor for ferroptosis execution, whose activity was increased in diabetic mice, and SPD could decrease the activity significantly (Figure 4F). Subsequently, we demonstrated that the remaining three indicators had the opposite trend to iron activity (Figure 4G-I).

**Exogenous SPD inhibits oxidative stress (OS) and endoplasmic reticulum stress (ERS) in cardiomyocytes**
Excessive ROS deposition can activate ferroptosis-related pathways of cells by a series of cascade reactions\cite{20, 21}. In our study, the ROS detection showed that SPD could reduce the ROS levels in HG-treated cardiomyocytes and Western blot results revealed that HG could upregulate Prdx-1, Tfr-1, p-p38 and p-JNK expression, and SPD could significantly downregulate the abovementioned proteins in cardiomyocytes (Figure 5A-B). Research demonstrated that ERS could induce ferroptosis via activation of ATF4-CHOP pathway\cite{20}. We then observed that the expression of GRP94, GRP78, ATF4 and CHOP increased in HG group, and these protein expressions significantly decreased in HG+SPD group (Figure 5C).

**Pannexin-1 regulates the P2X7 expression level in HG-treated cardiomyocytes**

Immunofluorescence results showed that compared with WT group, the Pannexin-1 fluorescence intensity significantly increased in the T2D group, and significantly decreased in the T2D+SPD group compared to T2D group (Figure 6A), which was consistent with the trend of western blot results (Figure 6B). Hereafter, purinergic receptor P2X7 was determined in the heart tissue, and P2X7 expression was upregulated in the db/db mice, indicating more ATP emerged in myocardium. Notably, SPD could significantly inhibit P2X7 expression (Figure 6C). We also observed that HG could increase the levels of extracellular ATP and decrease intracellular ATP levels in cardiomyocytes, treatment with SPD could reverse the abovementioned changes (Figure 6D-E). Recently, it has been reported that ferroptosis is closely related to Pannexin-1\cite{22}, and we used erastin (a special ferroptosis inducer) to treat with cardiomyocytes at different time point, and the results showed that erastin could upregulate the Pannexin-1 expression, which indicated that Pannexin-1 was involved in ferroptosis in cardiomyocytes (Figure 6F). Further experiments confirmed Pannexin-1-siRNA and SPD could both downregulate P2X7 expression level and extracellular ATP level in HG-treated in cardiomyocytes.

**Exogenous SPD suppresses ferroptosis via inhibiting ERK1/2-HO-1 pathway**

To further study the mechanism of spermidine alleviating diabetic cardiomyopathy, we used P2X7 specific inhibitor (A-438079) and ERK1/2 signaling pathway blocker (LY3214996) to treat with cardiomyocytes separately. The results showed that HG could activate p-ERK1/2 expression. Conversely, A-438079 and SPD could significantly reduce its expression (Figure 7A). Furthermore, we demonstrated that HO-1 expression was downregulated, the expression of NCOA-4 and PTH-1 were upregulated in HG group, and treatment with LY3214996 or SPD could obtain the opposite trend (Figure 7B). BODIPY probe results showed that HG could significantly increase the intracellular lipid ROS level and treatment with Pannexin-1-siRNA, A-438079, LY3214996, Fer-1 and SPD had markedly opponent effects (Figure 7C). In addition, iron assay showed that the variation tendencies of iron were consistent with the BODIPY probe results (Figure 7D).

**Discussion**

In present study, we selected db/db mouse, a model of type 2 diabetes as the research object, whose genetic background is C57BL/6 mouse diabetes gene knocked out and even insulin intervention fails to
control hyperglycemia in db/db mouse. Therefore, db/db homozygous mice are widely used in the study of endocrine defects, neurological diseases and cardiac diseases induced by abnormal glucose and lipid metabolism[23, 24]. At 1, 4, 8, 12 weeks, db/db mouse showed persistent hyperglycemia, and there was no downward trend after SPD injection. At 12 weeks, insulin activity increased significantly in T2D group, and serum TC and TG content levels increased significantly, the result was consistent with trends in insulin resistance test, which indicated that the type 2 diabetes mouse model was successfully established, however, SPD had no effects on the above indicators.

At 12 weeks, echocardiography displayed that EF and FS were decreased, LVIDs and LVIDd were increased in the T2D group, indicating heart systolic dysfunction. Further myocardial injury marker enzyme test confirmed LDH, CK-MB and cTnl levels were increased in the blood serum, and SPD could attenuate the above indicators of heart damage. In T2D group and T2D + SPD group, db/db mice showed a significant increase of body weight, however, treatment with SPD could decreased the HW/TL ratio and we suspected it was due to remodeling of the heart and increasing the myocardial extracellular matrix. This speculation is supported by cardiac morphology. In T2D group, Masson and Sirius red staining showed large amounts of collagen deposition in the interstitial areas, TEM results presented obvious myofilament dissolution and mitochondrial edema, HE staining displayed db/db mouse heart eventuated degeneration and necrosis of cardiomyocytes, and SPD could inhibit these changes effectively. These results demonstrated that SPD could alleviate high glucose-induced myocardial damages.

To further explore the mechanism of DCM in db/db mice, we used proteomics to analyze the differential expression of proteins in cardiac tissue between C57BL/6 and db/db mouse, and enrich in KEGG pathways. The results showed that DCM was closely related to ferroptosis pathway, MAPK signaling pathway and gap junction, which indicated the next research direction of our project.

ACSL-4 and GPX-4 is the central regulator of ferroptosis, and they are often used as a marker of ferroptosis[25, 26]. In vivo, western blot results showed that ODC and ACSL-4 expression were downregulated and SSAT and GPX-4 expression were upregulated significantly in T2D group, the opposite trend appeared in T2D + SPD group, which was consistent with the proteomics results, and then we also observed the same variation tendency in vitro. Excess Fe^{2+} and cysteine deprivation are often linked to mitochondrial membrane hyperpolarization and lipid peroxide accumulation, leading to ferroptosis[27]. In our study, mitochondrial membrane potential (ΔΨm) increased markedly in HG group, and treatment with SPD could reduce the above index. Moreover, increase of Iron level and decrease of GPX-4 activity, GSH level and GSH/GSSG ratio in db/db mice could also demonstrate that ferroptosis was involved in DCM, and SPD could significantly reverse the abovementioned changes.

Oxidative stress damage is proposed as a main inducer of ferroptosis[28], and Prdx1 is a member of the Prdxs (peroxiredoxins) family, has been implicated as an antioxidant and redox signaling protein, acting as an antioxidant effect, and its expression is promoted when cells undergo oxidative stress[21, 29]. TfR1 is an important iron regulatory protein, mediating most of the cellular iron uptake by binding iron transferrin at the cell surface, which is internalized by receptor-mediated endocytosis, permitting the
release and reduction of the iron in endosomes and transport of the released iron into the cytosol, and ferroptosis agonist can upregulate its expression to active p38 and JNK signaling pathway [30]. Our findings demonstrated that HG could increase ROS level, the expression of Prdx1, TfR1, p-p38 and p-JNK in cardiomyocytes, and SPD had the opposite effects. Recent studies have shown that ERS (Endoplasmic reticulum stress) is involved in ferroptosis and aggravates cardiomyocytes injury[20], subsequently, we observed that GRP94, GRP78, ATF-4 and Chop expression were upregulated in HG group and downregulated in HG + SPD group.

Pannexin-1 belongs to the ATP-releasing pathway family and its function is regulation of ATP flow out of the cell[31]. By immunofluorescence, we observed Pannexin-1 expression significantly increased in T2D group and decreased in T2D + SPD group, fitting well with the expression trends that we have observed with the in vitro experiments. P2X7 receptor is a family of purinergic G protein coupled receptors, localizes to the cell membrane and are stimulated by ATP[32], we found that P2X7 upregulated in the heart tissue of db/db mouse, which intimated the increasing leakage of endogenous ATP and treatment of SPD could reduce the expression of P2X7. To verify whether there was ATP leakage, we detected the endogenous and exogenous ATP concentration of cardiomyocytes, and the changes in the content of the two showed an opposite trend. That is to say, the endogenous ATP was markedly decreased in HG group, but exogenous ATP was significantly increased, and the opposite tendency was observed in the HG + SPD group. Subsequently, we used erastin (ferroptosis agonist) to treat with cardiomyocytes, the expression of Pannexin-1 emerged time-dependent, indicating ferroptosis could promote its expression. Furthermore, we also observed that both Pannexin1-siRNA and SPD could downregulate P2X7 expression, and reduce the concentration of the exogenous ATP in cardiomyocyte culture medium. Altogether, the abovementioned results further elucidated HG could induce ferroptosis and activate Pannexin-1 expression, resulting in more leakage of ATP in cardiomyocytes and SPD appeared effective protection.

Our proteomic analyses have confirmed that MAPK signaling pathway is involved in the pathological process of DCM, and previous studies have shown that ferroptosis could activate the MAPK pathway through ERK1/2 [33, 34], we demonstrated that A-438079 (a specific inhibitor of P2X7) could restrain ERK1/2 signaling pathway, and SPD had the same effect. Research has found that heme oxygenase-1 (HO-1), nuclear receptor coactivator 4 (NCOA-4) and ferritin light chain 1 (FTH-1) were involved in regulating ferroptosis [35–39]. Western blot analysis showed that HG could suppress the HO-1 expression, promote NCOA-4 and FTH-1 expression. Conversely, treatment with SPD and LY3214996 (ERK1/2 signaling pathway inhibitor) had the opposite effects. Finally, both BODIPY probe and iron assay results certified that treatment with Pannexin-1-siRNA, A-438079, LY3214996, Fer-1 and SPD had the inhibition effects of HG-induced ferroptosis.

Conclusions

Taken as a whole, based on the aforementioned experimental results and present literature, it is hypothesized that in db/db mouse, persistent hyperglycemic stimulation leads to cardiomyocyte damages (ROS and ERS) and activation of ferroptosis pathway, and then upregulates the Pannexin-1 to
open ATP outflow channel, resulting in an increase of extracellular ATP. Subsequently, increased ATP as a paracrine molecule combines to P2X7 receptors to activate ERK1/2 signaling pathway which regulate NCOA-4-mediated ferroptinophagy and antioxidant gene HO-1 expression. Conversely, SPD can reverse the above molecular regulation process (see Fig. 7E). Hopefully, our results could provide novel targets and an experimental basis for the prevention and treatment of diabetic cardiomyopathy.

**Materials And Methods**

**Experimental animals**

Homozygous eight-week-old male db/db mice (22 ± 0.5 g) on a C57BL/6 background were provided by the Animal Research Institute of Mudanjiang Medical University (MMU), and the study was approved by the MMU Medical Science Ethics Committee. All mice were maintained on a 12-h light/dark cycle and fed with a standard chow and clean water ad libitum. The mice were randomly divided into four groups (n = 8 per group): (1) WT group: eight-week-old male C57BL/6 mice were injected with stroke-physiological saline solution buffer (SPSS); (2) WT+SPD group: C57BL/6 mice intraperitoneal injection of SPD (Sigma, St. Louis, MO, USA) dissolved in SPSS (10 mg/kg); (3) T2D group: db/db mice; (4) T2D+SPD group: db/db mice intraperitoneal injection of SPD (10 mg/kg in SPSS), the dosing interval of animals in each group was every other day. All mice in the four groups were sacrificed in week 12, and determined relevant experimental indexes according to the protocols.

**Cell culture**

Primary neonatal mouse cardiomyocytes were isolated from the hearts of 1-3-day old C57BL/6 mice. In brief, in a sterile operating table, the hearts were quickly removed, immediately placed in pre-chilled D-hanks solution. All hearts in the petri dish were washed and transferred to a 15 ml centrifuge tube, added 3 ml D-Hanks and 5 ml 0.25 % trypsin, sealed and placed on a shaker at 4 °C overnight. The next day, added 5 ml DMEM containing 10 % fetal bovine serum (FBS) and 1 % penicillin and streptomycin, and then abandoned DMEM, added 7 ml type II collagenase (0.08 %, prepared with serum-free DMEM). Then put it on a shaker, 37 °C, 160 r/min, 10 min, and collected the supernatant DMEM in a 50 ml centrifuge tube (store at 4 °C). The abovementioned process was repeated 3-5 times until all hearts were completely digested. The cells in the 50 ml centrifuge tube were collected by centrifugation at 4 °C, 800 r/min, 5 min. Subsequently, added the medium and mixed well, inoculated in petri dishes, and 2 hours of incubation, the unattached cells were cardiomyocytes. The cardiomyocytes were planted in another petri dish and maintained at 37 °C in a 5 % CO₂ humidified incubator. The media of cells was changed three times per week. Cardiomyocytes were treated with high glucose (HG, 40 mM), SPD (10 μM), Erastin (0.25 μM), P2X7 specific inhibitor (A-438079, 3 μM), LY3214996 (20 μM), according to the experiment protocol.

**Serum measurements**

Blood glucose was measured from the mouse tail vein blood by a blood glucose meter (ACCU-CHEK, Roche, Germany). In week 12, blood samples which taken from the medial canthal vein were centrifuged
and serum was used to detect relevant indicators. Serum insulin levels were determined by ELISA kit (Tongwei, Shanghai, China). Serum levels of triacylglycerol (TG) and total cholesterol (TC) were analyzed using a standard biochemistry panel (Beyotime, Shanghai, China). Lactate dehydrogenase (LDH), creatine kinase isoenzyme (CK-MB) and cardiac troponin-I (cTnI) in the blood serum were measured by using commercially available kits (Jiancheng Institute of Bioengineering, Nanjing, China). All Kits were operated according to the manufacturer's instructions.

**Glucose tolerance tests**

Mice were intraperitoneally injected with D-glucose (2 g/kg mass). Tail vein blood was collected and blood glucose was detected using a glucometer (ACCU-CHEK, Roche, Germany).

**Iron measurements**

Heart tissue and cardiomyocytes were homogenized with PBS and the supernatant was collected after centrifugation. The iron levels of tissue and cells were detected by the Iron Assay Kit (ab83366, Abcam) according to the manufacturer's instructions.

**GPX-4 activity and GSH level measurements**

As described method in the previous protocol[40], GPX-4 activity was detected by using phosphatidylcholine hydroperoxide as a substrate. Total GSH levels of the mouse heart tissue were determined using GSH/GSSG Assay Kit (Beyotime, Shanghai, China)

**Histology analysis**

After anesthesia, the heart was quickly excised and washed with pre-cooled PBS buffer. The cardiac tissue was fixed in 10 % buffered paraformaldehyde, embedded in paraffin, sliced at 4 millimeters and used for morphological observation. Hematoxylin and eosin staining (HE), Masson trichrome staining, and Sirius red staining were conducted according to the dyeing protocol, tissue-stained sections were observed using computer-assisted color image analysis system (Leica Microscope DM2700M, Gemany).

**Echocardiographic analysis**

A Vivid 7 Dimension echocardiography machine was used to assess cardiac function and dimensions (Mylab Delta-vet, Esaote, Italy). All the mice were anesthetized with 2 % isoflurane to perform echocardiography. Left ventricular ejection fraction (EF), left ventricular fractional shortening (FS), left ventricular internal dimension systole (LVIDs) and left ventricular internal dimension diastole (LVIDd) were measured.

**Transmission electron microscopy (TEM)**

The ultrastructural analysis was performed as previously described[41]. Briefly, heart tissue was fixed with 2.5 % glutaraldehyde overnight at 4 °C, fixed in 1 % osmium tetroxide for 2 h. Subsequently, heart
tissue was dehydrated using a graded ethanol series, embedded in epoxy resin, and observed using a H-7650 transmission electron microscope (Hitachi, Japan).

Bioinformatic analysis

Heart samples were lysed and trypsin digested according to LC-MS/MS analysis procedure. Mass spectral data were retrieved using Proteome Discoverer (v2.4.1.15). Searching database is Mus_musculus_10090_SP_20201214.fasta (17063 sequences). A heat map was drawn by using the “gplots” R-package. KEGG enrichment analysis, enrichment of gene ontology (GO) biological process, cellular component, and molecular function terms were analyzed by using DAVIDs Functional Annotation Chart tool (Version 6.8) [42, 43].

Mitochondrial membrane potential (Δψm) analysis

Δψm was determined using a JC1 Mitochondrial Membrane Potential Assay Kit (Beyotime, Shanghai). In brief, after treatment with HG and SPD, cardiomyocytes were incubated with 10 μM JC1 for 30 mins at 37 °C in the dark. The images were then taken with laser scanning confocal microscope (FV1000-IX81, OLYMPUS) at an excitation 488 nm wavelength and emission 530 nm wavelengths and analyzed with Image J 1.46 software. Red fluorescence was JC1 aggregate, green fluorescence was JC1 monomer in the mitochondria.

ROS and Lipid ROS measurements

Oxidative stress was detected using a ROS kit (Beyotime, Shanghai). Briefly, diluted DCFH-DA with serum-free medium at 1:1000 to make the final concentration 10 μM, removed the cardiomyocytes culture medium and added an appropriate volume of diluted DCFH-DA, incubated in 37 °C condition for 20 minutes. The cells were washed three times with serum-free cell culture medium to fully remove DCFH-DA, and then used laser scanning confocal microscope (FV1000-IX81, OLYMPUS) at an excitation 488 nm wavelength and emission 525 nm wavelength to take photograph and analyzed with Image J 1.46 software. Lipid ROS was detected by using BODIPY 581/591 C11 fluorescent probe (D3861, Invitrogen) as the previous protocol[44].

Immunofluorescence analysis

Frozen sections of heart tissue were washed, fixed, and permeabilized. Subsequently, the slices were immunolabelled with specific primary antibodies (Pannexin-1 at 1:50 ratio) overnight at 4 °C. After rinsing, the slices were incubated with corresponding fluorescent-conjugated secondary antibody (goat anti-Rabbit IgG, diluted 1:500, abclonal, Wuhan), stained nuclei with DAPI, and observed using laser scanning confocal microscope (FV1000-IX81, OLYMPUS).

ATP analysis
The concentration of ATP in cardiomyocytes lysate was measured using ATP Assay Kit (Beyotime Biotechnology, Shanghai) and intracellular ATP was measured according to the instructions provided by the manufacturer.

To detect the concentration of extracellular ATP, the culture medium was processed by a special method[45]. Briefly, cardiomyocytes were treated with different experimental protocol for 24 h, the medium was collected and ATP release was measured by the luciferin–luciferase assay with an ATP Assay Kit (Beyotime Biotechnology, Shanghai), following the manufacturer's instructions [17].

**siRNA transfection**

The cardiomyocytes were seeded in a 35 mm petri dish treated with Pannexin-1 short interfering RNAs (sc-61287, Santa Cruz Biotechnology), following the experimental protocol. In brief, Cells were transfected by siRNA using PolyJet™ (Signagen), Con-siRNA, Pannexin1-siRNA and the transfection reagent were incubated for 12 h with serum-free medium to inhibit the relevant protein expression according to the manufacturer’s instructions, and replaced with normal medium containing 10 % FBS to continue to incubate for 12 h, and then the next experiment could be processed.

**Western blot analysis**

The mouse heart tissue and cardiomyocytes were homogenized in 0.5 ml of RIPA (1:1000 PMSF, Solarbio Science, Beijing) buffer using small tubes and vibrated for every 10 min for 5 seconds at 4 °C, the above process repeats 4 times. Solubilized proteins were centrifugated at 13,500 rpm for 25 min the supernatant was then collected. The protein concentration of each sample was quantified using the BCA Protein Assay kit (Beyotime, Shanghai). Protein lysates of each group were separated by electrophoresis with SDS-PAGE and electro-transferred onto a PVDF membrane (Millipore). Non-specific proteins on membranes were blocked with 5 % non-fat dried milk for 2 h at room temperature, the membranes were incubated overnight with the following primary antibodies (at a 1:1000 dilution, 4 °C): P2X7, ACSL-4, GPX-4, FTH-1, HO-1, NCOA-4, (ABclonal Technology, Wuhan, China); Prdx-1, Tfr-1, p-p38, t-p38, p-JNK, t-JNK, GRP94, GRP78, ATF-4, Chop (Proteintech, Wuhan, China); ODC, SSAT, ERK1/2, p-ERK1/2, Pannexin-1, β-actin and β-tubulin (Santa Cruz Biotechnology). And then the membranes were incubated with anti-mouse/anti-rabbit IgG antibody (ABclonal Technology, Wuhan, China) at a 1:10000 dilution for 1 h at room temperature. The specific complex was determined by an enhanced chemiluminescent (ECL) kit (Meilun, Dalian, China) and a multiplex fluorescent imaging system (ProteinSimple, CA).

**Statistical analyses**

All experiments were replicated at least three times independently. All data were expressed as mean value ± standard error of mean (SEM). Statistical analysis was performed by two-tailed Student t test or one-way ANOVA, followed by the Bonferroni multiple comparison test using GraphPad Prism 8.0. P-value < 0.05 was considered statistically significant.
Abbreviations

DCM, diabetic cardiomyopathy; DM, diabetes mellitus; SPD, spermidine; ODC, ornithine decarboxylase; SSAT, SPD/spermine N1-acetyltransferase; PAs, Polyamines; TC, total cholesterol; TG, Triglyceride; HE, hematoxylin and eosin; EF, Left ventricular ejection fraction; FS, Left ventricular fractional shortening; ERS, Endoplasmic reticulum stress

Declarations

Acknowledgments

Not applicable.

Author contributions

HY conceived and supervised the study; HY, CX and JS designed experiments; HY, JX, YL, YH, FW, SZ and XG performed experiments; YL provided new tools and reagents; HY analyzed data; HY wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Funding

This research is supported by the Basic Scientific Research Business Research Project of Heilongjiang (No.2021-KYYWF-0466).

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. The names of the repository/repositories and accession number(s) can be found at: Proteomics identifications database (PRIDE database) with accession PXD036364.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details
1 School of Basic Medical Sciences, Mudanjiang Medical University, Mudanjiang, China.

2 School of Medical Imaging, Mudanjiang Medical University, Mudanjiang, China.

3 Animal Research Institute, Research Department, Mudanjiang Medical University, Mudanjiang, China.

4 School of Stomatology, Mudanjiang Medical University, Mudanjiang, China.  

5 The Fifth Affiliated Hospital of Southern Medical University, Guangzhou, China.  

6 Department of Pathophysiology, Harbin Medical University, Harbin, China.

# These authors contributed equally

**References**


**Figures**

**Figure 1**

Successful establishment of type 2 diabetic mouse model
Db/db mouse was selected as the research object of T2D model, and the related indexes were determined 12 weeks. (A) scheme of the in vivo experiment, (B) blood glucose concentration, (C) insulin resistance test, (D) serum insulin concentration, (E) triacylglycerol, (F) total cholesterol. *P < 0.05 vs. WT group (n = 8).

Figure 2

SPD alleviates diabetic myocardial damage in db/db mice

(A) echocardiography, (B) left ventricular ejection fraction, (C) left ventricular fractional shortening, (D) left ventricular internal dimension systole, (E) left ventricular internal dimension diastole, (F) representative
images of H&E staining and transmission electron microscopy, (G) representative Masson's trichrome and Sirius red staining of heart tissues, (H) myocardial tissue fibrosis area statistics, (I) serum lactate dehydrogenase concentration, (J) serum CK-MB concentration, (K) serum cTnI concentration, (L) body weight, (M) the ratio of heart weight to tibia length. *P < 0.05 vs. WT group; #P < 0.05 vs. T2D group (n = 8).

**Figure 3**

**Proteomic analysis of cardiac tissues from C57BL/6 mice and db/db mice.**

(A) heat map showing the quantitative protein expression in cardiac tissues from C57BL/6 mice and db/db mice, (B-C) gene ontology analysis between C57BL/6 mice and db/db mice, (D) subcellular distribution of the differentially expressed proteins, (E) KEGG enrichment analysis for all the aberrantly
expressed proteins, bubble diagrams displaying the top 10 KEGG pathways. The Y-axis and X-axis represent the names of the enriched pathways and the ratio of enrichment, respectively. The size of the bubbles indicates the number of differentially expressed proteins in each pathway.

Figure 4

SPD inhibits ferroptosis pathway in vivo and vitro

According to the analysis of proteomic results, diabetic cardiomyopathy is involved in ferroptosis pathway and related detections were performed. (A-B) representative western blot of ODC and SSAT in comparison with β-actin expression in mice and cardiomyocytes, (C-D) the expression of ACSL-4 and GPX-4 in cardiomyocytes and mice, (E) mitochondrial membrane potential (Δψm) was measured using JC-1 staining and photos were taken using fluorescence microscopy, (F-I) heart tissue were collected in 12 weeks to measure iron, GPX-4 activity, GSH level, and GSH/GSSG ratio. *P < 0.05 vs. WT/Control group; #P < 0.05 vs. T2D/HG group (n ≥ 3)
Figure 5

SPD suppresses oxidative stress (OS) and endoplasmic reticulum stress (ERS) in cardiomyocytes

(A) intracellular ROS level detection was determined by fluorescence microscopy, (B) representative western blot of Prdx-1, TfR-1, p-p38 and p-JNK in cardiomyocytes, (C) the ER related protein expression of GRP94, GRP78, ATF-4 and Chop were determined by western blot. *P < 0.05 vs. Control group; #P < 0.05 vs. HG group (n ≥ 3)
Figure 6

The expression of P2X7 were regulated by Pannexin-1 in HG-treated cardiomyocytes

(A) the expression of Pannexin-1 in myocardial tissue was detected by immunofluorescence, (B) the expression of Pannexin-1 in cardiomyocytes was determined by western blot, (C) the P2X7 expression in myocardial tissue was detected by western blot, (D) ATP concentration in the medium of cardiomyocytes, (E) the concentration of intracellular ATP was detected by chemiluminescence in cardiomyocytes, (F) cardiomyocytes were treated with erastin and Pannexin-1 expression was detected in different time point, (G) representative western blot of Pannexin-1 in comparison with β-actin expression, (H) representative western blot of P2X7 in comparison with β-actin expression in cardiomyocytes, (I) ATP concentration in the medium of cardiomyocytes. *P < 0.05 vs. WT/Control group/Control-siRNA, #P < 0.05 vs. T2D/HG group (n ≥ 3)
**Figure 7**

**SPD restrains ferroptosis via inhibiting ERK1/2-HO-1 pathway**

(A) the ERK1/2 signaling pathway related protein expression was determined by western blot, (B) the expression of HO-1, NCOA-4 and PTH-1 were evaluated by western blot, (C) intracellular lipid ROS was stained by BODIPY probe, (D) iron content level of cardiomyocytes, (E) Schematic diagram showing the mechanism of SPD alleviating diabetic cardiomyopathy. *P < 0.05 vs. Control group; #P < 0.05 vs. HG group (n ≥ 3)